ORIGINAL ARTICLE

The role of neoangiogenesis and vascular endothelial growth factor in the development of carpal tunnel syndrome in patients with diabetes

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Abstract

Objective: Carpal tunnel syndrome (CTS) is an entrapment neuropathy which is caused by the disruption of blood supply in the median nerve under transverse carpal ligament. Systemic factors facilitate the formation of the syndrome. In this study, neovascularization in the subsynovial tissue and proliferative activity in the stroma are analyzed within the cases of diabetic and idiopathic CTS.

Materials and Methods: Subsynovial connective tissue samples of 30 diabetes mellitus patients with CTS and 30 patients with idiopathic CTS were evaluated. Vascular endothelial growth factor (VEGF), CD31, CD34, Factor VIII-related antigen, and smooth muscle actin (SMA) was used to make a comparative study of neovascularization. Proliferative index was assessed using anti-Ki-67 antibody.

Results: As a result of the proliferation of endothelial elements, *de novo* blood vessel formations in the subsynovial tissue were assessed by vascular markers. Significant neovascularization was seen in diabetic group for VEGF, CD31, SMA ($P < 0.01$); and for CD34 ($P < 0.05$) when compared with idiopathic CTS group. In addition, more intense positive staining for CD34, SMA ($P < 0.01$); and for VEGF ($P < 0.05$) was found at isolated stromal cells of diabetic CTS group against idiopathic CTS group. Significantly high proliferative index in subsynovial connective tissue with Ki-67 was observed the diabetic group ($P < 0.01$).

Conclusion: VEGF expression has an importance within CTS pathogenesis. Increased ischemia-reperfusion damage, neoangiogenesis, and VEGF expression has an important role frequently CTS occurrence in diabetic patients. Our study supports enhancement in VEGF expression similar to changes in diabetic nephropathy and retinopathy in the neovascularization within the subsynovial connective tissue in the cases of diabetes.

Key words: Carpal tunnel syndrome, diabetes mellitus, ischemia-reperfusion, neoangiogenesis, vascular endothelial growth factor

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Introduction

Carpal tunnel syndrome (CTS) is the most common entrapment neuropathy.[1,2] It occurs on the palmar side

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of the wrist where the median nerve becomes compressed in the carpal tunnel. CTS symptoms include pain in the hand, loss of strength, atrophy of the hand muscles, and dysesthesia.[3]

Increased pressure within the carpal tunnel was suspected of CTS and it depends either on compression or on the volume increase.[4-6] However, the pathophysiology of CTS has not been fully understood. The most common histomorphological change in CTS is noninflammatory fibrosis in subsynovial connective tissue surrounding flexor tendons.[1,2,5,7] Thickening of the tendon sheet and vessel wall, intimal hyperplasia, edema, thrombosis are other histomorphological changes.[1,2,8] Recently, in the etiology of CTS the focus has been ischemia-reperfusion damage according to vascular changes in subsynovial connective tissue.[2,4,9]

Neovascularization in subsynovial connective tissue has been presented using vascular markers such as vascular endothelial growth factor (VEGF). VEGF is not only a regulator of angiogenesis but also a major contributor to the formation of CTS by increasing vascular permeability.[1,10]

CTS is often seen as idiopathic, systemic causes also facilitate the compression.[11] Diabetes mellitus (DM), rheumatoid arthritis, myxedema, acromegaly, and amyloidosis are just a few of the diseases that lead to CTS.[11] DM patients constitute a large proportion of CTS patients.

The effect of DM on nerve metabolism is not the only cause for CTS. We hypothesized that ischemic damage and neoangiogenesis in patients with diabetes may be more explicit and diabetes may facilitate the formation of CTS by accelerating histomorphological changes in subsynovial connective tissue.

In our study, we analyzed neovascularization in the subsynovial tissue and proliferative activity in the stroma and presented within the cases of diabetic and idiopathic CTS.

Materials and Methods

This study designed as Level 3 retrospective cohort study.

