Histochemical and immunohistochemical investigation of the effects of Sambucus nigra on mast cells and VEGF in diabetic rat spleen

Research Article

Elif Tuğba Ergel[1a]
Tuğrul Ertuğrul[2b]

[1]Samsun Bafra State Hospital, Samsun, Türkiye
[2]Department of Histology and Embryology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Türkiye

ORCİD-
[a]0000-0001-6734-4834
[b]0000-0002-9310-1200

Correspondence
Tuğrul ERTUĞRUL
tugrulertugrul06@hotmail.com

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ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to disturbed insulin secretion or insulin function. The purpose of this study was to look into the effect of Sambucus nigra (S. nigra) extract on mast cell number and immunohistochemical expression of VEGF-immune positive cells in experimental diabetic rat spleen. Thirty-two male were used in this study. Control group, Diabetes group, S. nigra group and Diabetes + S. nigra group. When the groups were evaluated, the least number of mast cells (MC) was detected in the control group, whereas the highest number of MCs was observed in the diabetes group. The MC numbers in the S. nigra and diabetes + S. nigra groups were close. The application of S. nigra to diabetic rats resulted in a considerable reduction in the number of MCs, which was a notable finding in our investigation. The number of immune positive VEGF cells increased only in the diabetes group when the groups were examined individually. VEGF expression was similar in the control group, S. nigra group, and diabetes + S. nigra group. As a remarkable finding in our study, it was observed that the application of S. nigra caused a decrease in the number of immune-positive VEGF cells in diabetic rats. As a result, this study showed that S. nigra caused a decrease in the number of MC and immune-positive VEGF cells that increase with diabetes. These findings suggest that MCs may be both a source and a target of VEGFs.

Keywords: Diabetes, mast cell, Sambucus nigra, VEGF

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to disturbed insulin secretion or insulin function (Darenskaya et al., 2021). It has been reported that the development of chronic hyperglycemia in diabetes is accompanied by damage, dysfunction, and failure of various organs and tissues, and the development of micro and macrovascular complications (Harding et al., 2019).

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that plays important role in both normal physiological as well as pathological vasculogenesis and angiogenesis (Leung et al., 1989). It is known to help preserve tissue barrier functions, as well as neuroprotection, endothelial cell proliferation, and migration. Furthermore, VEGF receptors are located on various cells and govern cell survival and physiological function (Huang et al., 2012). Additionally, experimental evidence shows that VEGF plays a central role in mediating diabetes-induced disorders in many organs. It has also been reported that it may cause diabetic complications by affecting glucose levels simultaneously (De Vriese et al., 2001).

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Mast cells (MCs) are multifunctional immune cells that play a role in many different health and disease states. The local microenvironment has a direct impact on MC maturation and function. They are known to be able to perceive and respond to different stimuli by releasing a physiologically active sequence mediator (Galli et al., 2011). MCs might be activated by pattern-recognition receptors or tissue injury, and they can express the FcR1 and Fc receptors, allowing them to respond to adaptive immune system targets (Dwyer et al., 2016). Furthermore, MCs are a significant source of cytokines, chemokines, and growth factors. It has been known that VEGF is present in the cytoplasmic granules of MCs and that these cells can secrete VEGF both on their own and in response to activation (McHale et al., 2019). MCs are thought to be important cells that can function in pro- or anti-inflammatory roles in various immune processes such as pathogen clearance and autoimmune diseases (Kumar & Sharma, 2010). Also, MCs are known to play essential roles in preventing obesity and diabetes in experimental animal models (Sismanopoulos et al., 2012).

Natural products are known to provide a numerous supply of bioactive substances that can be employed in medicine. Many plants are used medicinally to prevent and delay illness development and progression, increase health span, and improve quality of life (Yang et al., 2020). Sambucus nigra (S. nigra) is used in traditional medicine because of its rich chemical composition, which contains many bioactive components, including vitamins, minerals, terpenes, sterols, and polyphenols (Ferreira et al., 2020). S. nigra has been also shown to contain water-soluble chemicals that promote intramuscular glucose absorption and utilization and activate insulin release (Gray et al., 2000).

