Effects of black cumin seed oil on oxidative stress and expression of membrane-cytoskeleton linker proteins, radixin, and moesin in streptozotocin-induced diabetic rat liver

Ugur Seker1, Seval Kaya2, Sevgi Irtegun Kandemir3, Dila Sener4, Ozlem Unay Demirel5, Yusuf Nergiz2

1Department of Histology and Embryology, Harran University School of Medicine, Sanliurfa, Turkey; 2Department of Histology and Embryology, Dicle University School of Medicine, Diyarbakir, Turkey; 3Department of Medical Biology, Dicle University School of Medicine, Diyarbakir, Turkey; 4Department of Histology and Embryology, Bahcesehir University School of Medicine, Istanbul, Turkey; 5Department of Medical Biochemistry, Bahcesehir University School of Medicine, Istanbul, Turkey

Abstract

Background and Aim: This study examined the effects of black cumin seed oil treatment on oxidative stress and the expression of radixin and moesin in the liver of experimental diabetic rats.

Materials and Methods: Eighteen rats were divided into 3 equal groups (control, diabetes, treatment). The control group was not exposed to any experimental treatment. Streptozotocin was administered to the rats in the diabetes and treatment groups. A 2.5 mL/kg dose of black cumin seed oil was administered daily for 56 days to the treatment group. At the conclusion of the experiment, the blood level of malondialdehyde (MDA) and glutathione (GSH) was measured. The expression level and the cellular distribution of radixin and moesin in the liver were analyzed.

Results: The plasma MDA (3.05±0.45 nmol/mL) and GSH (78.49±20.45 μmol/L) levels in the diabetes group were significantly different (p<0.01) from the levels observed in the control group (MDA: 1.09±0.31 nmol/mL, GSH: 277.29±17.02 μmol/L) and the treatment group (MDA: 1.40±0.53 nmol/mL, GSH: 132.22±11.81 μmol/L). Immunohistochemistry and western blotting analyses indicated that while the level of radixin was not significantly between the groups (p=0.05) and moesin expression was significantly downregulated (p<0.05) in the experimental group, the treatment was ineffective.

Conclusion: The administered dose was sufficient to prevent oxidative stress, but was not sufficient to alleviate the effects of diabetes on moesin expression in hepatic sinusoidal cells.

Keywords: Diabetes; liver; moesin; radixin; rat.

Introduction

Diabetes is one of the most common metabolic diseases worldwide, and the incidence continues to increase. It has been reported that approximately 451 million people suffered from diabetes-related complications in 2017, and that figure is estimated to increase to 693 million by 2045.[1] In diabetic conditions, the effects on lipid and protein metabolism can lead to life threatening complications, such as nephropathy, retinopathy, hepatopathy, vasculopathy, and neuropathy.[2] Both morphological and biochemical degeneration in liver may be observed. Increased oxidative stress, apoptotic cell death, and an increased level of hepatic enzymes in blood demonstrate reduced organ function and injury. Diabetes-related liver injury has been associated with various cellular mechanisms.[3-8] Several experimental studies have been performed to find the optimal treatment method for insulin-dependent diabetic liver injury with the fewest side effects and lowest cost, and the large number diabetes-related articles demonstrate the topicality of the current study. Some of this research has examined the possibility of alleviating streptozotocin (STZ)-induced diabetic complications with herbal medicine. Black cumin (Nigella sativa) seed oil and its major compound, thymoquinone, has exhibited anti-hyperglycemic activity in STZ-induced diabetes, and the results have suggested that the treatment had a protective effect on diabetes-related organ complications.[6,7] Diabetes can lead to cellular cytoskeleton changes, which may affect cellular functions.[8] The cellular skeleton consists of filamentous proteins, including actin filaments, which are cross-linked to the plasma membrane by members of the ezrin-radixin-moesin (ERM) protein family.[9] ERM proteins have specific tissue expression levels and functions. For example, radixin is expressed in the liver, and moesin is expressed in the spleen, lungs, kidneys, lymphocytes, and vascular endothelial cells.[10,11] Radixin is typically expressed in hepatocytes and bile canaliculi to act as a scaffold protein for multidrug resistance-associated protein 2.[12] Previous studies have reported that moesin activates hepatic stellate cells during fibrosis formation in ischemic liver injury.[13,14] In diabetes, expression of the third member of the ERM protein family, ezrin, is reduced in podocytes of the kidney. It has been suggested that ERM proteins contribute to the pathogenesis of diabetic nephropathy.[15] The available literature on relationships between ERM family members and diabetic liver injury is very limited. The objective of this study was to investigate the effects of black cumin seed oil on oxidative stress status and expression-distribution of the cytoskeletal proteins, radixin and moesin, in the liver of experimental STZ-induced diabetic rats.
Materials and Methods