Subsynovial connective tissue samples of 30 DM patients and 30 other patients with idiopathic CTS, who were operated on for CTS between the years of 2011 and 2012, were evaluated retrospectively. The informed consent form for this surgical procedure was obtained from all of the patients included in the study. Since, this was a retrospective study IRB approval was not necessary for our institution. Archival tissue blocks were retrieved and stained immunohistochemically for this study.

To confirm the diagnosis of CTS in patients, stories of paresthesia along with median nerve trace, hand pain during the day or night were taken into consideration. In addition, positive findings that include sensibility and provocative tests during the physical examination were used to confirm CTS.

According to the evaluation results of blood biochemistry, patients whose hemoglobin A1c results are above 7% and with the diagnosis of DM at least 5 years before, were included to study.

On all patients with suspected CTS, electromyography was performed. The majority of patients, who were diagnosed with CTS as a result of physical examination mentioned above and laboratory findings, had received splints, nonsteroidal anti-inflammatory drugs, Vitamin B12 treatment. Despite this treatment, patients with ongoing complaints were operated. Standard open carpal tunnel release was performed by a single surgeon. In peripheral nerve surgery, patients were operated under local anesthesia with the minimally invasive technique. On the palmar side of the hand, 1 cm vertical incision from the wrist bend to fit the imaginary line between the middle finger and ring finger was made. With the help of retractor, a broader view of the proximal and distal directions was achieved by using the elasticity of the skin. Skin incision and subcutaneous incision were made respectively. Having seen the transverse carpal ligament colored bright pearl, median nerve was exposed by cutting about 3–5 cm from proximal to distal. About 0.5 mm^3 subsynovial connective tissue was excised from each case. About 1 cc saline solution was given to perineurium to decrease the hydrostatic pressure. Thus, the carpal tunnel was decompressed.

Connective tissue samples within 10% buffered formalin were immediately sent to histopathology laboratory. Paraffin-embedded blocks were prepared from tissue samples and 4 μ-thick sections were taken. Standard hematoxylin and eosin (H and E) staining was performed. The procedure was performed at room temperature.

We used VEGF, CD31, CD34, Factor VIII related antigen, smooth muscle actin (SMA) as vascular markers to make a comparative study of ischemic changes and neovascularization in subsynovial connective tissue. Furthermore, Ki-67 was used to compare proliferative index in subsynovial connective tissue in both patient groups. Immunohistochemical staining was performed for VEGF, CD31, CD34, Factor VIII related antigen, anti-SMA. Proliferative index was assessed using the anti-Ki-67 antibody. For immunohistochemical studies, 4 μ-thick sections were used on the positively charged slides. Sections were deparaffinized with xylene in the oven at 60°C. Then using descending concentrations of ethanol, examples

were rehydrated (absolute alcohol–alcohol - 96% to 90% alcohol - 70% alcohol).

Preparations were cleaned under running tap water and distilled water. Then antigen retrieval was applied. For antigen retrieval, citrate buffer (pH 6) was applied to preparations for 5 min under pressure. Respectively, hydrogen peroxide, phosphate buffered saline (PBS pH 7.4), ultra V block after the primary antibody solution were applied.

Preparations were kept in primary antibody for CD31 for 2 h and for CD34, Ki67, Factor VIII-related antigen, VEGF, α-SMA for an hour.

Slides, which were kept, respectively in PBS, amplifier, PBS, horseradish peroxidase polymer, PBS, 3-amino 9-ethylcarbazole chromogen, running tap water, hematoxylin dye, running tap water and distilled water, were dried at room temperature and closed by lamella.

In all of the immunohistochemical studies, micro polymer system kit was used. Negative control for immunohistochemistry was processed without the primary antibody. Olympus CX41 light microscope was used in the assessment of H and E stained preparations and immunohistochemical studies and photo shooting. In the histomorphological examination of H and E stained preparations, studies were performed in eight different and randomly selected areas in (20X lens).[2] VEGF, CD31, CD34, α-smooth muscle actin, Factor VIII-related antigen evaluation, again studies were performed in eight different areas randomly selected by means of 20X lens. In all these areas, staining intensity was classified as follows:

- Grade 0: Not stained
- Grade 1: Mildly stained
- Grade 2: Moderately stained
- Grade 3: Intensely stained.[5]

Immunohistochemical assessment of VEGF, CD34, CD31, SMA was performed separately for the vessel walls and isolated stromal cells. Factor VIII-related antigen is positive only in the vessel walls and assessment was made for the vessel walls.