Although there are studies describing the relationship between MCs and VEGF cytokine, there are few studies on the effect of S. nigra on MC distribution and VEGF expression in diabetic spleen tissue. This study aimed to investigate the effects of S. nigra on MC and VEGF immune-positive cell distribution in rat spleen in a model of STZ-induced diabetes.

MATERIAL and METHOD

Animal material

Thirty-two male rats weighing 250-300 g were used in this study. The rats were housed in conventional cages with 12 hours of light and 12 hours of darkness in a 21-23°C ambient temperature environment, and they were fed tap water and ad libitum food. The rats used in the study were randomly divided into four groups equal in number. Experimental groups: Group 1: Control group, Group 2: Diabetes group, Group 3: S. nigra group and Group 4: Diabetes + S. nigra group.

In our study, to form a diabetes model in animals in diabetes and diabetes + S. nigra group, animals received a single dose intraperitoneal (i.p.) injection of streptozotocin (STZ; 50 mg/kg). It was prepared by dissolving 450 mg of STZ (SO130; Sigma-Aldrich, USA) in 10 mL of distilled water (Çetin et al., 2013). After confirming the occurrence of diabetes in rats, S. nigra at a dose of 1.5 mg/kg (Bidian et al., 2021) was administered by oral gavage to all animals in the S. nigra group and diabetes + S. nigra group for 14 days. During the experiment, no application was made to the rats in the control group.

Following the experiment, the rats were sacrificed, and spleen tissue samples were taken. The spleen tissue samples were fixed in a 10% formaldehyde solution, then passed through standard histological tissue processing and blocked in paraffin.

Determination of blood glucose levels

A glucometer (PlusMED Accuro) was used to take blood from the hungry animals' tail vein 8 hours before the start of the trial to determine

their blood glucose level preprandial. Animals involved in the study with a glucose level of 300 mg/dL had their preprandial blood glucose level measured for 8 hours on the 3rd day of STZ practice.

Mast cell histochemistry

To count the MCs, ten serial cross-sections of 5 µm thickness were obtained from the prepared blocks at 30 µm intervals and stained with toluidine blue (92-31-9; Sigma-Aldrich, %0,5 ve pH=0.5) (Enerback, 1966). To determine the numerical distribution of MCs in the prepared serial crosssections, cell counts were performed with a 100 square ocular micrometer. At a magnification of 40x, the MCs in the ocular graticule were counted in per unit. For each piece of spleen tissue, the cells were counted in ten randomly selected regions. All these data were then converted to the number of MCs per 1 mm^2 unit area.

Immunohistochemical staining

To determine the expression of VEGF in 5 µm thick tissue sections taken from spleen tissue, one of the immunohistochemical methods, the "streptavidin-biotin-complex method," was used (True, 1990). The primary antibody utilized in immunohistochemistry was mouse monoclonal VEGF (1/800 dilution, Santa Cruz Biotechnology, sc-7269). Antibody diluent reagent solution was used for reconstitution (Zymed 00-3118). As a secondary antibody, the Histostain® Plus kit was employed (Zymed kit: 85-6743). To reveal the antigen in the tissues, after the deparaffinization process, the sections were taken into a citrate buffer solution and heated in a microwave oven at 700 watts. The same procedure was performed three times, each for 5 minutes. At the end of the process, the sections in the citrate buffer solution were left to cool at room temperature for 20 minutes. Sections washed with Phosphate Buffered Saline (PBS) were incubated in 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity. After the tissues removed from the PBS solution were thoroughly dried, the serum in the kit was dripped onto them to prevent non-specific protein binding. The blocking solution of the Histostain® Plus kit was used as protein blocking solution. The primary antibody was then dripped over the sections, which were then stored at +4 °C overnight. Only PBS solution was used in negative control group tissues. After washing, the sections were dripped with biotinylated secondary antibody and then incubated in streptavidin-horseradish peroxidase complex. In the last step, 3,3'-diaminobenzidine (DAB) was used as the chromogen (Zymed, 31079800) and the preparations were counterstained with hematoxylin and sealed with enthallan.

Immunohistochemical examination

In the immunohistochemical examination, the distribution of VEGF-positive cells was analyzed semiquantitatively. The following criteria were used in the evaluation: no stained cells in the scanned area no positive cell (–), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++) (Ertuğrul et al., 2021).

