ORIGINAL ARTICLE

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The Immunomodulatory Effects of Ginger (Zingiber officinale) Extract on CD4 and CD8 Expression in Spleen of Diabetic Rats

Diyabetik Sıçanların Dalağında CD4 ve CD8 Ekspresyonu Üzerine Zencefil (Zingiber officinale) Ekstraktının İmmünmodülatör Etkileri

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ABSTRACT

Aim: This study aims to examine the changes of the ginger extract treatment on CD4 and CD8 expressions in the spleen tissue of rats, in which the experimental diabetes was induced with streptozotocin.

Materials and Methods: Forty Wistar albino rats were divided into 5 groups: control, sham, ginger, diabetes, diabetes+ginger. The spleen tissue sections were stained by Crossman's triple staining and the streptavidin-biotin peroxidase complex method. Diabetes was induced by 50 mg/kg streptozotocin injection intraperitoneally in the diabetes and diabetes+ginger groups. Ginger extract was administered to the ginger and diabetes+ginger groups at dose of 200 mg/kg for 30 days. Statistical measurements were analyzed in order to determine whether there was a difference between the groups in terms of weight of the spleen, the number of immunopositive CD4 and CD8 cells, and ratio of CD4/CD8.

Results: It was observed that the numerous lymph follicles were atrophied and most of the follicles did not have a germinal center in the diabetes group. Also, it was determined that ginger extract reduced degenerative changes. While it was observed that CD4 and CD8 expression was intense in the diabetes group, the intensity of CD4 and CD8 expression was decreased in the diabetes+ginger group compared to the diabetes group. In addition, it was determined that the spleen weight decreased in the diabetes group and increased in the diabetes+ginger group, which was similar to the control and sham groups.

Conclusion: In this study, it was revealed that ginger exerted a protective effect against STZ-induced diabetes in rats and had immunostimulatory effect in diabetic experimental model. Also, it can be used therapeutically in spleen problems and diabetes-related immune dysfunction.

Keywords: CD4, CD8, spleen, diabetes, ginger

ÖZ

Amaç: Bu çalışmada, zencefil ekstrakt uygulaması ile streptozotosin ile deneysel diyabet oluşturulan ratların dalak dokusunda CD4 ve CD8 salınımındaki değişiklerin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmamızda toplam kırk adet Wistar albino ırkı rat kullanıldı. Ratlar; kontrol, sham, zencefil, diyabet, diyabet+zencefil olmak üzere 5 gruba ayrıldı. Dalak doku kesitlerine Crossman'ın üçlü boyama ve streptavidin-biotin peroksidaz kompleks boyama yöntemleri uygulandı. Diyabet ve diyabet+zencefil gruplarına 50 mg/kg streptozotosinin intraperitoneal yolla enjeksiyonu ile diyabet indüklendi. Zencefil ekstraktı, zencefil ve diyabet+zencefil gruplarına 200 mg/kg dozunda 30 gün süreyle uygulandı. Gruplar arasında dalak ağırlığı, CD4 ve CD8 immünopozitif hücre sayısı ve CD4/CD8 oranı istatistiksel olarak analiz edildi.

Bulgular: Diyabet grubunda çok sayıda lenf folikülünün atrofiye uğradığı ve çoğu folikülün de germinal merkezinin olmadığı görüldü. Ayrıca, zencefil ekstraktının dejeneratif değişiklikleri azalttığı tespit edildi. Diyabet grubunda CD4 ve CD8 salınımının yoğun olduğu gözlenirken, diyabet+zencefil grubunda CD4 ve CD8 salınım yoğunluğunun diyabet grubuna göre azaldığı görüldü. Ayrıca diyabet grubunda azalan dalak ağırlığının diyabet+zencefil grubunda arttığı kontrol ve sham gruplarıyla benzer değerde olduğu tespit edildi.

Sonuç: Zencefil ekstraktının deneysel yolla diyabet oluşturulan modelde immün sistemi uyarıcı etkiye sahip olduğu görülmüştür. Elde edilen sonuçlara göre, zencefil diyabetle ilişkili dalak sorunlarında ve bağışıklık fonksiyon bozukluğunda tedavi edici olarak kullanılabilir.

