

The Immunohistological Comparison of the Number of Gingival Blood Vessels Between Type 2 Diabetes Mellitus and Chronic Periodontitis Patients

Tip 2 Diabetes Mellitus ve Erişkin Periodontitis Hastalarında Dişeti Damar Sayısının İmmünohistokimyasal Olarak Karşılaştırılması

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ABSTRACT

Aim: The study was designed for comparatively analyze the number of vessels in gingival biopsies from type 2 Diabetes Mellitus (DM) with or without periodontitis and from systemically healthy subject with or without periodontitis.

Subjects and Methods: A total of 88 individuals, 24 being type 2 DM patients with periodontitis (DP), 21 being type 2 DM patients with periodontal health (DH), 25 patients with chronic periodontitis (CP) and 18 subjects with clinical and systemic health (C), were included in the study. Biopsies from attached gingiva of all patients were obtained from maxillary canine and premolars. Sections were immunohistochemically stained for CD34 positive cells and hematoxylin-eosin.

Results: The results indicated that in DP groups, the mean number of blood vessels was found to be significantly higher than that of DH, CP and C groups ($p \leq 0.05$). In the CP group, number of blood vessels was higher than DH and C groups ($p \leq 0.05$) and there was no statistically significant difference between DH and C groups ($p > 0.05$). When the number of blood vessels are considered within groups, comparisons

ÖZET

Amaç: Bu araştırma, Tip 2 Diabetes Mellitus (DM) hastalarında periodontitis varlığında ve yokluğunda dişetindeki damar sayısının sistemik açıdan sağlıklı periodontitisli ve periodontal sağlığa sahip bireylerle karşılaştırmak üzere planlandı.

Bireyler ve Yöntem: 24 Tip 2 DM'li ve periodontitisli (DP), 21 Tip 2 DM'li periodontal sağlıklı (DH), 25 kronik periodontitisli (CP) ve 18 hem periodontal hem de sistemik açıdan sağlıklı (C) toplam 88 bireyle yürütüldü. Örnekler hematoksilen-eozin ve immünohistokimyasal olarak CD34 ile boyandı.

DP grubunun mikrovasküler yoğunluk değeri DH, CP ve C gruplarından, yüksek bulundu ($p \leq 0.05$). CP değerleri de DH, C den yüksekti ($p \leq 0.05$) ama DH ve C grupları arasında önemli bir fark yoktu ($p > 0.05$). DP ve CP hastalarında 2. ve 3. derece iltihap varlığında kaydedilen ortalama damar sayısı değerleri arasında anlamlı bir fark izlenmedi ($p > 0.05$). DP hastalarında diyabetin neden olduğu komplikasyonlarla damar sayısı arasında ilişki tespit edilemedi.

between 2nd or 3rd grade gingival inflammation in DP and CP groups revealed no significant difference ($p>0.05$). In diabetic patients, no significant relationship was detected between the number of blood vessels and presence of complications, cardiovascular diseases and hypertension, caused by DM.

Conclusions: The results of the study showed that the number of blood vessels in gingival connective tissue is significantly higher in well-controlled diabetic patients with periodontitis than non-diabetic patients with periodontitis.

KEYWORDS

Diabetes mellitus, Periodontitis, Angiogenesis; Microvascular density, Histopathology

ANAHTAR KELİMELEER

Diabetes mellitus, Periodontitis, Anjiogenezis, Damar yoğunluğu, Histopatoloji

INTRODUCTION

The association between periodontal diseases and diabetes has been addressed in several reports and it has generally been accepted that periodontal disease is more prevalent and more severe in diabetic persons than in non-diabetic ones¹⁻⁶. Although the exact mechanism is not clarified yet, multiple interactions seem to contribute to the pathogenesis of periodontitis associated with DM. Microangiopathy^{7,8}, alteration in collagen metabolism^{9,10} and advanced glycosylated end products (AGE) formation^{11,12} and altered host response^{13,14} have all been suggested to contribute to the susceptibility of individuals with DM to periodontitis.