Statistical analysis

The Shapiro-Wilk test was employed to determine normality. One-way analysis of variance (ANOVA) was performed to assess the data depending on the normality of the data, and Duncan's test was employed to identify any differences within groups. Obtained data are shown as mean ± standard error of the mean (mean ± SEM). A p-value of <0.05 was considered statistically significant

RESULTS

Histochemical findings

MCs were metachromatically stained with toluidine blue in the spleen tissue of all groups, and their granules could not be distinguished individually. Morphological differences were not observed among the MCs in the whole

group, and cells were determined to be round and oval with different sizes. When the location of MCs in the spleen tissue is examined, they are rarely observed in the subcapsular region. It was remarkable that MCs were localized in the red pulp sinusoids. MCs were rarely found in the lymph follicles of the white pulp in our study. Moreover, MCs were not seen in the splenic cords. While MCs, which were found individually in the white pulp, were predominantly seen in the red pulp, either alone or groups.

When the groups were evaluated, the least number of MCs was detected in the control group, whereas the highest number of MCs was observed in the diabetes group. The MC numbers in the S. nigra and diabetes + S. nigra groups were close. The application of S. nigra to diabetic rats resulted in a considerable reduction in the number of MCs, which was a notable finding in our investigation. Table 1 shows the mean number of MCs in each group after staining toluidine blue (Figure 1).