Anahtar Kelimeler: CD4, CD8, dalak, diyabet, zencefil

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INTRODUCTION

Diabetes mellitus (DM) is a lifelong chronic disease that occurs with the body's inability to produce insulin, the absence of insulin, or the rise in blood sugar as a result of the body's inability to use insulin[1,2]. There are 2 types: type 1 DM (T1DM), which occurs mostly in childhood, and type 2 DM (T2DM), the most common type which occurs in advanced ages[2]. Immune balance is closely related to diabetes[3]. T2DM negatively affects the functioning of immune cells and increases the risk of infection. Insulin deficiency has also been observed to cause deterioration in CD4 and CD8 function[4].

Immunomodulators function as an immunostimulant in cases of immune deficiency and as an immunosuppressant in abnormally increased immunity to protect the body against harm and infection[5,6]. Studies with natural products that act as regulators by increasing or decreasing immunity are increasing[3,7,8]. Ginger (Zingiber officinale) has been used both as a spice and as a therapeutic since ancient times. The therapeutic property of ginger can be attributed to the fact that it has about 400 different components, mainly gingerols, shogaols, zingerone and paradols[5,9].

Immunity is classified as innate natural and acquired immunity. The spleen is the largest secondary lymphoid organ involved in the immunity of the organism. It hosts a large number of cells that play a role in defense, such as T and B lymphocytes, dendritic cells, macrophages and natural killer cells[10]. T lymphocytes are responsible for cellular immunity and they include two subgroups as CD4 and CD8[11].

CD4 and CD8 are very important in the pathogenesis of diabetes. Particularly in T1DM, an increase in the number of these cells or deterioration in their functions are effective. At the same time, these cells cause the destruction of islets β-cell. The increase in the number of CD4 positive cells with dysfunction causes autoimmune destruction of islet of β-cells in pancreas[12]. Therefore, CD4 T lymphocytes play an important role in the pathogenesis of T1DM[13]. T2DM, which is characterized by hyperglycemia and hyperinsulinemia, causes an increased risk for viral infection, melanoma or other different diseases. Also, it has been declared that CD8 T lymphocytes express the insulin receptor and are adversely affected by insulin and blood sugar increase. In addition, insulin is a direct influence on the function of CD8 T lymphocytes[4].

This study aimed to determine immunomodulatory effects of ethanolic extract of ginger on CD4 and CD8 expression in the spleen tissue of experimental diabetic rats.

MATERIALS AND METHODS

The study was conducted using 40 female, 206±6 g, and 4-month aged Wistar albino rats obtained from The Health and Experimental Center of Tekirdağ Namık Kemal University. All experiments were approved by the Tekirdağ Namık Kemal University Ethics Committee for Animal Experiments (meeting no: 09.11.2022/1153).

The rats were not used in any previous studies and they were housed at standard cages under temperature controlled room (22±2 °C) and were maintained on a 12-hour light/dark cycle and fed with a standard rat pellet diet and water ad libitum.

Preparation Ethanolic Extract of Ginger

Ginger fresh rhizomes were obtained from a local store and authenticated at the Department of Botany in Tekirdağ Namık Kemal University. Gingers were firstly washed and dried in a dark room. Dried ginger rhizomes were mechanically pulverized in a porcelain mortar. The resulting powder mixture was kept in 95% alcohol for 24 hours, then the mixture was filtered. This process was repeated 3 times in total. All prepared mixtures were collected together and alcohol was removed in the low speed evaporator. The prepared extract was stored in refrigerator at 4 °C. Ginger extract 200 mg/kg/bw/day was given orally to experimental rats for 30 days according to the previously mentioned methodology[14].

Induction of Diabetes

50 mg/kg of streptozotocin (STZ) (Sigma, st. Louis, MO, USA) was dissolved in 0.1 M citrate buffer (pH 4.5). Intraperitoneal injection (i.p.i.) was performed after an overnight fasting. After 3 days of the application, fasting blood glucose values were measured from the tail vein of the rats using the Accu-Chek Instant glucometer (Roche). Blood glucose values >250 mg/dL were considered an indicator for developing diabetes and rats were included in the diabetic groups[15].

Experimental Animals and Design

The experiment rats were divided into 5 groups and each group included eight animals. The total experiment protocol was maintained for 30 days. The experimental groups were as follows:

Control (n=8): No application was made (untreated group).

Sham group (n=8): Tween 80 was given to rats by oral gavage.

Ginger group (n=8): Fresh ginger extract (prepared daily) was given by oral gavage at the dose of 200 mg/kg for 30 days.