To assess the proliferative index with Ki-67, 1000 nuclei were counted in each biopsy specimen and the proliferative index was evaluated as the number of positive reacting cells out of 100 cell nucleus (%).[1]

Mann–Whitney U-test was used to compare immunohistochemical parameters of diabetic and idiopathic CTS groups.

Results

In the majority of tissue samples taken from both patient groups, noninflammatory fibrosis was observed in subsynovial connective tissue when the general histomorphological evaluation was made using H and E stained sections. Tissue edema was seen in varying degrees. Vascular proliferation, vascular hypertrophy, and intimal thickening were observed. When tissue samples belonging to both groups of patients was compared, histomorphological changes including vascular changes, tissue edema and fibrosis were found to be more intense in the cases of patients with diabetic CTS [Figure 1].

As a result of the proliferation of endothelial elements, *de novo* blood vessel formations in the subsynovial tissue were assessed by vascular markers. In both biopsy groups, positive staining was observed in vascular endothelial and isolated stromal cells through VEGF, CD31, CD34, and SMA.

Factor VIII-related antigen was positive in the endothelial cells of neoformed vessels. No reaction was observed in isolated stromal cells for Factor VIII-related antigen.

In the cases of diabetic CTS and idiopathic CTS, neovascularization compared to immunohistochemical markers;

Immunohistochemical staining was detected in endothelial cells for VEGF, CD31, CD34, and SMA. Significant neovascularization was seen in diabetic group for these four markers compared with idiopathic CTS group ($P < 0.01$ for VEGF, CD31, and SMA), ($P < 0.05$ for CD34).

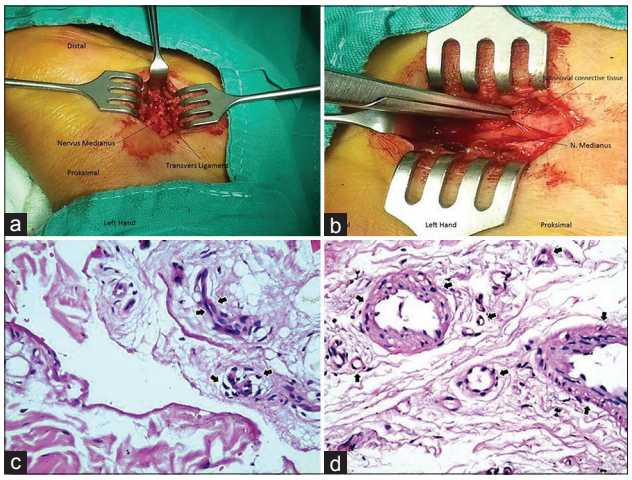

Figure 1: (a) Operating image, (b) operating image closely appearance, (c) idiopathic carpal tunnel syndrome, relatively mild vascular changes (H and E, ×400), (d) diabetic carpal tunnel syndrome, severe vascular changes, vascular hypertrophy, intimal thickening in the subsinovyal tissue (H and E, ×400)