Experimental Design

The experimental process used in the study is illustrated in Figure 1. The research was approved by the Local Experimental Animal Ethics Committee of Dicle University (date: 28.03.2019, number: 2019/04), and all of the procedures were performed according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Eighteen adult male Wistar Albino rats weighing 283.61±27.93 g supplied by the Health Sciences Application and Research Center of Dicle University were divided into 3 groups: control (n=6), diabetes (n=6), and treatment (n=6). All of the rats were housed in polycarbonate laboratory animal cages with standard pellet feed and water available ad libitum. Standard room temperature (21±3°C) and humidity (50±5%) were maintained, as well as a 12-hour day and night cycle. A 1-week adaption period was observed before beginning the experimental process.

Induction of Experimental Diabetes

The blood glucose level of the animals was measured from the tail vein with a portable glucometer prior to initiating the experiment. The rats in the control group were not subjected to any treatment and were sacrificed at the conclusion for comparison. A single intraperitoneal injection of 45 mg/kg STZ dissolved in freshly prepared ice-cold 0.1 M citrate buffer was administered to the rats in the diabetes and treatment groups to induce diabetes. Water with 10% glucose was provided ad libitum for 48 hours to avoid hypoglycemia. The blood glucose level was measured 72 hours after the STZ injection and a value of >240 mg/dL was considered diabetic. A daily dose of 2.5 mL/kg of commercially produced black cumin seed oil was orally administered to the rats in the treatment group. Previous research has indicated that this dosage of the plant extract provided anti-hyperglycemic and hepatoprotective effects.[16,17] The experiment continued for 56 days following confirmation of experimental diabetes, after which the animals were euthanized. Blood was withdrawn from heart and liver tissue samples and the blood was centrifuged and stored at -20°C for further analysis. Excised liver tissue samples were fixed in Bouin’s fixative for histopathological examination and frozen at -80°C for western blotting.

Biochemical Analysis

The blood samples were immediately centrifuged at a force of 1370 xg at 4°C for 10 minutes and the supernatant was carefully collected. The plasma was mixed with thiobarbituric acid and boiled at 95°C for 30 minutes. In order to measure the level of malondialdehyde (MDA) level, the by-products of lipid peroxidation - thiobarbituric acid reactive substances - were measured using the process described by Yagi et al.[18] The extinction coefficient of 1.56×105 M−1 cm−1 was used to determine the MDA equivalent expressed as nmol/mL. Glutathione (GSH) was measured using a modified version of the protocol previously described using Ellman’s reagent. The supernatant was mixed with sodium phosphate dihydrate solution and dithio-bis nitrobenzoic acid. Following thorough mixture, the absorbance was quickly measured at 412 nm. The GSH level was obtained using the 13,600 M−1 cm−1 extinction coefficient and the result was expressed as μmol/L.[19]

Tissue Processing and Immunohistochemistry

The fixed tissue samples were washed under tap water, dehydrated with a portable glucometer prior to initiating the experiment. The rats in the control group were not subjected to any treatment and were sacrificed at the conclusion for comparison. The experiment continued for 56 days following confirmation of experimental diabetes, after which the animals were euthanized. Blood was withdrawn from heart and liver tissue samples and the blood was centrifuged and stored at -20°C for further analysis. Excised liver tissue samples were fixed in Bouin’s fixative for histopathological examination and frozen at -80°C for western blotting.

Figure 1. Illustration of the study design protocol and the results.
Western Blotting
Liver samples (60 mg) frozen with liquid nitrogen were pulverized, mixed with 300 µL lysis buffer (cat no: 1861284; Thermo Scientific, Waltham, MA, USA), and incubated on ice for 1 hour. The total protein value of each sample was measured with a bicinchoninic acid kit (cat no: 21071; iNiRON Biotechnology, Seongnam, Gyeonggi, South Korea.). The protein lysate was mixed with 2X Laemmli buffer and boiled at 95°C for 5 minutes. A total of 25 µg of protein sample was then separated in 10% tris-glycine extended stain-free gel at 200 V for 20 minutes. The separated proteins were transferred to a polyvinylidene fluoride membrane and blocked for 1 hour at room temperature in 5% skim milk dissolved in phosphate buffered saline with Tween (PBS-T) (Twen: ICI Americas, Wilmington, DE, USA). The membranes were incubated with 1:1000 diluted radixin (cat no: ab52495; Abcam, Cambridge, UK), moesin (cat no: ab151542; Abcam, Cambridge, UK), and β-actin (cat no: sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies for 2 hours at room temperature. The membranes were washed in PBS-T and incubated with 1:10000 diluted secondary antibodies for 1 hour at room temperature. Immunoblotting was performed using enhanced chemiluminescence substrate (cat no: 1705061; Bio-Rad Laboratories, Hercules, CA, USA). The bands were monitored with chemiluminescence imaging equipment (ChemiDoc MP; Bio-Rad Laboratories, Hercules, CA, USA) and captured with Image Lab 6.1 Software (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of the bands was quantitated using Image-J software Version 1.46 (US National Institutes of Health, Bethesda, MD, USA).

