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ORIGINAL ARTICLE

Hypoxia and collagen crosslinking in the healthy and affected sites of periodontitis patients

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ABSTRACT

Summary: Present study suggests that diseased sites of periodontitis with stage 3 grade B and C had decreased fibroblast cell density, hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF) expressions while increased inflammatory cell counts compared to both healthy sites of the periodontitis patients and healthy controls. Collagen maturation enzymes also decreased in the diseased sites.

Objective: The present study aimed at determining markers of hypoxia and collagen crosslinking in healthy and diseased gingiva from healthy individuals and periodontitis patients.

Methods: Group-1; healthy individuals, Group-2; healthy sites of periodontitis patients-stage 3 grade B, (H-GradeB) Group-3; diseased sites of periodontitis patients-stage 3 grade B, (D-GradeB). Group-4; healthy sites of periodontitis patients-stage 3 grade C, (H-GradeC). Group-5; diseased sites of periodontitis patients-stage 3 grade C, (D-GradeC). Plaque index (PI), gingival index (GI) and clinical attachment levels (CALs) were recorded. Gingival biopsies were obtained. Fibroblast and inflammatory cells were counted. HIF-1α, prolyl hydroxylase (PH), VEGF, lysyl oxidase (LOX) and lysyl hydroxylase (LH) levels were determined via immunohistochemistry.

Results: Fibroblast cell counts were lower in D-GradeC and D-GradeB than other groups. C group had highest fibroblast cell counts. Inflammatory cell counts were highest in the D-GradeC and lowest in C group. HIF-1α levels were highest in C group and decreased in diseased sites. Lowest value was observed in D-GradeC group. VEGF, PH, and LH levels were higher in the control group compared to other groups. LOX levels were similar in the groups except for D-GradeC. LOX levels were similar in the groups except for D-GradeC which is significantly lower than those of the control group and healthy sites.

Conclusions: The results revealed that diseased sites of periodontitis patients had decreased fibroblast cells, HIF and VEGF expressions while increased inflammatory cells. Collagen crosslinking tend to decrease with disease regardless of stage and grade of disease.

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Hypoxia; collagen crosslinking; periodontitis; VEGF

Introduction

Periodontitis is the irreversible destructive disease of the periodontium which is triggered by dysbiotic oral microbiota. Development and progress of the disease vary with accompanying systemic, genetic or environmental factors [1,2]. Once started, inflammation increases host-derived proteinases, and periodontal attachment loss with apical migration of junctional epithelium and destruction of connective tissue fibres and alveolar bone occur [2,3]. Clinical manifestation of the periodontitis and symptoms differ with the age of the patient and severity, distribution, and the location of the periodontal lesions [3,4]. The most recent classification of the periodontitis was established considering the severity, extent, complexity, future risk and systemic effects of periodontitis [3]. However, the future risk of periodontal lesion development and existing periodontal status of unaffected sites in periodontitis patients were not clarified.

Periodontal infection and inflammation are commonly accompanied by other pathologic conditions such as hypoxia, which is the general or local depletion of the oxygen [5–7]. Hypoxia accelerates periodontal inflammation and inflammation also causes hypoxic alterations [5,8]. In this regard, gingival tissues exhibiting inflammation was reported to be hypoxic while healthy gingiva had normal oxygen levels [5]. A possible mechanism was suggested to be the pro-inflammatory pathways including nuclear factor KB (NF-KB), receptor activator of NF-KB (RANKL), interleukin (IL)-1β production induced by *Porphyromonas gingivalis* [5,9]. Along with *P. gingivalis, Fusobacterium nucleatum* also contributes to local oxygen depletion and mediate hypoxia-induced inflammation in periodontium by up-regulating HIF, inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF)-α, and IL-1β [10]. In addition to stimulating a pro-inflammatory response, hypoxia also alters cell behaviour

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with decreased proliferation, migration and differentiation in the human periodontal ligament and gingival cells [7,11,12].

Hypoxic alterations in tissues occur through the transcriptional regulator protein of hypoxia, hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) and overexpression of HIF-1α is associated with chronic inflammatory diseases such as diabetes, rheumatoid arthritis and periodontitis [8,13,14]. HIF-1α undergoes posttranslational modifications, hydroxylation, acetylation and phosphorylation [15]. In normoxic conditions, prolyl hydroxylase (PH) cross-links proline to HIF-1α and HIF is rapidly degraded by ubiquitin ligase enzymes, however, in hypoxic conditions, PH cannot transfer proline to HIF and degradation of HIF is prevented allowing HIF to accumulate [13,15]. Therefore, detection of HIF-1α levels could be an indicator of increased hypoxic state and inflammation in periodontium [10,16,17]. Accordingly, the HIF-1α and VEGF levels in periodontitis and gingivitis were found to be elevated while healthy gingiva had relatively low expressions of HIF-1α and VEGF [17]. Furthermore, Afacan et al. [8] showed increased HIF-1α, VEGF and TNF-α levels as indicators of periodontitis with different disease severity and extent.