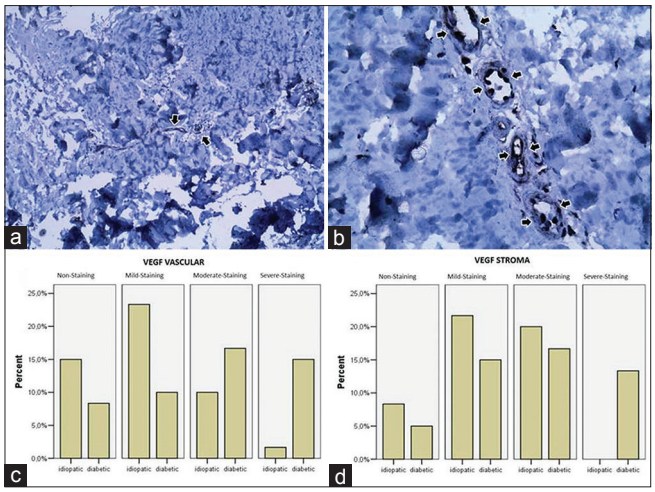

Figure 2: (a) Idiopathic carpal tunnel syndrome, neoformational vascular structures and small number of stromal cells around the vascular structures positive for the vascular endothelial growth factor. Vascular endothelial growth factor, ×10, (b) diabetic carpal tunnel syndrome, a great number of neoformational vascular structures positive for vascular endothelial growth factor. Vascular endothelial growth factor, ×10, (c) vascular endothelial growth factor vascular reaction, (d) vascular endothelial growth factor stromal reaction

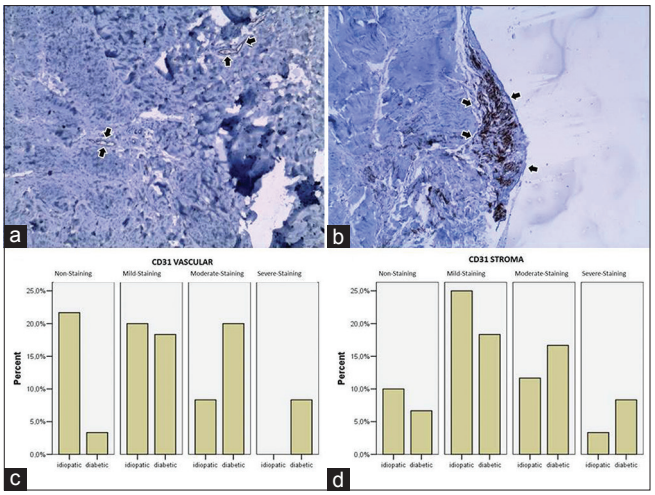

Figure 3: (a) Idiopathic carpal tunnel syndrome, a small number of vascular structures is seen with CD31. CD31, ×10, (b) diabetic carpal tunnel syndrome, positive staining for CD31 in intensively proliferating vessels and a small amount of stromal cells. CD31, ×10, (c) CD31 vascular reaction, (d) CD31 stromal reaction

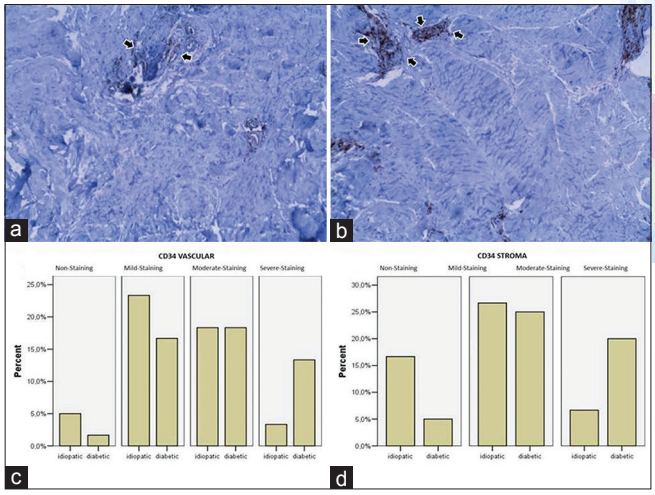

Figure 4: (a) Idiopathic carpal tunnel syndrome, relatively a small number of vessels is seen in the subsinovyal connective tissue with CD34. CD34, ×10, (b) diabetic carpal tunnel syndrome, increased number of vascular structures in the subsinovyal connective tissue with CD34. CD34, ×10, (c) CD34 vascular reaction, (d) CD34 stromal reaction

