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INVESTIGATION OF THE EFFICACY OF SAMBUCUS NIGRA ON THE EXPRESSION OF TLR4 AND IL-4 IN THE LUNG TISSUE OF DIABETIC RATS

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ABSTRACT

Sambucus nigra (S. nigra) is widely distributed in Europe, Asia, and North America; the plant has been used in folk medicine for centuries. Toll-like receptors (TLR) are transmembrane proteins found in various cells. Interleukin-4 (IL-4) is a pleiotropic cytokine that regulates cellular proliferation, and gene expression in various cell types. This study aimed to investigate the immunohistochemical expression of TLR4 and IL-4 in the lungs of healthy and diabetic rats treated with S. nigra. Rats were randomly divided into four groups (control, S. nigra, diabetes, diabetes + S. nigra), each containing eight rats. When the groups were generally evaluated, there was no significant difference in the control group, S. nigra group, and diabetes + S. nigra group. However, an increase in TLR4 immunoreaction intensity was determined in the lungs of diabetic rats. It was observed that IL-4 immunopositive cells were significantly lower in the diabetes group compared to the other groups but increased in the S. nigra group. S. nigra was found to increase the decreased IL-4 expression in the diabetes group compared to the control group. With the findings we obtained in our study, we observed that S. nigra could positively affect the immune system and be effective in metabolic disorders such as diabetes.

KEYWORDS:
Diabetes mellitus, Lung, Sambucus Nigra, Toll-like receptor 4, Interleukin-4

INTRODUCTION

Diabetes mellitus (DM) is a group of chronic metabolic diseases characterized by high blood glucose levels caused by the body's inability to produce insulin, resistance to the action of insulin, or both [1]. Because of its high prevalence and significant social and economic consequences, DM is one of the most important chronic diseases of the twenty-first century [2]. Retinopathy, nephropathy, neuropathy, periodontal disease, sexual dysfunction, and increased risk of cardiovascular disease are long-term consequences of DM [3]. Several studies have also reported an increased risk of lower respiratory tract infections such as pulmonary tuberculosis and pneumonia. In addition, diabetes is known to cause suppression of cytokines, abnormalities of phagocytosis, and immune cell dysfunction [4].

Sambucus nigra (S. nigra) is widely distributed in Europe, Asia, North Africa, and North America; the plant has been used in folk medicine for centuries. It contains a wide variety of anthocyanins, flavonoids, and other polyphenols [5]. Triterpenes, lectins, cyanogenic heterosides, and essential oils are also present in the chemical structure of S. nigra [6]. Since Hippocrates' time, this plant has been used in the development of numerous therapeutic agents to prevent and/or combat various ailments [7]. Various therapeutic properties have been attributed to S. nigra, including diuretic, diaphoretic, laxative, and hemostatic effects [8]. Previous studies have shown that S. nigra has antioxidant, antiviral, and antidiabetic properties [2]. Preparations from S. nigra have been shown in animal studies to improve glucose and lipid metabolism and diabetic osteoporosis status [9].

Toll-like receptors (TLRs) are transmembrane proteins found in various cells that stimulate cytokine production as part of innate immunity and recognize pathogen-associated molecular structures [10]. TLR4 can recognize exogenous pathogens by binding to bacterial lipopolysaccharide (LPS), stimulating the production of antimicrobial peptides, and triggering nonspecific immune responses in macrophages, such as activation of the nuclear factor kappa B (NFkB) pathway [11]. According to recent research, stimulation of TLR4 significantly upregulates pro-inflammatory and immunosuppressive cytokines as well as inflammatory factors [12]. TLR4 has also been shown to function as a sensor of damage-associated molecular patterns. Because of this property, it can recognize a variety of molecules released from damaged or dying tissue, as well as those actively released from intact cells in response to cellular stress [13]. Among TLRs, TLR4 plays a critical role in the pathogenesis of insulin resistance and inflammation in both experimental and clinical studies at DM [14].