Based on these reports, hypoxia can be considered as a contributor or at least modifier for the periodontal disease [5,7–11,16]. However, while the hypoxic alterations in the diseased tissues have been studied, the tissue changes related to severe and mild periodontal lesions and changes in the unaffected sites in periodontitis patients are not yet to be determined. Therefore, the present study hypothesized that unaffected sites in periodontitis patients would exhibit pathologic alterations even though there is no sign of clinical inflammation. And the present study aimed to evaluate hypoxia and related alterations in both diseased and healthy sites of periodontitis patients with different severity, extent, and distribution of periodontal lesions.

Materials and methods

The present study was designed as a cross-sectional clinical study. The study protocol was approved by the Medical Ethics Committee of Sivas Cumhuriyet University (#2016/12-09), and the research was conducted at Tokat Gaziosmanpaşa University Faculty of Dentistry Department of Periodontology. Written informed consent was obtained from all participants. All patients and healthy volunteers had a detailed oral and radiographic examination.

Forty-five participants were enrolled in the study. The study population consisted of 15 healthy individuals, 15 patients who were diagnosed as stage 3 grade B periodontitis and 15 patients diagnosed as stage 3 grade C periodontitis.

Group-1; healthy individuals (mean age 36.37 ± 4.53, 11 women, 4 men).
Group-2; healthy sites of periodontitis patients-stage 3 grade B, (H-GradeB) (mean age 40.37 ± 4.53, 10 women, 5 men).
Group-3; diseased sites of periodontitis patients-stage 3 grade B, (D-GradeB) (mean age 40.37 ± 4.53, 10 women, 5 men).
Group-4; healthy sites of periodontitis patients-stage 3 grade C, (H-GradeC) (mean age 35.25 ± 1.83, 11 women, 4 men).
Group-5; diseased sites of periodontitis patients-stage 3 grade C, (D-GradeC) (mean age 35.25 ± 1.83, 11 women, 4 men).

All participants were systemically healthy individuals with no tobacco use, drug use, pregnancy or lactation. Neither of the individuals had previous antibiotic therapy nor previous periodontal treatment within six months.

Periodontitis was diagnosed based on the criteria defined by the 2017 International World Workshop for a Classification of Periodontal Diseases and Conditions. The grade determination was based on longitudinal evaluation (evaluation of the patients over 5 years), evaluation of %bone loss/age, and the amount of plaque/tissue destruction [3]. Biopsy specimens obtained from participants were collected by gingivectomy or crown lengthening procedure in the routine treatment protocol or before orthodontically indicated tooth extraction. All biopsies were obtained from posterior maxillary teeth (#4, #5, #6 and #7).

Periodontal clinical parameters

Full-mouth clinical periodontal measurements were recorded. Full mouth clinical attachment level (CAL), plaque index (PI) [18], gingival index (GI) [19] measurements at six sites per tooth (mesial, middle and distal aspects of both buccal and lingual/palatal surfaces) were performed. CAL levels were measured via a Willams-type periodontal probe (Hu-Friedy Co., Chicago, IL, USA). CAL was calculated as the distance in millimeters from the cementoenamel junction to the bottom of the periodontal pocket. One tooth for the affected site and one tooth for the unaffected site were chosen in each periodontitis patient. The site selection was based on clinical measurements. The gingiva around teeth with probing depth and attachment loss over 6 mm were chosen for affected sites and the sites exhibiting probing depth below 3 mm without attachment loss were considered as unaffected sites.

Collection of gingival samples

Gingival biopsies were collected by either gingivectomy/crown lengthening procedures from patients with impaired gingival topography or before tooth extraction indicated for orthodontic treatment. All samples were taken from the maxillary posterior region of the individuals. Collected samples were stored in 10% neutral buffered formalin for 48 hours and embedded in paraffin for histological analysis.

Histopathological evaluation

All samples went through histological tissue processing. Firstly, dehydration was performed through ethanol series, and then tissues were cleared with xylene and embedded in

paraffin. A single examiner (F.G.) who was unaware of the identity of the samples performed all histological evaluations. About 5 μm continuous sections were obtained and hematoxylin and eosin (H&E) staining and immunohistochemistry were performed. All sections were analysed using a light microscope[1]. Fibroblast and inflammatory cells were counted in the H&E stained slides.

Determination of fibroblasts and inflammatory cells

For the evaluation of inflammation, an area of 10.000 μm^2 connective tissue neighbouring gingival epithelium was marked, and the total inflammatory cells (neutrophil, lymphocyte, eosinophil and macrophage cells) within the frame were counted. The fibroblasts were also counted likewise. The measurements were performed from three different points, and the mean of these three measurements was recorded [20].