Threshold Analysis of Immunostaining
Sixteen randomly selected liver lobules containing a central vein were used to quantify the intensity of the antigen in each group and used for threshold analysis. The total lobule and DAB positive areas were measured using Image-J Version 1.46 software, the results were converted to a ratio indicating the immunopositivity intensity for each lobule, and the results were analyzed statistically.

Immunoblotting Quantification
The immunoblotting band intensity and thickness were analyzed using Image-J Version 1.46 software. The plot lane plug-in was used to measure band intensity within a rectangle. The peak of each plot was enclosed, and the software calculated a percentage automatically. The thickness for each interest protein band was compared according to β-actin band thickness. The measurement was repeated using 3 randomly selected exposure times and the results were analyzed statistically.

Statistical Analysis
Due to the limited sample size and the distribution of the values of each group, the non-parametric Kruskal-Wallis test was used to evaluate statistical significance. Multiple comparisons between groups were evaluated with Tamhane’s T2 test. The results were expressed as mean±SD and p<0.05 was considered significant.

Results
Biochemical Analysis
The lowest lipid peroxidation was measured in the control group, and the MDA level demonstrated a significant increase (p<0.01) in the diabetes group. The mean plasma lipid peroxidation level in the black cumin seed oil-treated animals had declined and was similar to that of the controls (p>0.05). The mean GSH level of the control group was the highest, and it was dramatically lower (p<0.01) in the diabetes group. The mean plasma lipid peroxidation level in the black cumin seed oil-treated animals had declined and was similar to that of the controls (p>0.05). The mean GSH level of the control group was the highest, and it was dramatically lower (p<0.01) in the diabetes group. The mean GSH level of the treatment group was between that of the control and diabetes groups, and the difference was significant (p<0.01). The biochemical analysis results are shown in Table 1 and Figure 2.

| Control | Diabetes | Treatment |
|---------|----------|-----------|
| MDA (nmol/mL) | 1.09±0.31a | 3.05±0.45b | 1.40±0.53b | <0.01 |
| GSH (μmol/L) | 277.29±17.02a | 78.49±20.45b | 132.22±11.81c | <0.01 |

Statistical significance of group comparisons: a, b: P<0.01; c–e P<0.01; GSH: Glutathione; MDA: Malondialdehyde.

Immunohistochemical and Threshold Analyses Results
Microscopic investigation indicated that hepatocytes were intensely radixin-positive. Immunopositivity was also observed in the sinusoidal endothelial cells and hepatic stellate cells of the control group. In particular, radixin positivity was evident in the plasma membrane, and cytosolic accumulation of this protein was observed in some hepatocytes. The microscopic cellular distribution of radixin in the control, diabetes, and treatment groups was similar (Fig. 3). Threshold analysis of radixin indicated that the positivity was 46.13±13.61% in the control group, while it was 41.67±20.99% and 48.66±14.06% in the diabetes and treatment groups, respectively. The statistical analysis was consistent with light microscopic observations and there was no significant difference between groups (p>0.05). Hepatocytes were observed to be immunonegative for moesin; immunopositivity was only observed in hepatic sinusoidal cells and hepatic stellate cells (Fig. 4). Light microscopic evaluation indicated that the distribution in some hepatic lobules differed significantly between groups. The result of threshold analysis of moesin in the control group was 9.37±3.08%. Moesin immunopositiv-
Immunoblotting intensity was measured by comparing the band intensity of a protein of interest using β-actin as a loading control. The results of the statistical analyses demonstrated that the radixin and moesin band intensity ratio of the control group was 5.26±1.04 and 1.55±0.08, respectively. The ratios were 4.64±1.25 and 0.64±0.24, respectively, in the diabetes group, and 4.73±1.23 and 0.44±0.24 in the group treated with black cumin oil. The intensity ratio of radixin between groups was similar (p>0.05), but the moesin ratio was significantly lower (p<0.05) in the diabetes and treatment groups (Fig. 6).