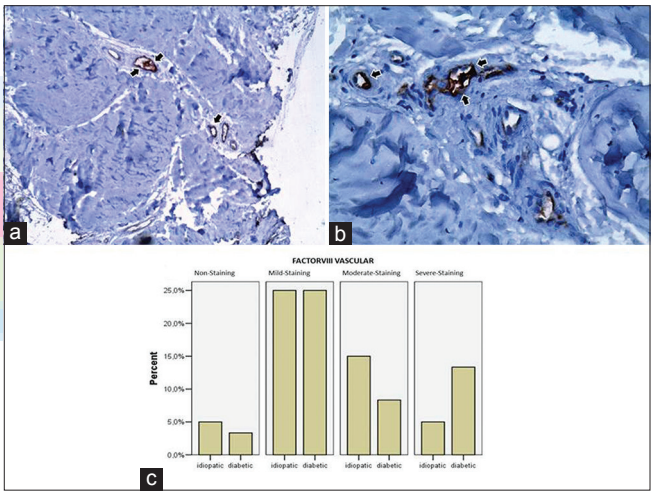

Figure 5: (a) Idiopathic carpal tunnel syndrome, a few number of vascular structures are seen with Factor VIII-related antigen. Factor VIII-related antigen, ×10, (b) diabetic carpal tunnel syndrome, neoangiogenesis, positive staining for Factor VIII-related antigen in the increased number of vascular structures. Factor VIII-related antigen, ×40, (c) Factor VIII vascular reaction

More intense positive staining for VEGF, CD34, SMA was found at isolated stromal cells of diabetic CTS group against idiopathic CTS group with immunohistochemical assessment ($P < 0.05$ for VEGF), ($P < 0.01$ for CD34 and SMA).

There was not statistically significant difference regarding neovascularization between diabetic and idiopathic CTS groups at immunohistochemical staining for Factor VIII ($P > 0.05$).

There was not statistically significant difference regarding immunohistochemical staining intensity for CD31 at isolated stromal cells between diabetic and idiopathic CTS groups ($P > 0.05$).

Considering the overall results, neovascularization in subsynovial connective tissue is observed significantly more intense in the cases of diabetic CTS. Graphic of percentages of vascular and stromal changes are [Figures 2-6]. VEGF expression (grades) in diabetic and idiopathic CTS cases is shown in Table 1.

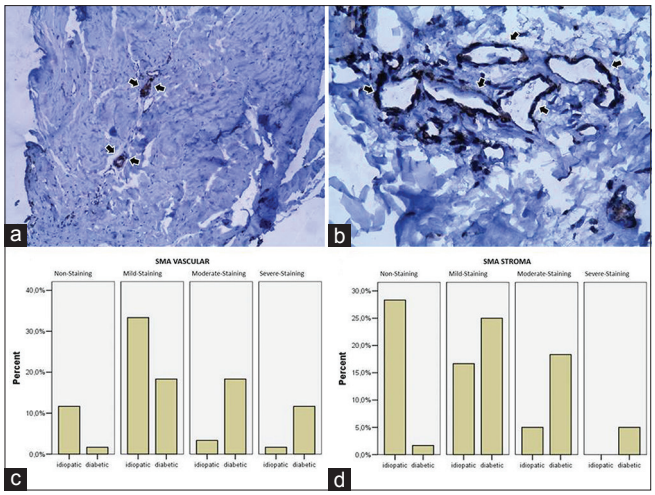

Figure 6: (a) Idiopathic carpal tunnel syndrome, relatively a small number of vessels is seen in the subsinovyal connective tissue with smooth muscle actin. Smooth muscle actin, ×10, (b) diabetic carpal tunnel syndrome, the positive reaction for smooth muscle actin is seen vascular walls and endothelial cells in the stroma. Smooth muscle actin, ×40, (c) smooth muscle actin vascular reaction, (d) smooth muscle actin stromal reaction