HIF-1α, VEGF, PH, LH and LOX immunohistochemistry

Three different slides for each parameter were randomly selected from the sections obtained from every individual. Immunohistochemistry was performed to indicate hypoxia-related alterations (Hypoxia-inducible factor [HIF]-1α, PH, and vascular endothelial growth factor [VEGF]) and collagen crosslinking enzymes (lysyl oxidase [LOX] and lysyl hydroxylase [LH]). For immunohistochemistry staining, slides were deparaffinized and dehydrated through ethanol series. After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for two h at 70 °C. To suppress endogenous peroxidase activity, slides were treated with 3% hydrogen peroxide. Then sections were incubated with normal rabbit serum for 30 min. After rabbit serum, samples were incubated with primary antibodies overnight. The antibodies and conditions used were as follows: goat polyclonal anti-HIF-1α antibody[2] (1:250), anti-VEGF antibody[3] (1:250), anti-PH antibody[4] (1:250), anti-LOX antibody[5] (1:250) and anti-LH antibody[6] (1:250). After phosphate buffer saline (PBS) wash three times for 5 min (3 × 5), the sections were incubated with biotinylated immunoglobulin G for 30 min and again washed 3 × 5 with PBS and reacted with a streptavidin-horseradish peroxidase-conjugated reagent for 30 min. Following 3 × 5 PBS wash, samples were incubated with AEC chromogen to visualize the immunoreactivity. Sections were counterstained with haematoxylin and analysed using light microscopy[7] [16,20].

Immunohistochemical semi-quantitative H SCORE analysis

Immunohistochemistry evaluation was performed from three different areas in each section. Stained sections were examined under 400× magnification. All cells in the selected area were marked as either 'unstained,' 'slightly stained,' 'mild stained' or 'dense stained' and counted. The H Score formula ($\sum Pi(i+1)$) was used to convert staining density and the number of stained/unstained cells to a numeric value. The evaluation was blind and mean of three evaluations were recorded. In this formula, *i* shows the staining intensity score and *Pi*: indicates the percentage of the stained cells [16,20].

Statistical analysis

A power analysis based on a previous study was performed before the study and 15 patients in each study group provided 85% power [20]. Data were presented as mean ± SD or percentage as appropriate. Results and statistical analysis were performed with digital software[8]. One sample KS test was used as a normality test. CAL values, fibroblast, and inflammatory cell counts, HIF-1α, VEGF, PH, LOX and LH H score values were evaluated with One-way ANOVA. PI and GI scores were evaluated with the chi-square test. $p < .05$ were considered statistically significant.

Results

The age and gender distribution among the healthy volunteers and periodontitis patients were similar ($p > .05$). PI values were lower in the healthy group compared to the other groups ($p < .05$) and similar in the healthy sites and diseased sites of the periodontitis patients ($p > .05$). GI values of the D-GradeB group were lower than the control group ($p < .05$) and the D-GradeC group had higher GI scores compared to the other groups ($p < .05$). CAL of the healthy individuals and healthy sites of the periodontitis patients were lower than those of the affected sites of the periodontitis patients ($p < .05$) (Table 1).

Table 1. Mean age, plaque index, gingival index, clinical attachment levels of the study groups.

Groups/ parameters (mean ± SD)	Healthy controls	Healthy sites in stage 3 grade B periodontitis (H-GradeB)	Affected sites in stage 3 grade B periodontitis (D-GradeB)	Healthy sites in stage 3 grade C periodontitis (H-GradeC)	Affected sites in stage 3 grade C periodontitis (D-GradeC)
Plaque index	0.50 ± 0.25	0.55 ± 0.30	**1.89 ± 0.70**[a]	0.50 ± 0.20	**1.92 ± 0.51**[a]
Gingival index	0.50 ± 0.20	0.55 ± 0.40	**1.80 ± 0.50**[a]	0.50 ± 0.45	**2.50 ± 0.53**[a,b,c,d]
Clinical attachment loss	2.30 ± 0.32	2.55 ± 0.45	**7.25 ± 0.70**[a,b]	**2.60 ± 0.35**[c]	**7.62 ± 0.91**[a,b,d]
Age	36.37 ± 4.53	40.37 ± 4.53	40.37 ± 4.53	35.25 ± 3.88	35.25 ± 3.88

[a] $p < .05$ vs. control group.
[b] $p < .05$ vs. H-GradeB group.
[c] $p < .05$ vs. D-GradeB group.
[d] $p < .05$ vs. H-GradeC group.