Certain vascular changes such as increased thickness of basement membrane in small vessels are frequently seen in diabetics, and these changes are essentially based on the impairment of carbohydrate, lipid and protein metabolism¹⁵. Electron microscopic studies of small vessels of the skin¹⁶, kidney^{17,18}, and retina¹⁹ revealed a thickening of basement membrane at the vessel wall. These vascular lesions may play an important pathogenic role in diabetic complications such as myocardial infarction, diabetic nephropathy, and proliferative retinopathy²⁰. The increased basement membrane thickness, deposition of fibrils in basement membrane and swelling of

Sonuç: Periodontitisi olan kontrol altındaki Tip 2 DM'li hastalarda diyabeti olmayanlara oranla daha yoğun mikrovaskülerite olabileceği sonucuna varıldı.

endothelium have been found in gingival biopsies from patients with DM^{8,21-23}. It has been hypothesized that the above changes may impede oxygen diffusion, metabolite waste elimination, leukocyte migration, and diffusion of immune factors and thus contribute to increased prevalence and severity of periodontitis in diabetic patients³. However, contrary to the above suggestions, Listgarten et al²⁴ were not able to correlate the thickened basement membrane of the gingival blood vessels to either the presence or absence of diabetes.

It may be considered that the increased level of cytokines in diabetic patients, which lead to an increase in the number of blood vessels in gingival connective tissue, may be one of the pathologic factors, which is responsible for the occurrence of more frequent and severe periodontitis in DM²⁵. There are some studies concerned with gingival vascular changes in diabetic patients with periodontitis; however, not much data is available on the quantity of blood vessels in such cases^{7,8,21,23}. The purpose of this study was to compare blood vessels number in gingival connective tissue between diabetics and non-diabetics. Thus, gingival tissues were obtained from type 2 diabetics with/without periodontitis and systemically healthy persons with/without periodontitis in order to compare the number of blood vessels.

MATERIALS AND METHODS

Study population

A total of 88 subjects (45 females, 43 males) were included in the study and constituted the following four groups: 1) 24 patients with type 2 DM and periodontitis (DP), 14 females, 10 males ranging in age from 46 to 52 (mean age 49.66 ± 1.54); 2) 21 patients with type 2 DM and clinical periodontal health (DH), 13 females, 8 males ranging in age from 48 to 56 (mean age 52.42 ± 1.92); 3) 25 patients diagnosed with chronic periodontitis (CP), 8 females, 17 males ranging in age from 39 to 44 (mean age 42.12 ± 1.36); 4) 18 subjects with systemic and periodontal health (C), 10 females, 8 males ranging in age from 36 to 47 (mean age 42.00 ± 2.77). Type 2 DM patients included in the present study were referred by Department of Endocrinology, School of Medicine, Hacettepe University while periodontitis patients and periodontally healthy subjects were selected from Department of Periodontology, Faculty of Dentistry, Hacettepe University. The investigation was approved by University of Hacettepe Ethics Committee.

Following data were recorded for DP and DH group; duration of diabetes and treatment, medicines and regimens, presence of complications such as hypertension and chronic vascular diseases and smoking. Fasting blood glucose, combination of fasting blood glucose plus a 2-hour test after glucose loading (2-hour postprandial) and glycohemoglobin tests were performed to determine current metabolic conditions of DM patients. Based on the understanding that an effective metabolic control may lead to an improvement of many complications of the disease, special care was taken to ensure that DM patients had similar metabolic control (HbA1c values ≤ 7.5).

In the periodontitis and control groups, patients with hypertension, a familial history of diabetes and impaired glucose tolerance were excluded from the study. These subjects were classified as having normal glucose tolerance if 2 hours plasma glucose concentration is $<140\text{mg/dl}^6$.

The criteria in the establishment of chronic periodontitis and clinically healthy gingiva were based on clinical measurements and radiographic alveolar bone loss. Clinical measurements included probing depth (PD), clinical attachment level (CAL), gingival index (GI)²⁶, plaque index (PI)²⁷ and bleeding on probing (BOP) were recorded on all of patients teeth. However, only the measurements of teeth adjacent to biopsy sites were analyzed statistically.