Discussion
A minimum of 17% of diabetes patients are predicted to have liver disease. Both clinical and experimental studies have demonstrated that oxidative stress plays a crucial role in diabetes-induced liver disease. It has been reported that diabetes may be linked to upregulation of inflammatory cytokines and activation of adaptive and innate immune responses.
cells linked to diabetic complications. The primary cause of liver injury in diabetic patients is thought to be a result of hyperglycemia-induced oxidative stress and other complications of carbohydrate, lipid, and protein metabolism. These complications boost the generation of excessive oxidative stress and the induction of inflammatory cascades. Widespread pathological changes, including structural degenerations, have been observed in cases of diabetes and increased oxidative stress. It has also been reported that the oxidative stress may provoke cellular cytoskeleton actin depolymerization cross-linked to the cellular membrane by a group of proteins, ezrin-radixin-moesin (ERM). In addition to the structural function, these proteins have been reported to contribute to cellular activities, such as cell migration, adhesion, cell polarization, motility, growth, fibrosis, and even membrane trafficking. Although there are numerous previously published articles related to STZ-induced diabetic liver complications, the current literature regarding the potential influence of radixin and moesin in diabetes is very limited. It has been reported that increased hepatic DNA fragmentation was thought to be a result of oxidative and glycative stress in STZ-induced diabetes. Gosh et al. found that STZ-induced diabetes provoked overexpression and activation of apoptotic proteins, such as p53, Bcl-2-associated X-protein, Bcl-XL/Bcl-2-associated death promoter, and cytochrome C. The authors concluded that the administration of the plant-derived antioxidant curcumin might be a successful strategy to protect the liver in STZ-induced experimental diabetes. Other studies have indicated that black cumin may function as an anti-hyperglycemic and antioxidant in STZ-induced diabetes. The anti-fibrotic activity of this plant has also been noted in a carbon tetrachloride-induced experimental liver injury model. Furthermore, Almatroodi et al. investigated potential protective and anti-hyperglycemic effects of the most prominent constituent of black cumin, thymoquinone, against STZ-induced diabetes, and their results indicated that STZ increased the blood glucose level and upregulated the serum level of liver function enzymes and lipid peroxidation. The findings suggested that the administration of thymoquinone reduced diabetic complications in laboratory animals. Interestingly, intense collagen accumulation has been linked with greater fibrosis formation in insulin-independent diabetes compared with normal circumstances. It has been established that radixin is expressed in liver hepatocytes in healthy individuals, and is necessary to maintain hepatocyte apical canalicular membrane function and structure. Kawase et al. observed that radixin expression in the liver decreased in response to inflammation. Another recently published article also noted that moesin phosphorylation was upregulated during hepatic stellate cell activation and fibrogenesis in an experimental animal liver injury model. Our immunostaining results regarding the cellular distribution of moesin in the liver were similar, however, our findings regarding the moesin expression level were somewhat different. The immunohistochemistry and immunoblotting results in our study indicated that moesin expression was significantly downregulated in an STZ-induced diabetic liver and upregulation was not observed in the rats treated with black cumin seed oil. The difference in the moesin level between the 2 studies might be a result of liver injury induction model. Karvar et al. generated liver fibrosis model using administration of carbon tetrachloride and common bile duct ligation, while our study was designed with a STZ-induced diabetic liver model that rarely results in fibrosis. There are some limitations to this study. First of all, we did not investigate the expression level of the third member of the ERM protein family, ezrin. Analysis of the expression level of all phosphorylated proteins would provide more details. Previously published articles have also investigated just 1 or 2 members of the ERM protein family members while investigating the role of these proteins on various cellular mechanisms. Nonetheless, our results provide some potentially valuable information about STZ-induced diabetic liver complications and potential contributions of these proteins to liver dysfunction as well as the effects of black cumin seed oil administration.

**Conclusion**

To the best of our knowledge, this is the first study to investigate the expression of radixin and moesin in an STZ-induced diabetic rat liver and the effects of black cumin seed oil treatment. In brief, results of our study indicated that black cumin seed is a strong antioxidant. In addition, in tissue level the Radixin expression has not been changed, but Moesin expression reduced in STZ induced diabetic liver and black cumin seed oil treatment was failed. We believe the lack of significant response in moesin expression might be related to an inadequate dose or greater sensitivity of hepatic sinusoidal cells to diabetic complications.

**Ethics Committee Approval:** The Dicle University Local Experimental Animal Ethics Committee granted approval for this study (date: 28.03.2019, number: 2019/04).