Fibroblast and inflammatory cell counts

Fibroblast cell counts were lower in D-GradeC and D-GradeB groups than those of the other groups ($p < .05$). The healthy group had the highest fibroblast cell counts compared to the other groups ($p < .05$) and the fibroblast counts in the healthy sites of the periodontitis patients were significantly lower than those of the control group ($p < .05$). However, diseased sites of the periodontitis groups had similar fibroblast cell counts ($p > .05$) (Figures 1 and 2, Table 2).

Inflammatory cell counts were highest in the D-GradeC, and lowest in the healthy group and the differences among the groups were significant ($p < .05$). D-GradeC periodontitis patients also had higher inflammatory cell counts compared to the H-GradeC, D-GradeB and H-GradeB groups ($p < .05$). Inflammatory cells increased both with the existence of periodontitis and the affected sites (Figures 1 and 2, Table 2).

HIF-1α, VEGF, PH, LOX and LH immunohistochemistry results

The immunohistochemistry results were presented in Table 2 and Figures 3 and 4. Hypoxia tends to decrease in the affected sites of the periodontitis, and HIF-1α levels of D-GradeC were significantly lower than those of the other groups ($p < .05$). Also, VEGF expressions also exhibited a similar pattern, as higher in the healthy groups and lower in the diseased groups. VEGF levels were the highest in the healthy controls and the healthy group, and H-GradeB group had

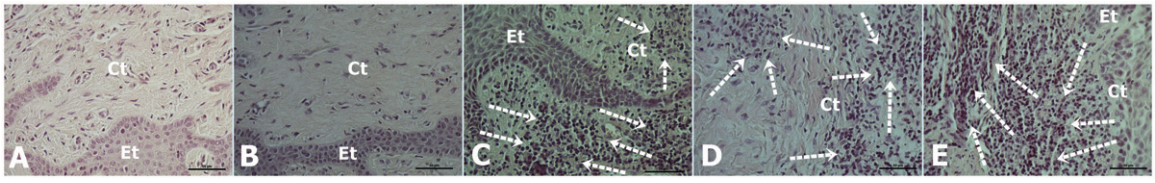

Figure 1. Representative histological images of the study groups. (A) Healthy control group, (B) healthy sites of periodontitis patients-stage 3 grade B, (C) diseased sites of periodontitis patients-stage 3 grade B, (D) healthy sites of periodontitis patients-stage 3 grade C, (E) diseased sites of periodontitis patients-stage 3 grade C. Interrupted white arrows indicate inflammatory cells. Ct: connective tissue; Et: Epithelial tissue.

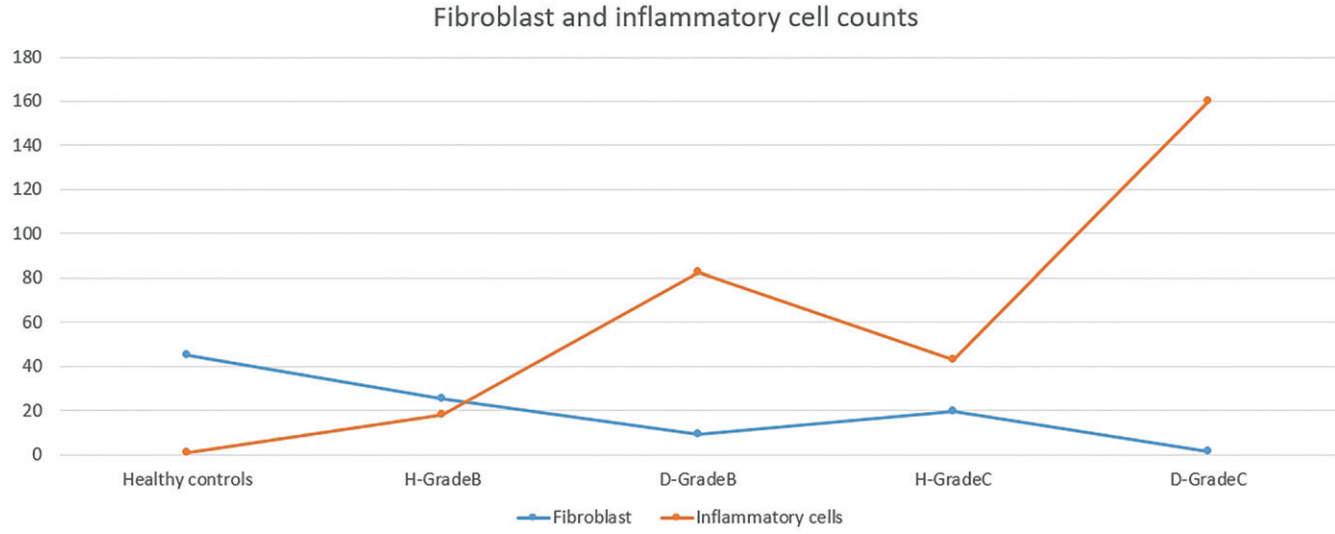

Figure 2. Graphic demonstration of fibroblast and inflammatory cell counts in the study groups. Healthy control group, H-GradeB: healthy sites of periodontitis patients-stage 3 grade B, D-GradeB: diseased sites of periodontitis patients-stage 3 grade B, H-GradeC: healthy sites of periodontitis patients-stage 3 grade C, D-GradeC: diseased sites of periodontitis patients-stage 3 grade C.