Periodontitis groups (DP and CP) had at least two or more sites of presenting PD ≥ 5 mm, CAL ≥ 4 mm and radiographic alveolar bone loss $\geq 25\%$ in one quadrant site. In periodontitis groups, only sites with ≥ 5 mm PD associated with a GI score of 2 or 3 were selected for sampling of gingiva. Gingiva was defined as clinically healthy when PD was $\leq 3\text{mm}$, with a GI score of 0. Special care was also taken to ensure that there was no evidence of BOP and any signs of radiographic alveolar bone loss. In all groups, gingival samples were obtained from the maxillary canine and premolar area.

The patients included in this study had no history of any periodontal treatment and/or medication within previous three months and they had at least 14 teeth.

Histopathological and Immunohistochemical Evaluations

Attached gingiva was obtained by sharp dissection with a scalpel under local anesthesia without vasoconstriction agents. The injection site was kept as distant as possible from the biopsy region to minimize any influence of injection on the tissue vasculature. The samples were fixed in 10% formaldehyde for 12-48 hours, embedded in paraffin, sectioned at $5\text{ }\mu\text{m}$ thickness and stained with hematoxylin and eosin to grade the present inflammatory status and to evaluate microvascular density immunohistochemically. Inflammatory status was classified into three grades based on the intensity and cell type, as follows:

1. No inflammatory reaction or few inflammatory cells in the connective tissue.

2. Presence of inflammatory reaction and prominent polymorphonuclear (PMN) leukocytes.

3. Presence of widespread mononuclear inflammatory cells (Plasma cells, lymphocytes, etc.).

During the immunohistochemical study, after deparaffinization and rehydration, 5-micron sections were incubated with hydrogen peroxide and CD34 antibody was applied (1/25) (Neo-markers, Westinghouse Dr., Fremont, CA 94539 USA) for 1 hour. After treating with second antibody and streptavidin peroxidase, sections were colored with diaminobenzidine and stained with hematoxylin.

After the immunohistochemistry, two observers who were aware of the patients' medical status simultaneously evaluated the sections. Discordances between these two observers were resolved by discussing and recounting in order to obtain a high interrater reliability. For every section, the endothelial cells that had a positive reaction with CD34 and constituted a lumen were counted at 10 HPF (High Power Field), (Fig.1). If the sections were not large enough to be counted at 10 HPF, maximum area that can be counted was investigated.

Statistical Analysis

The comparisons of changes among the groups (DP,DH,CP,C) were tested by "Kruskal

Wallis Variance Analysis" instead of by "One-way ANOVA" because the variances were not homogenous (The homogeneity of variances was tested by "Levene Test", $p \leq 0.05$). According to the results of "Kruskal Wallis Variance Analysis", if the differences were significant, "Mann-Whitney U Test" with the Bonferroni correction was used for pairwise comparison of the groups. The differences of the number of blood vessels in second and third degree inflammation between DP and CP groups were determined by t-test for unpaired samples. "t-test for independent sample" was used to assess differences in blood vessel numbers in DP and DH groups in respect to the absence and presence of systemic complications, and in DP and CP groups in respect to gender.

RESULTS

Descriptive statistics regarding clinical measurements and number of blood vessels for all groups are shown in Table I. The CAL, PD, BOP and GI values were similar in DP and CP groups with the exception of PI value ($p \leq 0.05$). In DP and CP groups, CAL, PD, BOP, PI and GI values were significantly higher than that of DH and C groups ($p \leq 0.05$). The mean CAL, PD, BOP and GI values were also similar in DH and C groups with the exception of PI value ($p \leq 0.05$) (Fig. 2).