Interleukin-4 (IL-4) is a pleiotropic cytokine that regulates cellular proliferation, apoptosis, and gene expression in various of cell types, including lymphocytes, macrophages, fibroblasts, epithelial cells, and endothelial cells [15]. It is known that IL-4 regulates chemotaxis and plays an important role in regulating inflammation and cell-mediated immune responses. [16]. As an immune regulator, IL-4 induces macrophage polarization, conversion of natural T helper cells to type 2 T helper cells (Th2), and stimulation and differentiation of B cells [17]. IL-4 is a cytokine with proliferative or antiproliferative properties depending on the cell type [18]. IL-4 has been shown to upregulate the antigen-presenting function of various cell populations, including B cells, macrophages, and dendritic cells [19]. Microenvironmental IL-4 can antagonize chronic inflammation in adipose tissues when insulin resistance occurs [20].

This study aimed to investigate the immunohistochemical expression of TLR4 and IL-4 in the lungs of healthy and diabetic rats treated with S. nigra.

MATERIALS AND METHODS

Animal material. Approval was received by the Animal Experiments Local Ethics Committee of Ondokuz Mayıs University (Decision no: OMU-HADYEK/2020-15). In this study, 32 male rats, weighing 250-300 g, that were used. The rats used in the study were harbored in a standard cage in 12 h light and 12 h dark environment at 22±2°C ambient temperature and fed by ad-libitum and tap water.

Experiment groups. It was prepared by dissolving 450 mg streptozotocin (STZ) (Sigma, S0130-1G) in 10 ml distilled water and administered to the diabetic groups [21]. 1st control group (n=8): no application was made. 2nd S. nigra group (n=8): S. nigra extract was administered at a dose of 15 mg/kg by oral gavage for 14 days [22]. 3. diabetes group (n=8): experimental diabetes was induced by a single intraperitoneal injection of STZ at a dose of 45 mg/kg. 4. diabetes + S. nigra group (n=8): experimental diabetes was induced by a single intraperitoneal injection of STZ (45 mg/kg) and S. nigra extract was administered to the STZ-induced diabetic rats (15 mg/kg for 14 days by oral gavage). Then, the necks of the rats in each group were torn out under anesthesia and lung tissue samples were collected for immunohistochemistry. The lung tissue samples were fixed in 10% formaldehyde solution, and the tissue sections were taken with a thickness of 5 µm from the prepared paraffin blocks.

Determination of blood glucose levels. The blood sample was collected from the tail vein of the hungry animals 8 hours before the start of the study using a glucometer (PlusMED Accuro) 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. From day 3 of STZ practice, S. nigra extract was administered by oral gavage for 14 days.

Immunohistochemistry. The lung sections 5 µm thick taken from paraffin blocks were stained immunohistochemically by using mouse monoclonal antibody TLR4 (1/500 dilution, Santa Cruz Biotechnology, sc-293072), and rabbit monoclonal IL-4 (1/500 dilution, Shanghai YL Biotech, YID2904) primary antibodies with Streptavidin biotin complex [23]. Histostain Plus (Zymed kit: 85-6743) kit was performed as secondary antibody. After deparaffinization, sections were heated in a microwave oven of 700 watts within citrate buffer (pH=6) solution for proteolysis. In order to block endogenous peroxidase activity, the tissues were incubated in 3% hydrogen peroxide solution. Following washing with phosphate buffer solution (PBS), serum in the kit was instilled to prevent nonspecific protein binding in sections. Primary antibody was applied on, and they were stored at +4 ^{0}C for overnight. Only PBS solution was process on negative control group tissues. Following the washing procedure, biotinylated secondary antibody was instilled into sections and incubated at streptavidin-horseradish peroxidise complex after washing. As the last stage, 3, 3'-diaminobenzidine (DAB) was used as chromogen and the preparations were covered with entellan by counterstaining was performed with hematoxylin.

Immunohistochemical examination. After immunohistochemical staining, preparates were examined under an examination microscope (Nikon Eclipse 50i) in terms of immunoreactivity. IL-4 positive cell distribution was evaluated semiquantitatively. The following criteria were employed in semiquantitative evaluation: no positive cell in the scanned area (-), 1-2 cells (±), 3-4 cells (+), and 5-6 cells (++) [24]. TLR4 staining was assessed semiquantitatively using a light microscope (Nikon Eclipse 50i): negative (-), weak reaction (+), moderate reaction (++), and strong reaction (+++) [25].