**Author Contributions:** Concept – US, SK, YN; Design – US, SK; Supervision – SIK, DS, OUD, YN; Fundings – US, SK, YN; Materials – US, SIK, DS, OUD; Data Collection and/or Processing – US, SIK, DS, OUD; Analysis and/or Interpretation – US, SIK; Literature Search – US, SK, SIK, DS, OUD, YN; Writing – US, SIK, DS; Critical Reviews – SIK, DS, OUD, YN.

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Financial Disclosure:** This research was partially supported by Scientific Research Projects Coordination of Dicle University (grant no: TIP.19.018).

**References**

1. Cho N, Shaw J, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract 2018;138:271-281. [CrossRef]
2. Amos AF, McCarty DJ, Zimmet P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 1997;14(Suppl 5):S1-S85. [CrossRef]
3. Harrison SA. Liver disease in patients with diabetes mellitus. J Clin Gastroenterol 2006;40(1):68-76. [CrossRef]
4. Manna P, Das J, Ghosh J, Sil PC. Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IkappaBalpha/NF-kappaB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunic acid. Free Radic Biol Med 2010 Jun 1;48(11):1465-1484. [CrossRef]
5. Rodriguez V, Plavnik L, de Talamoni NT. Naringin attenuates liver damage in streptozotocin-induced diabetic rats. Biomed Pharmacother 2018;105:95-102. [CrossRef]
6. Maksymchuk O, Shysh A, Rosohatska I, Chashchyn M. Quercetin prevents type 1 diabetic liver damage through inhibition of CYP2E1. Pharmacol Rep 2017;69(6):1386-1392. [CrossRef]
7. Meral I, Yener Z, Kahraman T, Mert N. Effect of Nigella sativa on glucose concentration, lipid peroxidation, anti-oxidant defence system and liver damage in experimentally-induced diabetic rabbits. J Vet Med A Physiol Pathol Clin Med 2001;48(10):593-599. [CrossRef]
8. Shimoni Y, Rattner JB. Type 1 diabetes leads to cytoskeleton changes that are reflected in insulin action on rat cardiac K(+) currents. Am J Physiol Endocrinol Metab 2001;281(3):E575-E585. [CrossRef]
114. [CrossRef]

11. Ingraffea J, Reczek D, Bretscher A. Distinct cell type-specific expression of ERM proteins. Cell 1993;72(3):1073-1084. [CrossRef]

10. Berryman M, Franck Z, Bretscher A. Ezrin is concentrated in the apical membrane-cortex interactions and signaling. Curr Opin Cell Biol 2011;23(4):377-382. [CrossRef]

9. Neisch AL, Fehon RG. Ezrin, Radixin and Moesin: key regulators of membrane-cortex interactions and signaling. Curr Opin Cell Biol 2011;23(4):377-382. [CrossRef]

8. Yagi K. Assay for blood plasma or serum. Methods Enzymol 1984;105:328-331.

7. Beutler E. Reduced glutathione (GSH). Red Cell Metabolism 1971:103-105.

6. Al-Hussaini AA, Sulaiman NM, Alenizi AS, Khan M. Prevalence of hepatopathy in type 1 diabetic children. BMC Pediatr 2012;12:160.

5. Almatroodi SA, Alnuqaydan AM, Alsahli MA, Khan AA, Rahmani AH. Thymoquinone, the most prominent constituent of Nigella sativa, attenuates liver damage in streptozotocin-induced diabetic rats via regulation of oxidative stress, inflammation and cyclooxygenase-2 protein expression. Appl Sci 2021;11(7):3223.

4. Kobt T, Gehrke N, Schad A, Nagel M, Wörns MA, Sprinzl MF, et al. Diabetic liver injury from streptozotocin is regulated through the caspase-8 homolog cFLIP involving activation of JNK2 and intrahepatic immunocompetent cells. Cell Death Dis 2013;4(7):e712. [CrossRef]

3. Allani PK, Sum T, Bhanarsi SG, Mukherjee SK, Sonee M. A comparative study of the effect of oxidative stress on the cytoskeleton in human cortical neurons. Toxicol Appl Pharmacol 2004;196(1):29-36. [CrossRef]

2. Louvet-Valée S. ERM proteins: from cellular architecture to cell signaling. Biology of the Cell 2000;92(5):305-316. [CrossRef]

1. Andican G, Burčak G. Oxidative damage to nuclear DNA in streptozotocin-diabetic rat liver. Clin Exp Pharmacol Physiol 2005;32(8):663-666. [CrossRef]

0. Ghosh S, Bhattacharyya S, Rashid K, Sil PC. Curcumin protects rat liver from streptozotocin-induced diabetic pathophysiology by counteracting reactive oxygen species and inhibiting the activation of p53 and MAPKs mediated stress response pathways. Toxicol Rep 2015;2:365-376. [CrossRef]