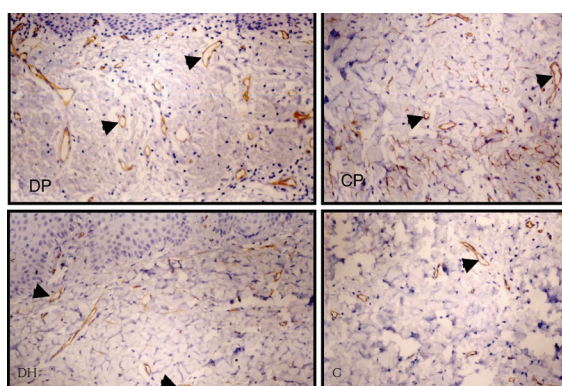


FIGURE 1

Photograph of blood vessels stained with CD34 in gingival connective tissue of DP group (original magnification X 40). DP, DM Patients with Periodontitis; DH, Periodontal Health; CP, Chronic Periodontitis; C, Systemic Health

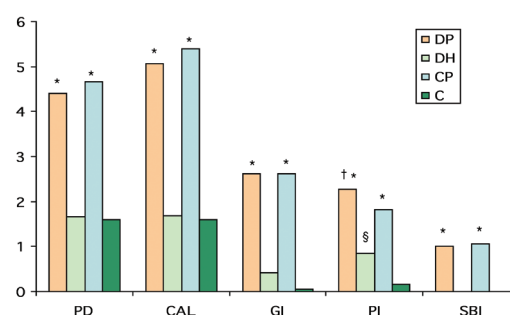


Figure 2. The comparisons of clinical parameters between groups.
* Significantly different from DH and C groups ($p \leq 0.05$)
† Significantly different from CP group ($p \leq 0.05$)
§ Significantly different from C group ($p \leq 0.05$)

FIGURE 2

The comparisons of clinical parameters between groups.
*Significantly different from DH and C groups ($p \leq 0.05$)
† Significantly different from CP group ($p \leq 0.05$)
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TABLE I

Clinical Parameters and Vessel Numbers of the Groups

Parameters	Groups	Arithmetic Mean ($\bar{x}$)	SD	SEM	Min.	Max.
Vessel Numbers	DP	16,148	3,319	0.677	14.746	17.549
	DH	10,314	2,028	0.442	9.391	11.238
	CP	13,509	4,131	0.826	11.803	15.214
	C	10,375	0.915	0.215	9.919	10.830
PI	DP	2,270	0.884	0.180	1.897	2.644
	DH	0.857	0.882	0.192	0.455	1.258
	CP	1,820	0.775	0.155	1.499	2.140
	C	0.166	0.297	0.007	0.189	0.314
GI	DP	2,625	0.536	0.109	2.398	2.851
	DH	0.428	0.426	0.093	0.234	0.622
	CP	2,620	0.389	0.078	2.459	2.780
	C	0.056	0.161	0.038	0.248	0.136
SBI	DP	1,000	0.000	0.000	1.000	1.000
	DH	0.000	0.000	0.000	0.000	0.000
	CP	1.060	0.410	0.083	0.888	1.231
	C	0.000	0.000	0.000	0.000	0.000
PD	DP	4,409	1,011	0.206	3.978	4.832
	DH	1,670	0.493	0.107	1.446	1.895
	CP	4,668	0.874	0.174	4.307	5.029
	C	1,599	0.310	0.073	1.444	1.753
CAL	DP	5,074	1,053	0.215	4.629	5.519
	DH	1,694	0.483	0.105	1.473	1.914
	CP	5,396	0.971	0.194	4.995	5.797
	C	1,599	0.310	0.073	1.444	1.753

SD, Standard Deviation; SEM, Standard Error of Mean; PI, Periodontal index; GI, Gingival index;

SBI, Sulcular bleeding index; PD, Pocket Depth; CAL, Clinical Attachment Level;

DP, DM Patients with Periodontitis; DH, Periodontal Health; CP, Chronic Periodontitis; C, Systemic Health

The differences in the number of blood vessels for all groups are shown in Table II. Blood vessels number in DP group was significantly higher than in those of DH, CP and C groups ($p \leq 0.05$) and it was also significantly higher in CP than in DH

and C groups ($p \leq 0.05$) (Fig. 3). No significant difference between DH and C groups was found in the number of blood vessels ($p > 0.05$).