The immunopositive cells were scored from 0 to 3 semi-quantitatively [26] as follows. A histoscore was derived from the IL-4 immunopositive cell distribution, 0: no positive cell in the scanned area (-), +1: 1-2 cells (±), +2: 3-4 cells (+), +3: 5-6 cells (++). TLR4 expression was acquired by the semi-quantitative method that taking staining intensity, 0: negative (-), +1: weak reaction (+), +2: moderate reaction (++), and +3: strong reaction (+++).

Statistical analysis. All data were analyzed using the SPSS 22.0 program and presented as mean ± standard error of the mean (SEM). The Levene's test

was used to ensure that all data was normally distributed before analysis. For data obtained from the immunohistochemical expression immunoreactivity, one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test were utilized. $p<0.05$ was considered as significant.

RESULTS

TLR4 expression. Immunohistochemical staining showed that bronchial, bronchiolar and alveolar epithelial cells exhibit TLR4 expression. Also, there were positive TLR4 reactions in smooth muscle cells surrounding the bronchi and bronchioles and smooth muscle cells of the blood vessels (Figure 1-A, 1-B, 1-C, 1-D). When the groups were generally evaluated, there was no significant difference in the control group, S. nigra group, and diabetes + S. nigra group. However, an increase in TLR4 immunoreaction intensity was observed in the lungs of diabetic rats. In addition, as a remarkable finding, it was determined that S. nigra reduced TLR4 expression, which was high in the diabetes group. The histoscore results of the semi-quantitative analysis between groups for TLR4 immunohistochemical staining are summarized in Table 1.

IL-4 expression. Table 1 summarizes the histoscore results of the semi-quantitative examination of IL-4 immunohistochemistry staining between groups. As a result of immunohistochemical staining, IL-4 positive cells were determined as brown colour in lung tissue. Immunopositive IL-4 cells were seen in the connective tissue surrounding the bronchi and bronchioles, as well as the interalveolar areas and pleura. Positive IL-4 cells were found more frequently in bronchial-associated lymphoid tissue (BALT) (Figure 2-A, 2-B, 2-C, 2-D). It was observed that IL-4 immunopositive cells were significantly lower in the diabetes group compared to the other groups, but increased in the S. nigra group. S. nigra was found to increase the decreased IL-4 expression in the diabetes group as compared to the control group.

DISCUSSION

The DM can affect multiple systems throughout the body, along with a host of complications. Many observations suggest that metabolic abnormalities in DM may be due to the overproduction of cytokines [27]. It has also been reported that DM may cause low elastic recoil of the lungs and functional abnormalities in the respiratory system such as lung volume and diffusion capacity [28]. Studies show that some natural extracts have beneficial effects on cellular and molecular mechanisms involved in immune system disorders in DM [9]. Recently, S. nigra has received significant attention as a functional compound in food applications such as natural preservatives or food supplements, particularly because of its antioxidant capacity. Furthermore, it is also known to stimulate insulin secretion [29]. When the effect of S. nigra on glucose transport and metabolism was studied, it was found to stimulate glucose uptake, glucose oxidation, and glycogen synthesis in a manner comparable to insulin [30]. The importance of finding alternative sources of antidiabetic agents and the limited number of studies on the invesgestion of S. nigra extracts to reduce diabetes complications indicate that more detailed studies on this topic are needed.

TLRs are important transmembrane proteins that transmit antigen recognition information from outside to inside the cell, which plays a role in the immune response [31]. TLR4 is closely associated with a variety of diabetic complications. For example, TLR4-mediated ursolic acid has been observed to reduce inflammation to prevent diabetic nephropathy [32]. TLR4 is a key molecule that triggers autoimmune damage to beta cells and may serve as an early indicator of beta-cell destruction [33]. However, as shown in lung injury, TLR4 has also been found to provide adequate protection against inflammatory tissue damage by promoting tissue repair and remodeling processes [34]. Clinical studies have also shown that the expression of TLR4 is increased in patients with DM compared to the control group [35]. Experimental studies on the pancreas of mice showed that TLR4 immunopositive islet area was significantly increased in the STZ group compared to the normal control group [36].