Table 2. Mean immunohistochemistry values of the HIF-1α, VEGF, PH, LOX and LH in the study groups.

Groups/ parameters (mean ± SD)	Healthy controls	Healthy sites in stage 3 grade B periodontitis (H-GradeB)	Affected sites in stage 3 grade B periodontitis (D-GradeB)	Healthy sites in stage 3 grade C periodontitis (H-GradeC)	Affected sites in stage 3 grade C periodontitis (D-GradeC)
Fibroblast counts	45.00 ± 6.63	**25.50 ± 3.89**[a]	**9.00 ± 7.67**[a,b]	**19.75 ± 7.86**[a,c]	**1.25 ± 1.75**[a,b,d]
İnflammatory cell counts	1.12 ± 1.35	**18.00 ± 2.80**[a]	**82.75 ± 12.74**[a,b]	**43.00 ± 10.39**[a,b,c]	**160.50 ± 40.80**[a,b,c,d]
HIF-1α	108.35 ± 2.07	106.97 ± 1.41	**99.32 ± 1.87**[a,b]	**107.84 ± 0.75**[c]	**91.14 ± 1.46**[a,b,c,d]
VEGF	122.73 ± 17.37	**112.80 ± 2.18**[a]	**98.74 ± 3.73**[a,b]	**102.69 ± 1.54**[a]	**98.25 ± 4.09**[a,b]
PH	25.14 ± 4.39	**9.67 ± 1.89**[a]	**8.59 ± 7.13**[a]	**7.95 ± 4.10**[a]	**3.08 ± 4.25**[a,b]
LH	22.38 ± 1.82	**13.76 ± 1.39**[a]	**12.14 ± 2.79**[a]	**12.80 ± 2.36**[a]	**7.48 ± 2.13**[a,b]
LOX	7.90 ± 2.15	7.85 ± 2.08	4.76 ± 1.99	7.89 ± 2.94	**1.87 ± 1.58**[a,b,c,d]

[a] $p < .05$ vs. control group.
[b] $p < .05$ vs. H-GradeB group.
[c] $p < .05$ vs. D-GradeB group.
[d] $p < .05$ vs. H-GradeC group.