Diabetes group (n=8): This group was administered 50 mg/kg i.p.i. STZ.

Diabetes+ginger group (n=8): After diabetes was established, 200 mg/kg ginger extract was administered to this group by oral gavage for 30 days.

At the end of 30 days, the rats were euthanized under deep anesthesia and spleen tissues were removed.

Histological Procedure

Spleen tissues were fixed in 10% formalin for 48 hours. After fixation, the samples were processed for routine histological protocols and embedded in paraffin. The sections taken at 5 µm were deparaffinized in xylene, rehydrated through decreasing concentrations of ethanol and stained with Crossman's triple staining for histological examination[16].

Immunohistochemical Staining

The streptavidin biotin peroxidase complex (strepABC) method was applied to investigate CD4 and CD8 T lymphocytes immunoreactivity in the spleen. Sections of 5 µm thickness were collected on adhesive slides. The sections were processed in citrate buffer solution (pH 6.0) for 10 min in a microwave oven at 700 watts. Then, tissues were kept in 3% hydrogen peroxide (H_2O_2) for 10 min. Sections were incubated with anti-CD4 antibody (ab237722, diluted 1:150) and anti-CD8 antibody (OX-8) (ab33786, diluted 1:150). Sections were incubated with biotinylated goat anti-rabbit and rabbit anti-goat IgG for 30 min and peroxidase conjugated streptavidin (1:200) (P0397; Dako Corp., Carpinteria, CA, USA) for 10 min. After 3,3′-Diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/mL; Dako Corp.) was used as chromogen, Gill III hematoxylin was used as counterstaining. Sections were evaluated using light microscope (Olympus BX51, Tokyo, Japan). Rabbit serum without primer antibody served as the negative control. Evaluation of immunoreactivity of CD4 and CD8 were scored. Immunoreactive cells were categorized as having negative, slight, moderate, and intensive.

Assessment of Immunohistochemical Staining

The number of immunohistochemical staining of CD4 and CD8 were examined using light microscopy at X400 magnification and photographed. A total number of fifteen representative images were selected from each rat. The number of CD4 and CD8 positive and negative cells per unit area was calculated according to the following formula: (The number of positive cells/number of total cells counted in the field) x100. The number of CD4 and CD8 positive cells and ratio of CD8/CD8 were statistically analyzed[3,17,18].

Relative Spleen Weight

Fresh spleens were weighted immediately after rats were euthanized. Statistical method was used to compare change in spleen weight between groups.

Statistical Analysis

Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) (version 20.0, IBM, SPSS Inc., Chicago, USA). Data were examined for normality distribution and variance homogeneity with the Shapiro-Wilk's test. If normally distributed, the one-way ANOVA test was applied, and the differences between the groups were analyzed with the post-hoc Tukey test. The differences were considered significant at $p<0.05$, and the means and standard errors were calculated. In the study, nonparametric tests were used as the data did not provide normal assumptions. So, the differences between the groups were analyzed with the Kruskal-Wallis, and the Mann-Whitney U test was used between the groups. Also, the differences were considered significant at $p<0.05$, and the median values (minimum-maximum) were calculated.

RESULTS

Histological Results

The control, sham and ginger groups had normal histological structure of the spleen. The tissue was surrounded by a capsule from the outside. Trabeculae separated from the capsule extended to the parenchyma of the spleen. The spleen consisted of separated lymphoid follicles (white pulp) and was surrounded by highly vascular matrix (red pulp). The white pulp was formed of periarteriolar lymphatic sheath and marginal zones. Lymphoid follicles had germinal centers and central arteriole. The red pulp consisted of network of blood cells cords (Figure 1a-c). The diabetes group was seen to have atrophied lymphoid follicles. There was no germinal center in most of the follicles (Figure 1d). The diabetes+ginger group was observed as white and red pulps, similar to those in the control, sham or ginger groups. Most of the follicles had germinal center (Figure 1e).

Immunohistochemical Results

Specific CD4 and CD8 immunoreactions were seen in all groups. However, there was a difference between the groups in terms of the severity of immunoreaction-positive cells.