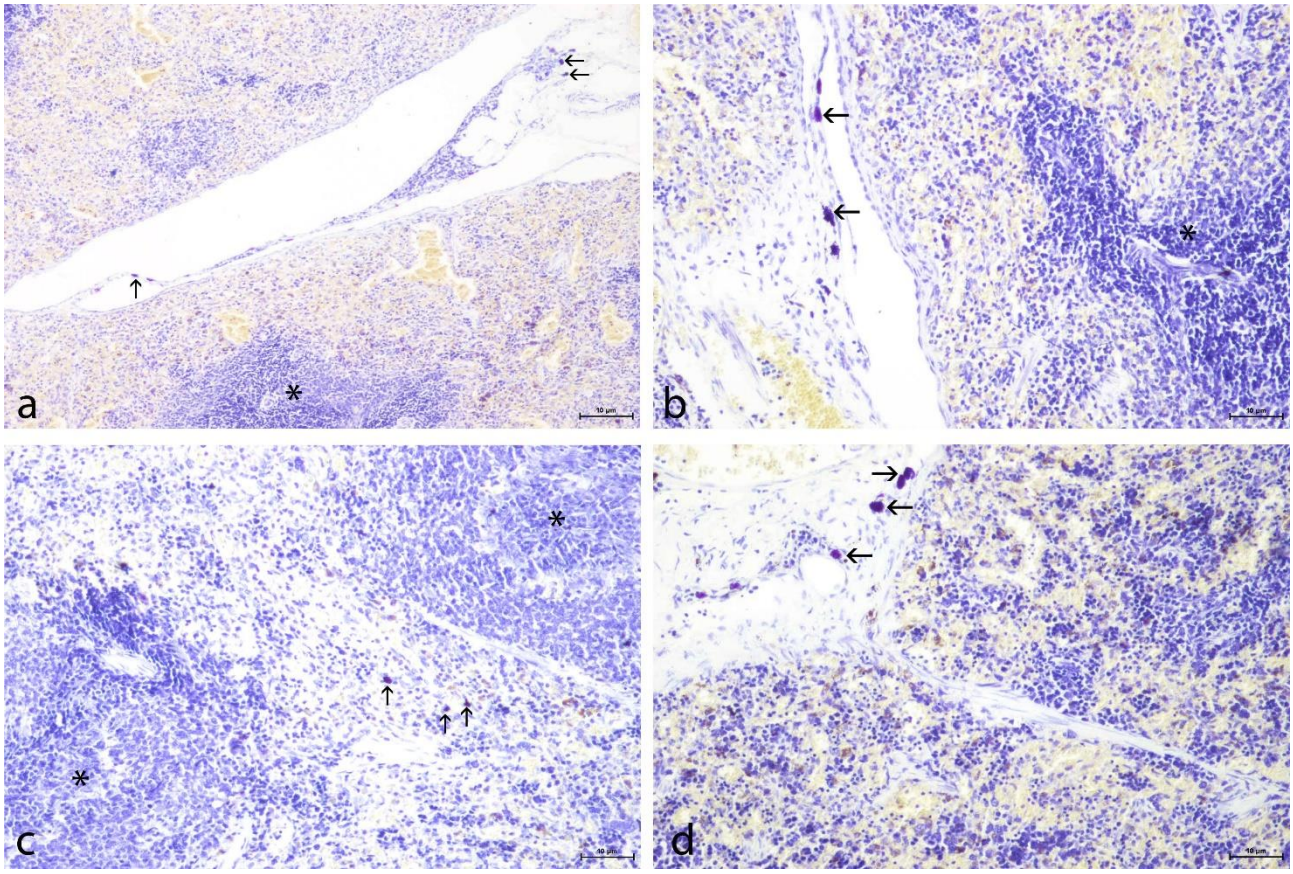

Figure 1. Toluidine blue staining. a. Control group, b. Diabetes group, c. S. nigra group, d. Diabetes + S. nigra group, (arrow): metachromatic mast cells, (asterix): white pulp, range bar, 10 µm.

Table 1. Mean count of MCs in groups after staining with toluidine blue.

Group	Mast cell count (× ±Sx /mm^2)	Minimum	Maximum
Control group	**20.45±0.73**	**17.20**	**21.40**
Diabetes group	**30.52±0.93[a]**	**26.40**	**32.40**
S. nigra gruop	**23.60±0.71[a]**	**20.20**	**25.20**
Diyabetes+S. nigra group	**24.72±1.17[a, b]**	**21.20**	**26.60**

[a]p<0.001 compared with the control group and [b]p<0.001 compared with the diabetes group (n=8).

Immunohistochemical findings

The density of VEGF-positive cells was evaluated semiquantitatively. Table 2 shows the immunohistochemical reactions in detail as well as a comparison of the groups. The positive

brown color VEGF immune-positive cells in the spleen tissue of all groups were characterized by different sizes, round or oval shapes. Intracytoplasmic stained VEGF immune-positive cell expression was observed in the spleen capsule, between lymph follicles and lymphatic cords in the red pulp areas. VEGF-positive cells were found in the red pulp, particularly in clusters. Positive cells were observed around the edges of lymph follicles and/or inside lymph follicles in the white pulp of the spleen on a very occasional basis.

When the groups were evaluated in detail among themselves, it was determined that the number of immune-positive VEGF cells increased only in the diabetes group. VEGF expression was similar in the control group, S. nigra group, and diabetes + S. nigra group. As a remarkable finding in our study, it was observed that the application of S. nigra caused a decrease in the number of immune-positive VEGF cells in diabetic rats (Figure 2).