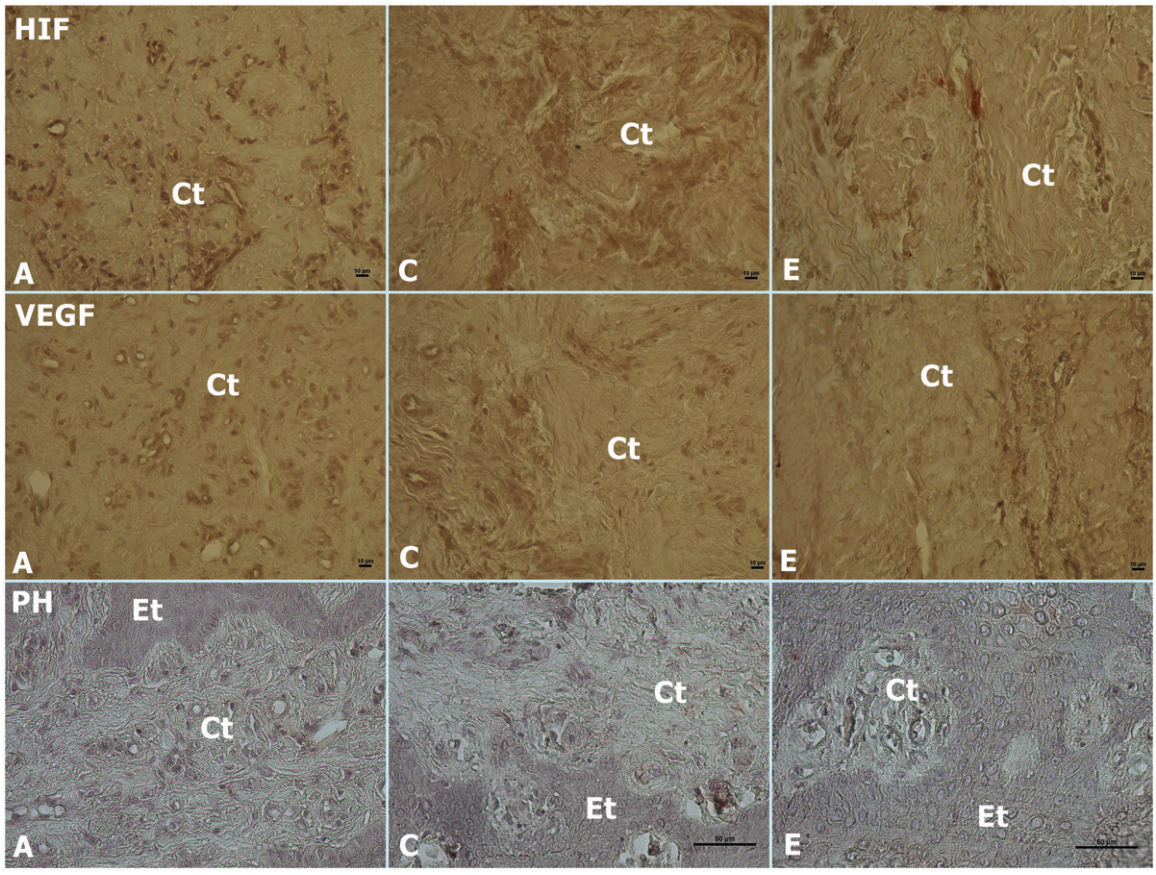

Figure 3. Representative Immunohistochemistry images of the HIF-1α, VEGF and PH in the study groups. (A) Healthy control group, (C) diseased sites of periodontitis patients-stage 3 grade B, (E) diseased sites of periodontitis patients-stage 3 grade C. HIF-1α: hypoxia-inducible factor-1α, VEGF: vascular endothelial growth factor, PH: prolyl hydroxylase, Ct: connective tissue, Et: Epithelial tissue.

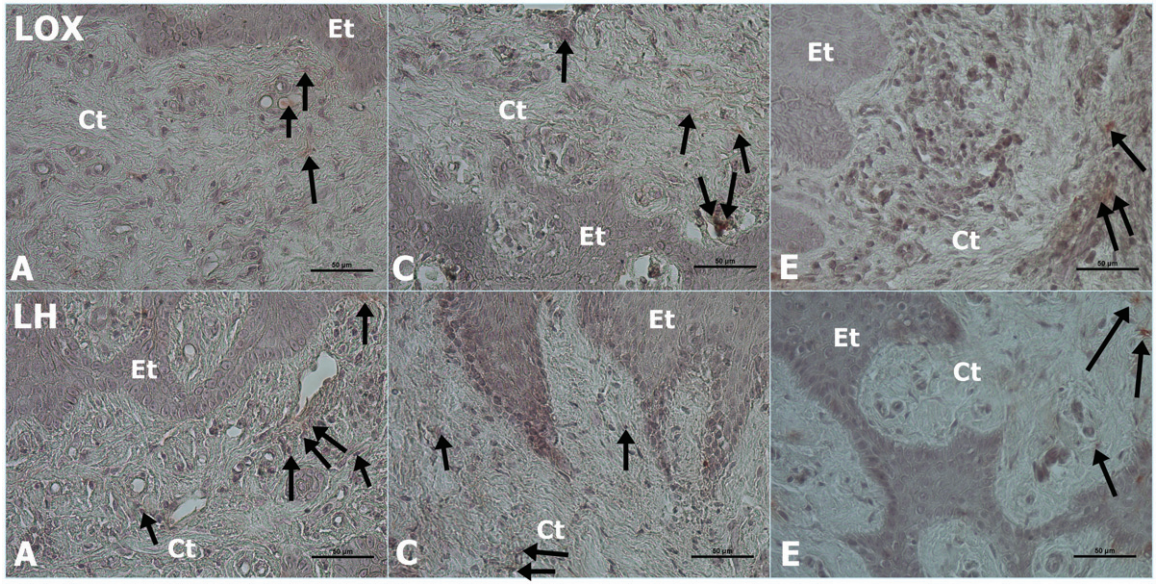

Figure 4. Representative Immunohistochemistry images of the LH and LOX evaluations in the study groups. (A) Healthy control group, (C) diseased sites of periodontitis patients-stage 3 grade B, (E) diseased sites of periodontitis patients-stage 3 grade C. Straight black arrows indicate cells with immunostaining. LH: lysyl hydroxylase; LOX: lysyl oxidase; Ct: connective tissue; Et: Epithelial tissue.

higher levels than those of the D-GradeB and D-GradeC groups ($p < .05$). Likewise, PH expressions were also higher in the healthy controls, and the values decreased with the existence of periodontitis lesions ($p < .05$). The D-GradeC group had lower PH levels compared to the healthy controls and H-GradeB group ($p < .05$). Other comparisons were found to be insignificant ($p > .05$).