TABLE 1
Summary of TLR4 and IL-4 immunohistochemical expression results

	TLR4	IL-4
Control group	0.94 ± 0.04	1.68 ± 0.04
S. nigra group	0.96 ± 0.05	2.24 ± 0.07^{a}
Diabetes group	$1.2 \pm 0.06^{*}$	1.23 ± 0.06^{a}
Diabetes+S. nigra group	$0.95 \pm 0.03^{\#}$	$1.64 \pm 0.03^{b, c}$

Data are expressed as mean ± SEM (one-way ANOVA followed by Tukey's post-hoc test, n=8 for each group).
$^{*}p<0.01$; when compared to control group and $^{\#}p<0.01$; when compared to diabet group.
$^{a}p<0.001$; when compared to control group, $^{b}p<0.001$; when compared to sambucus nigra group, and $^{c}p<0.001$; when compared to diabet group.

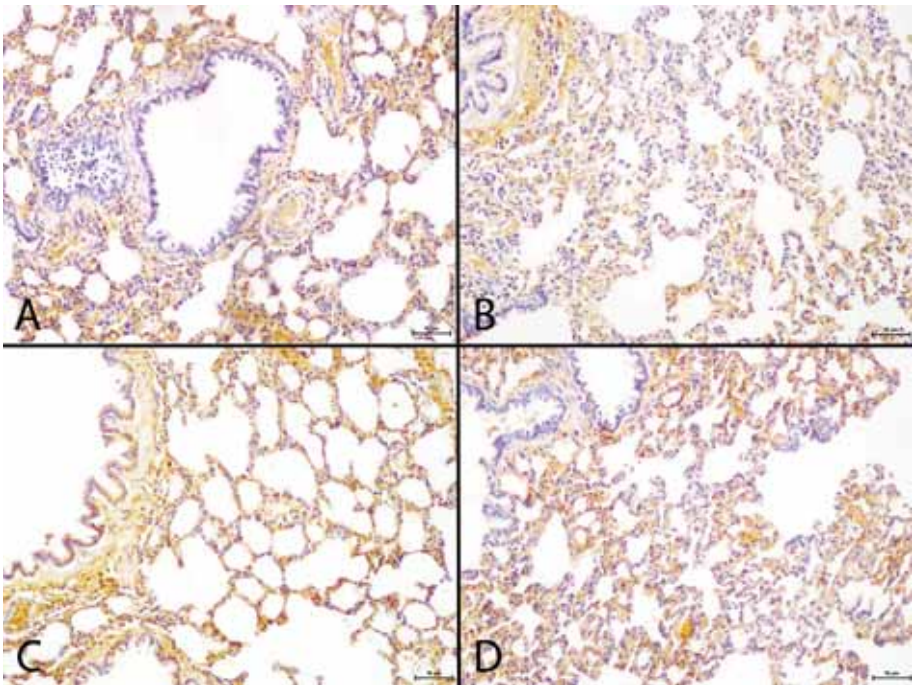

FIGURE 1
Toll-like receptor 4 immunostaining findings
A: Control group, B: S. nigra group, C: diabetes group,
D: diabetes + S. nigra group, x20; range bar, 10 µm.