CD4 and CD8 T lymphocytes were localized mostly in red pulp and few positive cells in white pulp. Slight reaction was observed in the control and sham groups in red pulp (Figure 2a, 2b, 3a, 3b). The ginger group was shown to have moderate reaction and a few slight reactions in the cells (Figure 2c, 3c). While intensive reaction was remarkable in the diabetes group (Figure 2d, 3d), moderate reaction was seen in the diabetes+ginger group (Figure 2e, 3e).

Assessment of Immunohistochemical Results

According to our findings, it was observed that the number of CD4 and CD8 immunopositive cells in the diabetes group showed significant increase compared to the control, sham and ginger groups ($p<0.0001$). After treated with ginger, it was observed that the diabetes+ginger group exhibited significant decrease as compared to the diabetes group ($p<0.0001$ for CD4 and CD8). All data of CD4 and CD8 are shown in Table 1.

In the present study, a highly significant increase was found in the CD4/CD8 ratio ($p=0.002$). There was a significant decrease in the diabetes group compared to the control ($p=0.002$) and sham groups ($p=0.001$), but there was a nonsignificant increase in the diabetes+ginger group ($p>0.05$) (Table 1).

Spleen Weight Results

When all the groups were compared in terms of spleen weight, a significant decrease was found in the diabetes group. Also, the diabetes+ginger group was compared to the diabetes group and a significant increase was detected in spleen weight ($p<0.05$). Comparison of mean values of spleen weight among the groups is shown in Figure 4.

DISCUSSION

A new one is added to the studies on immunological resources every day. The discovery of highly biostable immunomodulators without toxic side effects is great relevance. In particular, the immunomodulatory effects of plants have been a matter of curiosity that have proven biosafety in recent years[19]. In this study, we set out to investigate whether ginger has a protective effect on the spleen tissue, which has an important function on immunity in STZ induced diabetic rats.

Table 1. The number of CD4 and CD8 positive cells per unit area in all groups

	Control	Sham	Ginger	Diabetes	Diabetes+ginger
CD4	19.00	19.00	18.00	83.50[a]	21.00[bc]
CD8	15.50	16.00	16.00	80.00[a]	19.00[bc]
CD4/CD8	1.12	1.20	1.20	1.04[a]	1.12

[a]: $p<0.05$ diabetes group versus control group/diabetes group versus sham group, [b]: $p<0.05$ diabetes+ginger group versus diabetes group, [c]: $p<0.05$, diabetes+ginger group versus ginger group