Regarding LH, the differences among the groups were similar to those of the PH expressions. The highest value was in the healthy controls, and the levels decreased with both

grade B and Grade C diseases regardless of the site in the GradeB periodontitis patients. However, the diseased site of the Grade C periodontitis patients exhibited lower LH levels compared to the healthy sites of the Grade B periodontitis patients ($p < .05$). In terms of LOX levels, healthy controls, and the healthy sites of the grade B and C periodontitis patients had similar expressions, and the diseased site of the Grade C periodontitis patients had slightly lower levels, but the difference did not reach significance ($p > .05$). Additionally, D-GradeC group had lower LOX levels compared to the healthy controls and the healthy sites of the grade B and C periodontitis patients ($p < .05$).

Discussion

The present study is a histological cross-sectional study which evaluated the hypoxia and collagen crosslinking enzymes in stage 3 grade B and C periodontitis patients and healthy individuals. Both unaffected and affected sites of the periodontitis patients were examined, and the results revealed that fibroblast and inflammatory cell counts of the unaffected sites were different than those of the healthy controls were more evident in the unaffected sites of the grade C patients. HIF-1α expressions were lower in the diseased sites, and the decrease in the D-Grade C group was more visible. VEGF, PH and LH expressions followed a similar pattern as higher in the healthy samples and lower in the diseased samples while LOX levels only decreased in D-GradeC group.

Periodontitis can be considered as a site-specific disease; however, it certainly has systemic effects such as an up-regulated constant low-level inflammation and changes in the cytokine and growth factor levels [21]. The higher the severity of the periodontitis lesion the higher the tissue destruction and inflammation [3] and decreased number and function of gingival fibroblasts were also evident [22]. Apoptosis also contributes to the reduced fibroblast cell counts in periodontal inflammation [23]. Therefore, the lesions with severe inflammation and tissue destruction tend to have lower fibroblast cell density and higher inflammatory cell infiltration [22,24]. The fibroblast cell count results of the present study revealed that healthy individuals had the highest cell counts while D-GradeC group had the lowest fibroblast cell, counts. As for D-GradeB group, the cell counts were higher than those of the D-GradeC group and lower than the healthy sites of the diseased groups. Inflammatory cell counts exhibited an inverse pattern as the highest inflammatory cell counts were observed in the D-GradeC patients while the lowest cell counts were found in the healthy controls. The fibroblast and inflammatory cell counts were site-specific, and the gingival tissue samples of the grade C periodontitis patients had increased inflammatory cell counts compared to the equivalent grade B patients. Another major factor affecting the composition of gingiva is the aging process which alters certain matrix metalloproteinases (MMPs) and growth factors [25,26]. The proliferation, differentiation, and migration of the fibroblast cells were also reported to decrease with age [27]. However, there was no significant age difference among the groups, and the immunohistochemistry results indicated no associations which could be related to the age difference.

Hypoxia and its main regulator HIF-1α are involved in various chronic inflammatory diseases and infections either as the result or the cause of the pathology, and periodontitis is one of the diseases which hypoxia has been recently shown to participate in [7,10,13–15,17,28–31]. Hypoxia induces pro-inflammatory cytokines such as IL-1β and cytokines, in return, increase hypoxic state and accelerates existing inflammation, as observed in rheumatoid arthritis and periodontitis [7,12,30,31]. NF-KB and RANKL which are significant players in the inflammation contribute to hypoxia-induced periodontal inflammation and tissue destruction as well [9,12,32]. Even though hypoxia is strongly related to pathologic mechanisms, its main regulator, HIF-1α is still detectable in healthy gingiva even there is no sign of visible inflammation, which might indicate a persistent subclinical inflammation in healthy periodontium [5,8,20,31,33]. Recently Afacan et al. [8] demonstrated increased HIF-1α, VEGF and TNF-α levels in gingival crevicular fluid and saliva of periodontitis patients associated with the severity of the periodontal inflammation and attachment loss. In contrast, in a previous study of ours, there was no difference in HIF-1α expressions in gingival samples of patients with either periodontitis or diabetes and periodontitis [20]. However in the present study, in contrast to Afacan et al. [8] diseased sites of the grade B and C periodontitis patients (formerly chronic and aggressive periodontitis) had lower HIF-1α expressions compared to equivalent healthy sites. Healthy sites of the periodontitis patients had similar HIF-1α levels with healthy controls indicating that HIF expressions were not site-specific unlike Afacan et al.'s [8] report. However, evaluation of HIF-1α alone could be misleading; therefore VEGF, the mediator of HIF-1α-related alterations, and PH, an inhibitor of HIF-1α, levels were also determined. VEGF and PH were both decreased in the D-GradeB and D-GradeC groups, but the differences were not site-specific instead decreased with the existence of periodontitis lesions. The pathways and mediators play roles in periodontal inflammation might result in these decreased levels as the presence and activity of HIF-1α occurs through NF-κB pathway which also regulates other mechanisms such as apoptosis and inflammation [34]. Furthermore, PH is an oxygen-dependent inhibitor of HIF-1α which is also affected by iron levels [35], and there are several other oxygen-dependent and independent factors which could either trigger or inhibit HIF-1α [36].