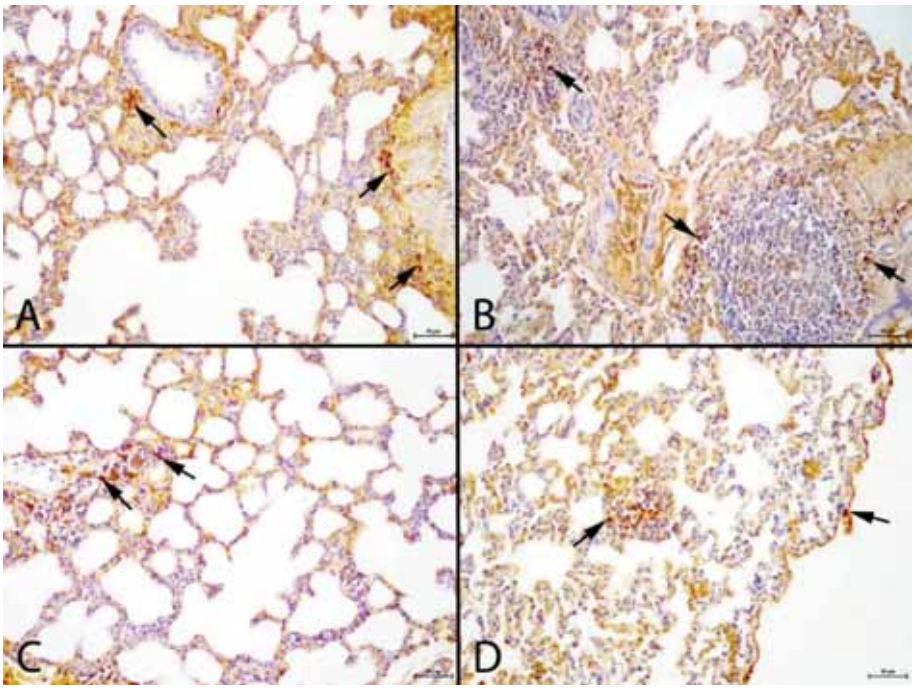

FIGURE 2
Interleukin-4 immunostaining findings
A: Control group, B: S. nigra group, C: diabetes group,
D: diabetes + S. nigra group, arrow: immunopositive IL-4 cells, x20; range bar, 10 µm.

At the end of two weeks in experimental DM rats with STZ, high TLR4 expression is reported in the glomerular basement membrane of the kidney, proximal convoluted tubule, and renal interstitial area with a significant difference compared to the control group [37]. The articles by Wang et al. [38] showed that TLR4 expression was significantly increased in retinal endothelial cells of STZ-induced diabetic mice 6 and 8 weeks after STZ injection compared with control mice. In addition, many studies found that TLR4 levels increased after STZ, injection and TLR4 played a role in the development of

cytokines in diabetes [39]. In our study, in parallel with the above studies, an increase in TLR4 expression was observed in the lungs of rats that had diabetes. Moreover, we found that S. nigra did not directly affect TLR4 expression. It was observed that the increased TLR4 expression decreased in diabetes + S. nigra group. Based on the current knowledge and the data in this study, we think that S. nigra could contribute to the enhancement of immune response and indirectly to the treatment of diabetes.

Studies have shown that IL-4 has the potential to alter the course of diabetes by regulating insulitis and maintaining normal glycemia levels [40]. It is known that exposure to high glucose concentration can significantly affect cytokine production [41]. The metabolic regulating roles of IL-4 were demonstrated in a study examining the relationships between cytokines/immune responses, insulin sensitivity, and lipid metabolism [42]. Notedly, previous studies have reported that IL-4 restores insulin sensitivity in insulin-resistant adipocytes through mechanisms unrelated to induced adipogenesis or de novo formation of lipid stores [43]. Evidence from studies showed that IL-4 promotes energy storage by increasing insulin-stimulated glucose uptake and lipid synthesis [44]. It has been observed in a study that mice with IL-4 overexpression show better glucose tolerance and insulin sensitivity by increasing insulin signaling. In addition, IL-4 may inhibit lipid accumulation in adipose tissue and indirectly mediate energy homeostasis by regulating adipokines derived from adipose tissue [45]. It has been shown that the IL-4 levels are significantly lower in experimental diabetic rats compared to the control group. Moreover, in the same study, black cumin and garlic with immunomodulatory effect were found to increase IL-4 levels [46]. In our study, although IL-4 immunoreactivity was found to be increased in the lungs of rats in the S. nigra group. Whereas, it was observed that diabetes caused a decrease in IL-4 expression. Furthermore, it was found that S. nigra enhanced the decreased IL-4 expression in diabetes. Our results show parallels with previous studies on IL-4 cytokine with diabetes and immunomodulatory agents. This may suggest that the increase in IL-4 expression plays an important role in the prevention of diabetic pathogenesis.

CONCLUSIONS

As a result, our research found that S. nigra administration did not significantly affect TLR4 expression, but it was observed that S. nigra reduced diabetes-induced TLR4 expression. Moreover, it was seen that S. nigra increased the IL-4 cytokine expression, which was decreased in the diabetes group, in the lung tissue. With the findings we obtained in our study, we observed that S. nigra could positively affect the immune system and be effective in metabolic disorders such as DM. The plants' usage in the treatment of numerous ailments will be encouraging for future and long-term uses, but further research is needed to demonstrate S. nigra's good influence on diabetic complications.

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