When the grade of inflammation was concerned, there was not a significant difference between

TABLE II

*The Differences of Vessel Numbers For all Groups
(Mann Whitney-U test)*

	U	p
DP-CP	169.0	0.0090*
DP-C	3.0	0.0001*
DH-CP	159.0	0.0220*
CP-C	113.0	0.0060*
DP-DH	15.5	0.0001*
DH-C	161.0	0.4430

*Statistically significant

DP, DM Patients with Periodontitis; DH, Periodontal Health;
CP, Chronic Periodontitis; C, Systemic Health

TABLE III

*The Comparisons of Vessel Numbers Between
2nd and 3rd Grade Inflammation Within DP and CP Groups*

Groups	2nd grd. inf.	3rd grd. inf.	t	p
	x ± SEM	x ± SEM		
DP	15.599±0.941	16.896±1.061	0.918	0.369
CP	13.248±1.121	14.408±1.211	0.702	0.490
t	1591	1545		
p	0.12	0.138		

DP, DM Patients with Periodontitis; CP, Chronic Periodontitis

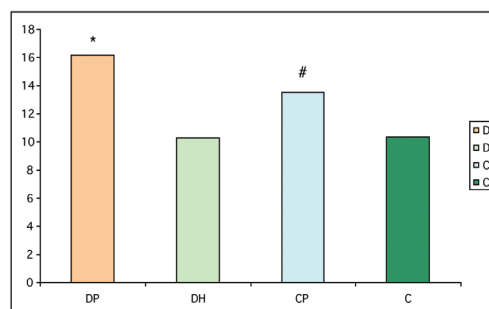


Figure 3. The comparisons of blood vessel numbers between groups.

* Significantly different from the others ($p \leq 0.05$)

Significantly different from DH and C groups ($p \leq 0.05$).

FIGURE 3

The comparisons of blood vessel numbers between groups.

*Significantly different from the others ($p \leq 0.05$)

Significantly different from DH and C groups ($p \leq 0.05$)

en the number of blood vessels of the patients with second and third grade inflammation in DP group ($p > 0.05$). When mean blood vessels number values of DP and CP groups with second and third grade inflammation were compared, no significant difference was found between the two groups ($p > 0.05$) (Table III).

In DP patients, no significant relationship was detected between the number of blood vessels and presence and absence of complications caused by DM (Table IV). In DP and CP groups, mean blood vessels number values between males and females were not significantly different.

TABLE IV

Comparison of Blood Vessel Numbers in DP and DH Groups Regarding the Absence (-) Presence (+) of Systemic Complications

Complication	N	Arithmetic Mean	SD	SEM	t	p
DP -	13	15,931	3,519	0.976	0.341	0.736
DP +	11	16,404	3,215	0.969		
DH -	9	10,289	2,002	0.667	0.049	0.962
DH +	12	10,334	2,136	0.616		

SD, Standard Deviation, SEM, Standard Error of Mean

DP, DM Patients with Periodontitis; DH, Periodontal Health

DISCUSSION

This study was performed to determine whether diabetic patients with periodontitis have different microvascular density in gingival connective tissues when compared to non-diabetics. The mean number of blood vessels in the gingival tissues of periodontitis patients with/without diabetes was higher than that of patients who had clinical periodontal health with/without diabetes. When the findings of type 2 DM patients with periodontitis were compared to the findings of chronic periodontitis patients, diabetics showed significantly higher number of blood vessels in the gingival connective tissues than chronic periodontitis patients.