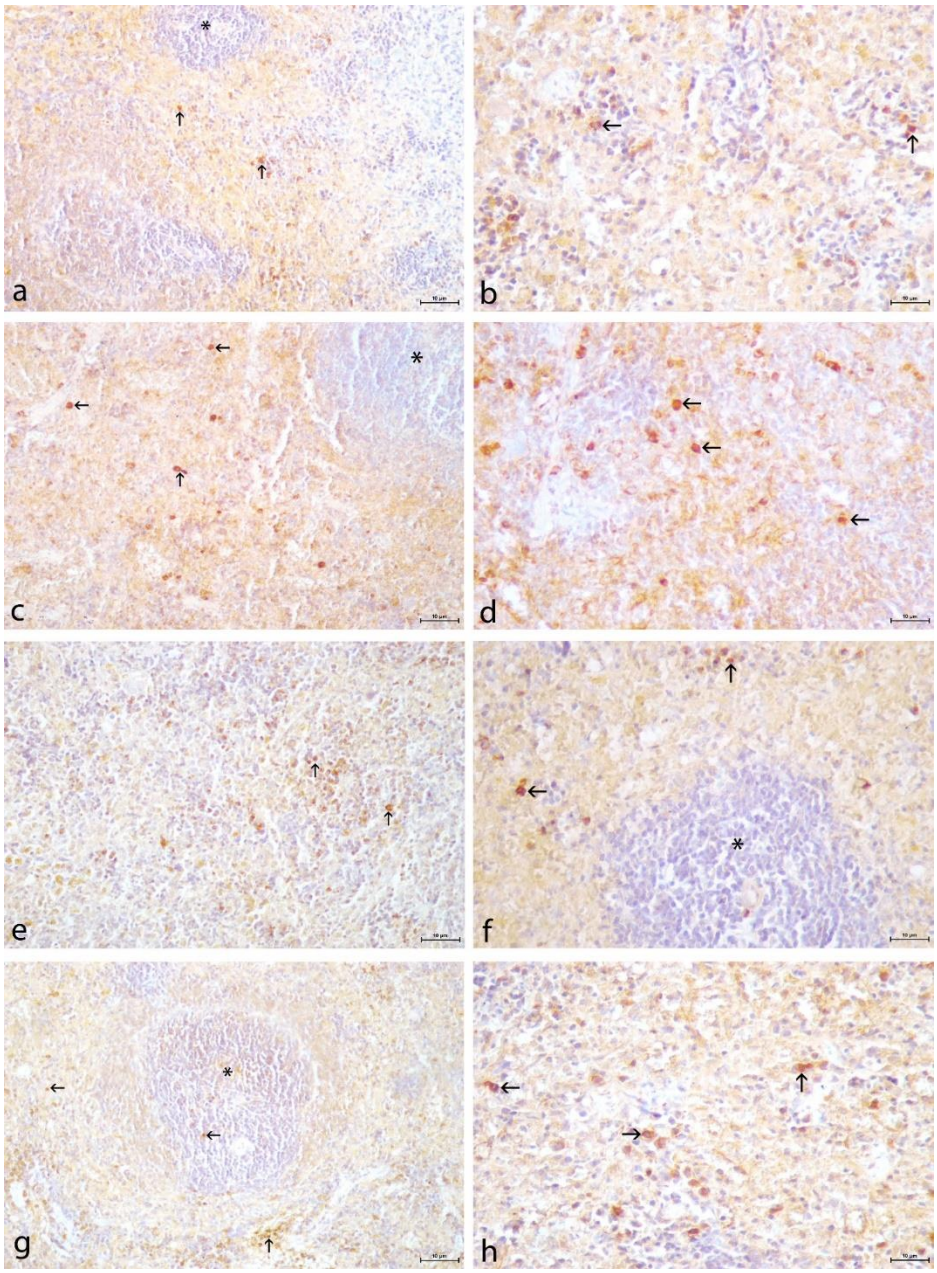

Figure 2. VEGF immunostaining. a-b. Control group, c-d. Diabetes group, e-f. S. nigra group, g-h Diabetes + S. nigra group, (arrow): VEGF immune-positive cell, (asterix): white pulp, range bar, 10 µm.

Table 2. Semiquantitative immunostaining score of VEGF positive cell reactivity in different groups' spleen tissue.

Group	VEGF immune-positive cells
Control group	++
Diabetes group	+++
S. nigra gruop	++
Diyabetes+S. nigra group	++

Note: no stained cells in the scanned area no positive cell (–), 1-2 cells (±), 3-4 cells (+), 5-6 cells (++), 7 and more cells (+++).

DISCUSSION

It has been suggested that increased IgE levels in the bloodstream of diabetic patients and IgE-mediated activation of MCs may play a role in the pathogenesis of diabetes (Svensson et al., 2012). In all layers of the oral mucosa and tongue, particularly in the lamina propria and around blood vessels, there has reportedly been a significant increase in MC after STZ administration in rats compared to control tissue (Batbayar et al., 2003). Çetin et al. (2013) showed that diabetic rats had a statistically significant increase in the number of MC in their heart tissue compared to non-diabetic rats. Martino et al. (2015) have shown that a greater number of MC infiltrates into pancreatic islets in samples from donors with diabetes than in models without diabetes in their study on human pancreatic tissue samples. Researchers have also investigated the effect of insulin on MC activation in rats with experimental diabetes. This study revealed an increase in the number of MCs in the lungs in the diabetes group and a decrease in the MCs in the lungs in the insulin-supplemented group (Cavalher-Machadove et al., 2004). The role of MCs in STZ-induced diabetes has been investigated using MC-deficient mice (W/W^v). The study found that W/W^v mice hyperglycemia accelerated, and 100% of the mice became diabetic compared to healthy mice. In addition, in this study, MC adaptive transfer before STZ administration has been observed to impart resistance to diabetes (Carlos et al., 2015). In a study, it was reported that natural polyphenols in S. nigra can modulate specific and non-specific immune defense in insulin deficiency diabetes (Badescu et al., 2015). The present study reports for the first time the potential effects of S. nigra on MC number and distribution in the spleens of diabetic rats. In this study, in parallel with the studies mentioned above, an increase in the number of MCs was observed in the spleen tissue of the rats whose diabetes model was formed. Consequently, the present study showed that S. nigra statistically decreased the increases in spleen MC numbers of diabetic rats. However, the number of MCs increased in the S. nigra group compared to the control group. The data of our study suggest that S. nigra, whose immunomodulatory effects are known, may influence MC number and distribution.