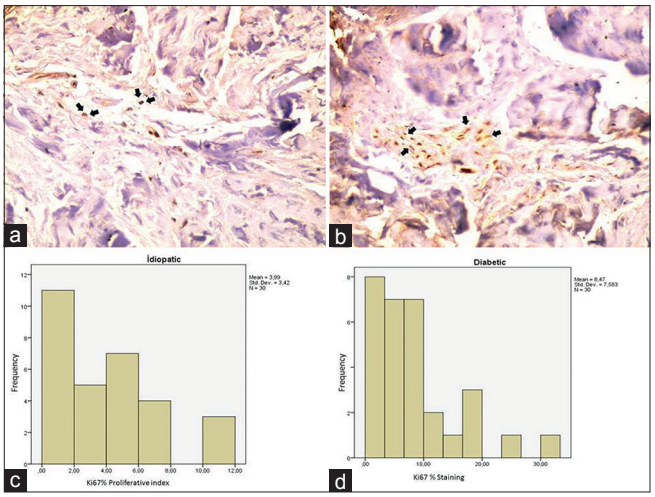

Figure 7: (a) idiopathic carpal tunnel syndrome, approximately 6% proliferative index is seen in the subsinovyal connective tissue for Ki-67. Ki-67, ×200, (b) diabetic carpal tunnel syndrome, approximately 15% proliferative index is seen in the subsinovyal connective tissue for Ki-67. Ki-67, ×200, (c) Ki-67% staining diabetic, (d) Ki-67% proliferative index idiopathic

Table 1: VEGF expression

Grades	Diabetik CTS				Idiopatic CTS			
	VEGF-vascular component		VEGF-stromal component		VEGF-vascular component		VEGF-stromal component	
	Cases	%	Cases	%	Cases	%	Cases	%
0	5	17	3	10	9	30	5	17
1	6	20	9	30	14	47	13	43
2	10	33	10	33	6	20	12	40
3	9	30	18	27	1	3	0	0

VEGF=Vascular endothelial growth factor; CTS=Carpal tunnel syndrome

That no statistically significant results were obtained in Factor VIII-related antigen with vascular component and CD31 with stromal cells may be explained as different immunohistochemical markers have different sensitivities to the newly formed vasculature.

While proliferative index in the diabetic group ranged between 32.4% and 0.4% in subsynovial connective tissue cells with Ki-67, the proliferative index in the idiopathic CTS group with Ki-67 ranged between 11.8% and 0.4%.

Significantly high proliferative index in subsynovial connective tissue with Ki-67 was observed the diabetic group compared to idiopathic CTS group ($P < 0.01$) [Figure 7].

Discussion

CTS develops as a result of compression and degeneration on the median nerve.[4,11] Extraneural and intraneural blood flow, axonal transport and conductivity on the median nerve deteriorate progressively.[4] It has been long discussed that the histological changes in CTS are pathognomonic.[12-20] Most of them show that severe changes in the small arteries (vascular hypertrophy and intimal thickening) and connective tissue (noninflammatory fibrosis) are seen in CTS.[1,3,6] They focus on neoangiogenesis through ischemia-reperfusion injury triggered by subsynovial fibrosis in etiology of CTS.[1,2,4-6,9,11,21] Formation of new vessel structures in the ischemic tissue is to achieve a new circulation model which is tried to prevent ischemic damage. However, the new vessel structures have pathological character and cannot prevent ischemia. Thus, the ineffective neoangiogenesis continues to progress.[1]

Angiogenesis is a multi-step process which involves many growth factors and cytokines (tumor necrosis factor-alpha, transforming Growth factor-beta, alpha fibroblast growth factor, beta fibroblast growth factor, interleukin6, VEGF).[4,21]

The probable source of endothelialization is the migration of endothelial precursor cells (EPCs).[4] EPCs originate from hemangioblast precursors in the bone marrow and positive for CD31 and CD34.[22] CD31 and CD34 are positive in endothelial-origin cells in vascular structures and stroma of subsynovial tissue in CTS[1] which supports neovascularization in subsynovial tissue. More intense staining with both markers in diabetic CTS cases indicates that neovascularization is more apparent in diabetic cases.

VEGF is one of the cytokines which stimulates EPCs.[21] Pericytes are cells that regulate angiogenesis, provide vessel

stability, control endothelial proliferation and express VEGF.[23,24] VEGF expression is held responsible for neoangiogenesis through ischemia-reperfusion in the subsynovial connective tissue in CTS.[10,25] VEGF regulates angiogenesis and also contributes to the development of symptoms in CTS by increasing vascular permeability.[10]

In both diabetic and idiopathic CTS group, we identified significant neovascularization with subsynovial connective tissue vascular markers (CD31, CD34, SMA, Factor VIII, VEGF).