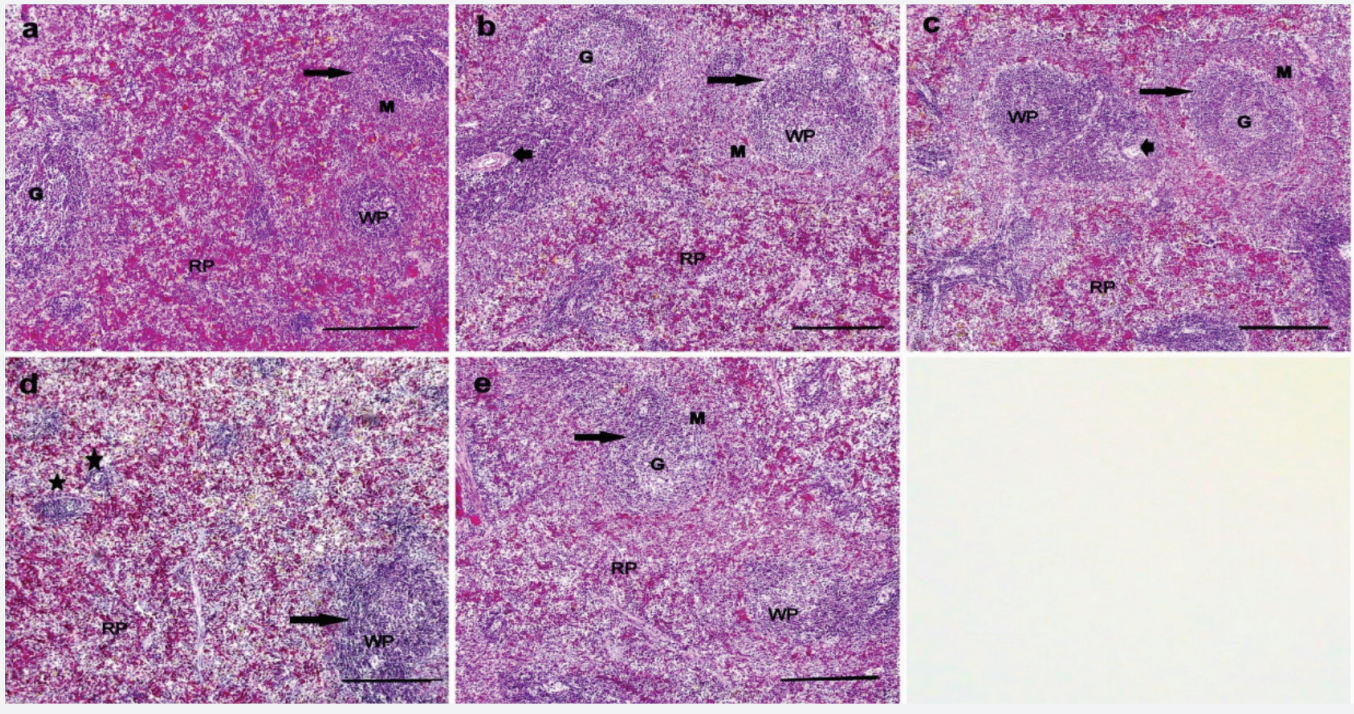

Figure 1. Histological structure of spleen tissue. The control (a), sham (b), ginger (c), diabetes (d), diabetes+ginger groups (e). Red pulp (RP), white pulp (WP), lymphoid follicles (arrow), germinal center (G), marginal zone (M), atrophied lymphoid follicles (star), periarteriolar lymphatic sheath (small arrow). Triple staining. Bar=100 µm

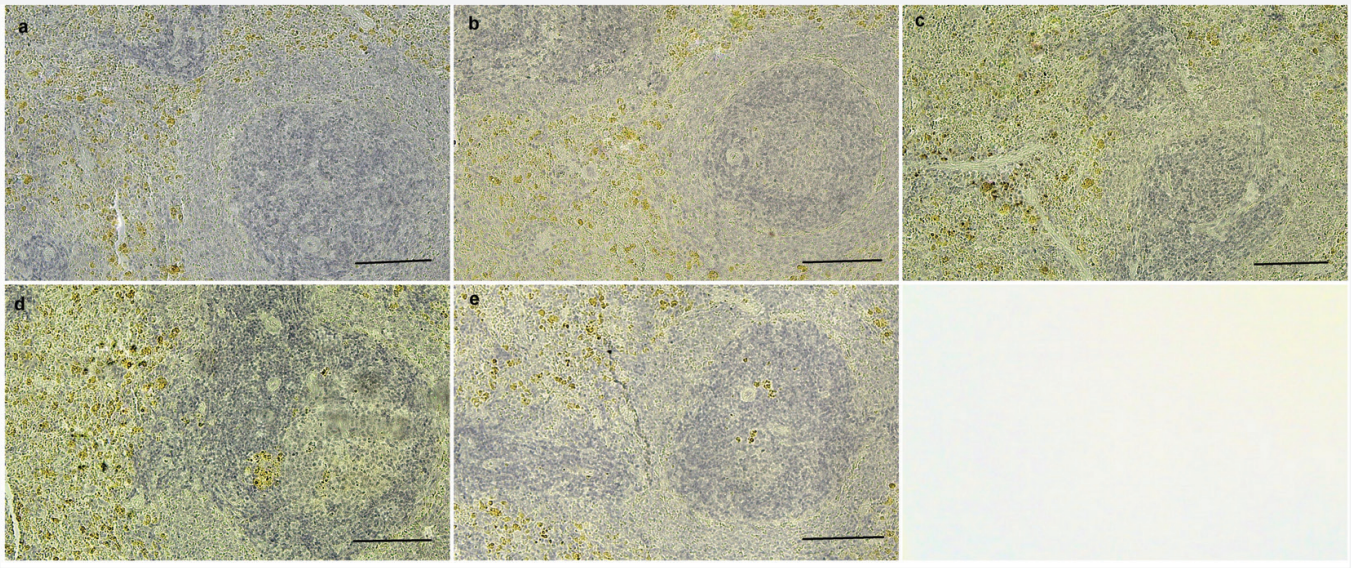

Figure 2. CD4 expression in spleen tissue. The control (a), sham (b), ginger (c), diabetes group (d), diabetes+ginger groups (e). Immunohistochemical staining. Bar=100 µm