VEGF is a potent regulatory cytokine that promotes vascular endothelial cell proliferation, differentiation, and migration (Apte et al., 2019). It has been specified that hyperglycemia-induced oxidative stress activates and increases VEGF expression, an angiogenic factor (Ozdemir et al., 2014). The role of VEGF in the pathophysiology of tissue dysfunction in diabetes was investigated, and for this purpose, STZ-induced diabetic rats were treated with monoclonal anti-VEGF antibodies. As a result of this study, it has been observed that inhibition of VEGF reduces tissue damage caused by diabetes (De Vriese et al., 2001). In a study conducted by Mıcılı et al. (2012), it was reported that there was an increase in VEGF expression in the glomerular and tubular areas of kidney tissue in rats with experimental diabetes. It has been shown that VEGF expression is increased in the retinal layers of rats with diabetic retinopathy (De Meloa et al., 2020). In the present study, we observed that

diabetes increased VEGF expression in the rat spleen. Our results are similar to previous studies. Additionally, in our research, it was also determined that S. nigra did not change the number of VEGF immune-positive cells in the spleen tissue. However, when the diabetes group and the S. nigra group were compared, it was observed that S. nigra semiquantitatively reduced VEGF expression. As a result, although the precise mechanisms remain unknown, these results suggest that S. nigra may reduce the possible increase in diabetes-induced VEGF immune-positive cells in spleen tissue.

MCs are reported to increase in number in their angiogenesis regions, and products such as histamine, fibroblast growth factor, VEGF, heparin, and tryptase secreted from their granules promote angiogenesis (Crivellato et al., 2010). As a result of a study on human dengue fever, it has been reported that the number of MCs and the levels of VEGF and MC-specific proteases tryptase and chymase in the circulation increased significantly compared to healthy individuals (Furuta et al., 2012). Liang et al. (2012) found an increase in the number of MCs in tissue sections stained with toluidine blue in pterygium syndrome compared to normal conjunctiva. Also, they have detected using the double immunostaining method that tryptase MCs and VEGF were expressed from the same cell. Tissue samples from the oral mucosa with leukoplakia lesions and healthy oral mucosa tissue samples were compared in a study. According to the findings, it has been reported that there was a parallel increase in the number of MCs and VEGF expression in the lesioned areas (Michailidou et al., 2012). In an experimental study with STZ, it has been found that both MC number and VEGF expression increased in mice groups with diabetes compared to healthy control groups in skin tissues (Nishikori et al., 2014). According to Gyurkovics et al., (2016) in experimental diabetes, it was observed that the number of MCs in gingiva increased significantly in rats. Furthermore, in this study, it has been shown that the expression of VEGF secreted from these MCs was also increased. A synergistic relationship was observed between MC number and VEGF expression in our study, especially in diabetes + S. nigra group. These findings suggest that MCs can be both a source and a target of VEGFs. In addition, the fact that MCs are strategically located in tissues, especially around vessels that have VEGF cytokine expression, suggests that they may play an essential role in angiogenic and tissue hemostasis.

CONCLUSION

In conclusion, the increased number of MCs and VEGF immune-positive cells in the spleens of experimental diabetic rats allowed us to suggest that MCs can synthesize and store VEGF in their granules. In addition, since there is not enough literature information about the effects of S. nigra on diabetes and especially on spleen tissue, it is thought that this study will contribute to the literature on this subject.

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