The finding in CP seemed to agree with earlier reports suggesting that the number of blood vessels in gingival connective tissues of periodontal pockets is higher than clinically healthy periodontal tissues. Zoelner and Hunter²⁸ reported that vessel profiles in deep connective tissues of periodontal pockets were increased in number with the development of the advanced periodontal lesion and suggested that this increase may be due to the increase in the number and in loop formation of blood vessels. Similarly, Bonakdar et al.²⁹ showed an increase in vascularity in subepithelial connective tissue from chronic periodontitis lesions. They suggested that possible factors in this vascular change might be due to an increase in the number of blood vessels, in their length and formation of new vessels. Johnson et al.³⁰ assessed changes in gingival concentrations of VEGF during initiation and progression of inflammation and compared them to the changes in the number of blood vessel and concentration of IL-6 recognized markers of periodontal disease severity. They suggested that VEGF might be a factor in initiation and progression of gingivitis to periodontitis, possibly by initiating expansion of the vascular network.

Considering that the inflammation and the severity of inflammation in gingival tissues might affect the number of blood vessels, we com-

pared DP and CP groups with respect to severity of inflammation (2nd and 3rd grade inflammation in histological view) both within and between groups. We did not find significant differences between diabetics and non-diabetics with periodontitis and similar results were obtained within the groups.

In our study, however DP and CP groups have shown similar clinical indices except for PI, the number of blood vessels in DP was higher than in CP. The amount of dental plaque was higher in DH group than in C group, and there was no statistically significant difference between these groups in the number of blood vessels. The finding that DH and C groups had similar blood vessels number may be because of bacterial plaque in these patients did not have sufficient pathologic contents and quantity causing inflammatory reactions in gingival tissues of diabetic patients. The potential of dental plaque to initiate periodontal disease depends on its contents and quality rather than its width or apico-occlusal dimension. However, the increased glucose level in the crevicular fluid of diabetic patients could change; the environment and quality of bacterial plaque that could account for the severity of periodontitis in diabetic patients.³¹ Several studies have focused on whether the dental plaque contents in diabetics are different from that of non-diabetics. Mandell et al.³² have found that bacterial profiles in the diseased sites of diabetics were similar to those of healthy adults with periodontal disease. Other authors have reported higher levels of specific microorganisms in juvenile diabetics.³³ PI scores may not be the only sufficient and dependable factor in explaining increased blood vessels number in DP because plaque index only expresses dental plaque quantity but it does not concern itself with the duration of plaque accumulation and pathogenicity of microorganisms. In addition to dental plaque, metabolic changes seen in diabetes may have affected vessels of gingival connective tissues, by various mechanisms. In our study, we did not use any parameters associated with these various mechanisms. For

this reason, we cannot suggest possible factors affecting the vasculature of gingival connective tissues in DP. Several studies have demonstrated a significant increase in capillary basal lamina in gingival connective tissue of diabetic patients as compared to that of non-diabetics and they have suggested that thickened capillary basal lamina may lead to insufficient oxygen diffusion and may impede metabolic waste elimination, resulting in physiologic imbalance and increasing the severity of periodontal disease^{3,21,22,34}. On the other hand, if there is a progressive narrowing of the lumen of a vessel, impaired and a delicate point of equilibrium of blood supply may emerge and there will be a need to establish collateral supply. Minchenko et al.³⁵ have suggested that hypoxia is a potent inducer and also a stimulator of VEGF expression in vivo. These factors may be responsible for increased number of vessels in diabetic patients with periodontitis.

Chan et al.⁷ suggested that diabetes and hypertensive cardiovascular disease affect the gingival vascular bed. This may lead to interferences with local blood supply, and thus be partially responsible for the increased periodontal disease. They did not mention periodontal status of patients in their study. In our study, we compared microvascular density of DP groups with hypertension and cardiovascular disease to ones without those complications and did not find any significant differences in the number of blood vessels between the groups.

In conclusion, the present study showed that well controlled diabetic patients with periodontitis had statistically increased number of vessels in gingival connective tissues than non-diabetic patients with periodontitis. Microvascular changes may be one of the early factors in the etiology of gingivitis or its progression to periodontitis, possibly by transporting proinflammatory cells and increasing endothelial surface, resulting in the production of cytokines and other progression factors for inflammation. Further studies are necessary on vascular changes in diabetic patients and antiangiogenic agents in prevention and treatment of periodontal diseases seen in diabetes.

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