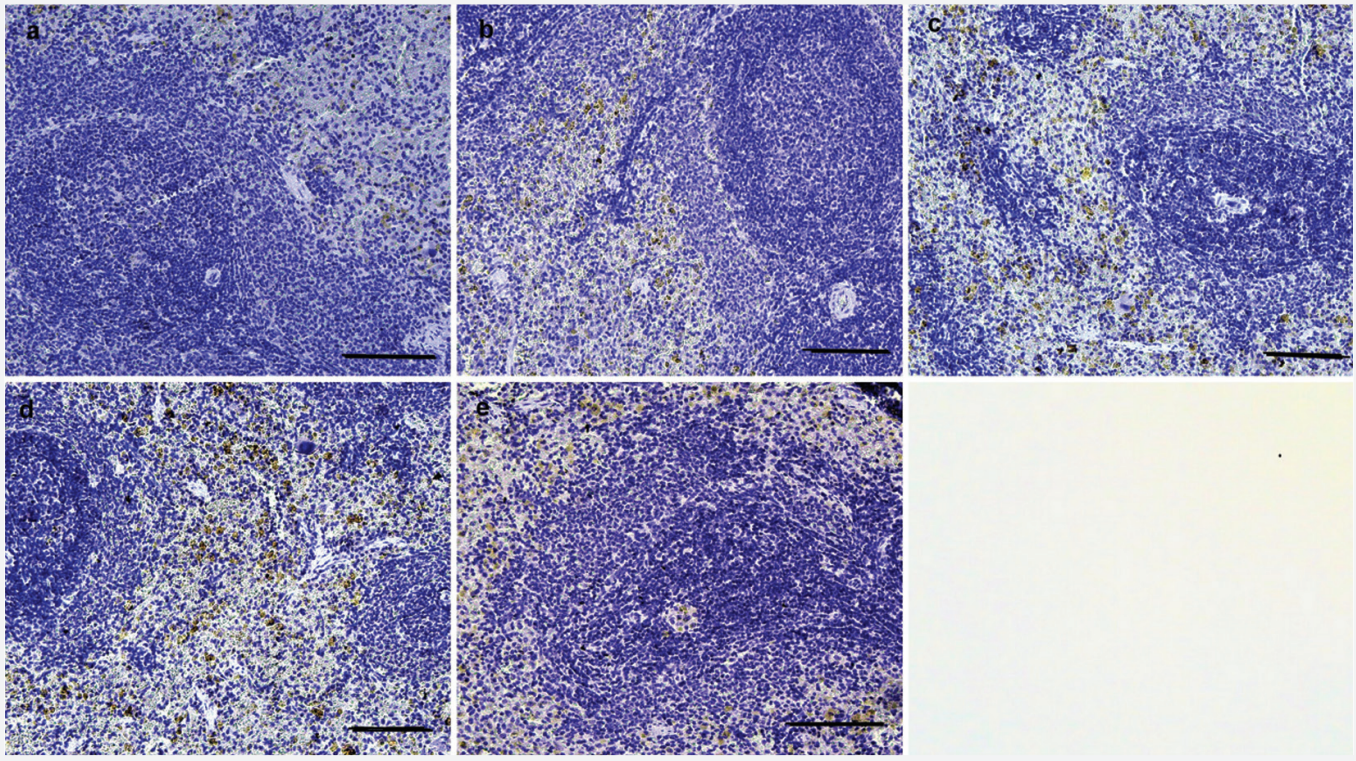

Figure 3. CD8 expression in spleen tissue. The control (a), sham (b), ginger (c), diabetes group (d), diabetes+ginger groups (e). Immunohistochemical staining. Bar=100 µm

Lymphoid organs such as the spleen are highly sensitive to various stresses, and exposure to disease causes splenic atrophy[18,20]. It was declared that germinal center of the lymphoid follicles was also dramatically reduced in diabetic rats[20,21]. Said et al.[3], (2020) declared that most follicles were atrophied with pyknotic nuclei in most of their cells. No germinal centers were seen in most of them. In animal models[3,22,23] and clinical studies[24], it was reported that ginger had a restorative effect against diabetes induced damage. In the current study, we administered ginger extract by oral gavage in diabetic rats for 30 days. We saw that ginger administration improved the histological and ultrastructural degenerative in the diabetic group. This improvement can be seen as increased number of lymph follicles in the white pulp and the appearance of the centrum germinativum.