A possible link between hypoxia and periodontal inflammation occur through LPS, and Toll-like receptors which are associated with dysbiotic microbiota and directly up-regulate HIF-1α and HIF-1β leading an increase in HIF-mediated biological activities even in the normoxic conditions and suppression of HIF-1α activity attenuates inflammation [9,10,32,37,38]. HIF-1α activity and related hypoxic alterations in the periodontium was regulated by PH which was reported to be under control of the TGFβ/Smad pathway [39]. Gölz et al. [9] reported up-regulated IL-1β, NF-KB, and MMP-1, -2, and -9 and cyclooxygenase (COX)-2 levels

accompanying increased HIF-1α and VEGF expressions in response to hypoxia. The severity of the tissue destruction was suggested to be correlated with hypoxia [5,7,8,38]. Song et al. [40] also demonstrated increased apoptosis and autophagy of PDL cells along with elevated MMP-8 and IL-1β levels. As for the present results, HIF-1α, VEGF and PH levels were found to be lower in the affected sites of the periodontitis patients. Hypoxia is a multifaceted concept that may be affected by many other factors and studies reported, and studies do not provide much information on hypoxia, except its contribution to periodontal disease [7,9,13,15,28,30,32,33,40]. HIF-1α, its mediator and inhibitor were all decreased in the presence of the disease. The lower levels of HIF-1α observed might result from the microbiota or inflammatory cytokines which were not evaluated in the present study.

Hypoxia can alter the homeostasis and composition of the connective tissue as reported in the previous studies [5,31,33,37,38] and a possible mechanism of hypoxia to do so was suggested to be the LOX enzyme [41]. Alterations in LOX levels induced by hypoxia and HIF-1α were associated with altered collagen crosslinking and fibrosis [42,43]. Covalent collagen crosslinking occurs via three enzymes, PH, LH and LOX and along with PH and LOX, the activity of LH is also affected by hypoxia indicating a direct modulation of collagen maturation by hypoxia [39,42–46]. However, the effect of hypoxia on these three enzymes are different as PH inhibits HIF-1α and related biochemical events, while LOX is down-regulated to cause fibrosis and LH is up-regulated to increase metastases risk of cancers [39,42–44,46]. Collagen crosslinking and hypoxia are relatively novel issues in periodontal research; however, Watanabe et al. [39] reported that PH2 elevation induced by TGF-β1 suppressed HIF-1α transcription and hypoxic changes in PDL cells. In a previous study of ours, LH2 and HIF-1α expressions in diabetic and non-diabetic periodontitis patients and healthy individuals were found to be similar [20]. In terms of LOX, though not related to hypoxia, LOX levels were reported to increase with inflammation [47,48]. All parameters evaluated in the present study tend to decrease with inflammation. Healthy controls exhibited higher expressions of PH and LH while the lowest expression was observed in the D-GradeC group. The differences were not site-specific. As for LOX, D-GradeC group had significantly lower expressions compared to the other groups. Decreased values in the study might be considered as a decrease in anabolic activity with periodontal inflammation in the collagen crosslinking.

Conclusions

Diseased sites of periodontitis patients with stage 3 Grade B and C had decreased fibroblast cell density, HIF and VEGF expressions while increased inflammatory cell counts. Collagen crosslinking markers tend to decrease with disease regardless of the stage and grade of the disease. Unaffected sites in periodontitis patients were not as healthy as the control group and might be considered as at risk for further disease development. Findings of this study should be interpreted by considering the current limitations of the study. Firstly, the present study is a histological study which evaluated markers of hypoxia and collagen crosslinking. Examination of different signal molecules and pathways with further biochemical analysis could provide a better explanation of the mechanism involved in hypoxia and collagen metabolism in gingival tissues.

Notes

1. Nikon Eclipse, E 600, Tokyo, Japan.
2. Abcam plc, Cambridge, UK.
3. Abcam plc, Cambridge, UK.
4. Abcam plc, Cambridge, UK.
5. Abcam plc, Cambridge, UK.
6. Abcam plc, Cambridge, UK.
7. Nikon Eclipse, E 600, Tokyo, Japan.
8. IBM SPSS, IBM Corporation, New York USA.

Disclosure statement

No potential conflict of interest was reported by the authors.

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