In the literature, we came across different studies about ischemia-reperfusion damage in DM cases, neoangiogenesis in ischemic tissue and the role of VEGF in neoangiogenesis. Some of them state the idea that compensatory microvascularity is insufficient as a response to ischemia.[22,26-28]

However, Yang *et al*.[29] lay emphasis on the role of VEGF expression enhancement in the pathogenesis of diabetic retinopathy and neovascularization, which develops in the diabetic retinopathy. They also stated that hyperglycemia, advanced glycation end products, and oxidative stress may enhance VEGF in diabetic cases.[29] Donato *et al*. stated that anti-VEGF medicine recommended in many different pathological conditions.[1] Zorena *et al*. recommended anti-VEGF medicine in diabetic retinopathy.[30]

In the early stages of diabetic nephropathy, enhancement in VEGF expression causes abnormal angiogenesis and mesangial expansion. As the disease progresses, the level of VEGF decreases resulting in loss of endothelial cells and capillary rarefaction.[31]

VEGF expression in ischemia-reperfusion damage in diabetes patients varies according to the stage of the disease and the anatomical localization affected by ischemia. In this regard, further molecular and immunohistochemical studies are essential.

We have not seen a study regarding the histomorphological changes in subsynovial connective tissue in diabetic CTS. However, based on our results, there was a statistically significant increase in neoangiogenesis in subsynovial connective tissue in diabetic CTS cases. VEGF expression was also significantly enhanced in diabetic patients. Ki-67 and the proliferative index are also significantly high in diabetic CTS cases. We thought that neovascularization and tissue proliferation, which developed as a response to hyperglycemia and metabolic reasons in ischemic diabetic cases, are rapid. Our study supports enhancement in VEGF expression similar to changes in diabetic nephropathy and retinopathy in the neovascularization within the subsynovial connective tissue in the cases of diabetes.

It has been stated in the literature that noninflammatory fibrosis and vascular proliferation in subsynovial connective tissue, vascular hypertrophy, intimal thickening was seen at idiopathic CTS cases.[1,2,5,10]

Donato *et al*. showed VEGF, CD31, CD34, α-SMA, and Factor VIII-related antigen expression at the subsynovial connective tissue and isolated stromal cells in idiopathic CTS.[1]

Hirata *et al*. indicated VEGF expression at subsynovial connective tissue in idiopathic CTS cases.[10]

However, none of them mentioned about diabetic CTS cases. We found similar immunohistochemically and histomorphologic findings with them in both diabetic and idiopathic CTS groups. Stromal fibrosis and vascular changes are more distinct in diabetic CTS group. Furthermore, VEGF expression was found more intense in diabetic CTS group against idiopathic CTS group.

Donato *et al*. found proliferative index as 0.5–10.0% with Ki-67 at subsynovial connective tissue in idiopathic CTS cases.[1] They did not evaluate proliferative index in diabetic CTS cases.[1] We found proliferative index as 0.4–11.8% with Ki-67 in idiopathic CTS cases and 0.4–32.4% in diabetic CTS group.

In CTS cases, vascular proliferation due to ischemia-reperfusion and neovascularization in the subsynovial connective tissue has impacts on the formation of symptoms related to CTS. In diabetic cases, neovascularization through ischemia-reperfusion is found to be more apparent compared to idiopathic cases. VEGF expression in subsynovial connective tissue is more intense in diabetic cases. We thought that anti-VEGF medicine, recommended recently to treat different diseases and to decelerate the histomorphological changes in subsynovial connective tissue in the cases of idiopathic CTS, has more promising results in diabetic cases. However, further study is needed on this issue. We also think that regulating blood glucose in DM may decelerate histomorphological and vascular changes in subsynovial connective tissue and accordingly the development of clinical complaints related to CTS.

Conclusion

Vascular proliferation due to ischemia-reperfusion damage and VEGF expression play significant role in CTS pathogenesis. Increased ischemia-reperfusion damage, neovascularisation, VEGF expression are seen more frequently in diabetic CTS cases compared with idiopathic CTS cases. Our study showed increased VEGF expression and neovascularisation within the subsynovial connective tissue in the cases of diabetes similar with changes in diabetic nephropathy and retinopathy.

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Conflicts of interest

There are no conflicts of interest.

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