Ginger is included in the list of safe herbs by the Food and Drug Administration. It has been reported that the use of up to 4 grams per day will not cause a pathological problem[9]. In a study conducted in patients with T2DM, it was reported that the use of ginger powder for 6-12 weeks at dosages up to 3 grams/day did not cause serious side effects[22]. It was declared that from 200 to 500 mg/kg/day for ginger extract had anti-inflammatory and antioxidative effects[9]. Also, ginger extract at the dose of 1000 mg/kg by oral administration is tolerated in pregnant rats. It was added that it exerted no adverse effects on pregnancy or the development of fetuses[25]. Before starting our experimental study, we examined many articles to design our experimental procedure, so we completed our work at the appropriate and effective dose (200 mg/kg) and time (30 days)[14]. Also, we found in our study that the dose and duration did not have serious adverse effects and complications in rats treated with ginger.

CD4 and CD8 T lymphocytes responsible for cellular immunity directly destroy harmful cells[26]. Many studies have emphasized that CD4 and CD8 T lymphocyte subtypes are effective in both T1DM and T2DM[27,28]. Miya et al.[27], (2018) investigated the effects of glucose loading on T lymphocytes in Japanese with T2DM and without diabetes. 75 g OGTT was applied to the participants. T lymphocytes were calculated at 60 and 120 minutes after fasting (12 hours, overnight). In the study, there was no difference in CD4 positive cell counts between the groups before glucose loading. However, they found that CD4 positive cell counts increased rapidly in both the T2DM and nondiabetic groups after 120 minutes of glucose loading.

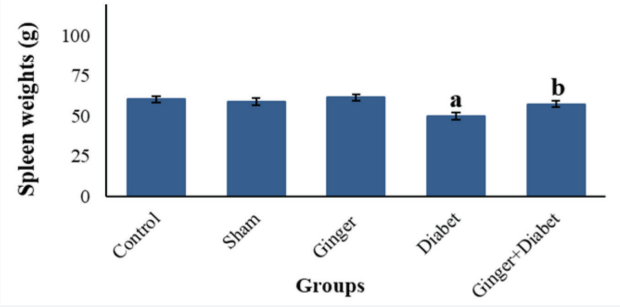

Figure 4. Comparison of the mean values of spleen weight among the groups. The control group-diabetes group p=0.007 (a), sham group-diabetes group p=0.05 (a), diabetes group-diabetes+ginger group p=0.021 (b)

The number of CD4 cells is important in the CD8 cell response. An increase in CD8 count causes an increase in CD4 count[29]. Li et al.[30], (2022) investigated CD4 expression in diabetic and normal mice. They revealed that while CD4 positive cells in the spleen, pancreas and kidney tissues were low in healthy mice, CD4 expression was significantly increased in organs of mice with DM. In the study, CD and CD8 positive cell counts of the diabetic group were found to be statistically higher than those of the control, sham and ginger groups.

Many serious side effects of synthetic drugs used in the treatment of immune system related diseases attract the attention of the consumers. Therefore, there is an increasing interest in therapeutic agents with fewer or no side effects[31]. Ginger is promising in this regard. The findings of the present study showed that ethanol extract of ginger activated T lymphocyte subtypes in term of CD4 and CD8 expression. This immunomodulatory activity of ginger may provide a future basis for the development of this plant as a source of immunoregulating substance in diabetes.

Study Limitations

Experimental diabetes was created in the study. After being treated with ginger, both the damage caused by diabetes and the anti-inflammatory effect of ginger were investigated in the spleen tissue. This study has a limitation. Immunohistochemical method was used only in this study. A more detailed evaluation can also be made using other methods such as western blot or polymerase chain reaction.

CONCLUSION

In the study, it was determined that CD4 and CD8 expression increased in rats with experimental diabetes. It was observed that the CD4 and CD8 expressions were equivalent to the control group, with the administration of ginger at 200 mg/kg for 30 days. It has been determined that ginger has an effect on eliminating the negative effects caused by diabetes and can be used as an immunomodulator.

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Ethics

Ethics Committee Approval: The study was approved by the Tekirdağ Namık Kemal University of Ethics Committee for Animal Experiments (meeting no: 09.11.2022/1153).

Informed Consent: Animal experiments.

Peer-review: Externally peer-reviewed.

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