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The role of Nrf2/SIRT1 pathway in the hepatoprotective effect of PEITC against HFD/STZ-induced diabetic liver disease

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Abstract

Aim: We intended to assess the hepatoprotective effects of phenethyl isothiocyanate (PEITC) against oxidative liver injury induced by a high-fat diet (HFD) and streptozotocin (STZ) diabetes through the Nuclear Factor E2-Related Factor 2 (Nrf2) and Sirtuin 1 (SIRT1) pathways in rats.

Materials and Methods: Thirty male Wistar Albino rats were separated into three groups: the control group, the second group (HFD+STZ) fed HFD and injected with STZ (35mg/kg b.w.), and the third group (HFD+ STZ+PEITC) fed an HFD, injected with STZ (35mg/kg b.w.), and given PEITC (40mg/kg b.w. by oral gavage). Feeding with HFD and PEITC was given for two weeks and continued one more week following STZ. Serum ALT and lipids levels, antioxidant enzyme activities, MDA, GST, SIRT1, NF-$\kappa\beta$, and Nrf2 levels were measured. Liver histological changes were detected.

Results: In comparison with the control group, in the HFD+STZ group, serum HDL levels, activities of hepatic antioxidant enzymes, Nrf2 activity, levels of GST, SIRT1, and NF-$\kappa\beta$ reduced and besides, serum ALT, TG, TC, LDL / VLDL, and hepatic MDA levels increased ($p < 0.05$). PEITC pre-administration led to improvement in these changes made by HFD-STZ ($p < 0.05$).

Conclusion: Our data presented that PEITC ameliorated hepatic injury and serum lipid profile induced by HFD and STZ via the activation of Nrf2 and SIRT1 pathways. We can suggest that PEITC could be a possible candidate agent against liver diseases.

Introduction

Diabetes Mellitus (DM) is a metabolic disease that causes various micro and macro complications characterized by hyperglycemia. Hyperglycemia, inflammation and dyslipidemia that occur with diabetes cause oxidative stress and damage to many organs [1]. It is known that there is a relationship between diabetes and liver diseases. Liver injury as a result of diabetes can lead in serious liver diseases such as non-alcoholic fatty liver disease (NAFLD) [2]. NAFLD is the liver appearance of the metabolic disorders. The incidence of NAFLD is higher in diabetes, hyperlipidemia and obesity [3]. Although there are still incompletely clarified areas, it is accepted that oxidative stress contributes to the development of NAFLD [4]. Thus, reducing oxidative stress in diabetes can diminish liver damage and the generation of serious liver diseases that increase mortality from DM [5].

The Nrf2 pathway has been recently investigated as a target in studies with DM. It is indicated that natural products can prevent liver injury induced diabetes via increasing the activation of Nrf2 that suppress oxidative stress, increase insulin sensitivity, and as well as inhibition of inflammatory pathways [6]. Nrf2 is a substantial transcription factor that modulates cytoprotective genes against the over-oxidative condition. It is normally accommodated in the cytoplasm as bound to a protein called Kelch-like ECH-associated protein 1 (Keap1) or INrf2, which is a cytosolic inhibitor. In cases of increased cellular oxidative stress, it cleaves from INrf2 and binds to the antioxidant response element (ARE) region in the nucleus. It leads to increasing the upregulation of many antioxidant and detoxification genes [7]. Additionally, Nrf2 has a critical role in inflammation via pro-inflammatory transcription factor NF-$\kappa\beta$ suppression [8].

Sirtuin 1, as a protein deacetylase, has a considerable role in the preservation of cellular oxidative stress by triggering the upregulation of Nrf2 [9]. It contributes to preventing fatty liver disease progression through enhancing antiox-

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idant capability and deacetylating NF-$\kappa\beta$ [10]. Nrf2 and SIRT1 could play an antioxidative role in when overproduction of free radicals.

Phenethyl isothiocyanate is a phytochemical in Cruciferae plants and is produced by hydrolysis of gluconasturtiin with myrosinase. PEITC suppresses oxidative stress by causing Nrf2 activation and protects to cell injury [11] and ameliorates the liver damage [12]. It also reported to inhibit adipogenesis and hepatosteatosis in high-fat diet induced obesity [13]. So, we hypothesized that activation of SIRT1 and Nrf2 by PEITC could contribute to hepatoprotective effect against liver injury caused by HFD and STZ-induced diabetes.

Materials and Methods

Animals and experimental design

This study was performed in compliance with the "Ethical Guidelines for Animal Use" after receiving the approval of the İnönü University, Faculty of Medicine, Local Ethics Committee (decision date: 16.04.2019 and number: 2018/A-49). The power analysis was performed using G*Power 3.1 (Dusseldorf Germany). Alpha coefficient was stated as 0.05, and the power was determined as 0.95 and the minimum number of sample required for the study was determined as 30 animals. Throughout the study, the rats were accommodated in rooms suitable for a 12-h light/dark cycle in 22-24°C at polypropylene cages and were fed ad libitum and tap water. The study included 30 male Wistar albino rats (6-8 weeks-old, weighing 150-250 g). The rats were randomly divided into 3 groups containing 10 rats each: Control group (C) were fed ad libitum. The second group (HFD+ STZ) received high-fat diet (HFD energy content: roughly 41,4% fat, 14,7% protein, and 38,7% carbohydrate) and injected with STZ (35mg/kg b.w; dissolved in 0.1 M citrate buffer, pH:4.5. Sigma-Aldrich, USA), and the third group (HFD+ STZ+PEITC) received high-fat diet and injected with STZ (35mg/kg b.w.), and PEITC (40mg/kg b.w. by oral gavage; dissolved in 1% DMSO solution. Sigma-Aldrich, USA) for two weeks [14]. Followed injection with STZ intraperitoneally, it was continued to give HFD and PEITC for approximately one week.

Sample collection

After one week of the STZ induction, the fasting blood glucose of the rats was measured. Blood glucose values of 300 mg/dL and above were accepted diabetes. The rat blood samples were collected under anesthesia with xylazine/ketamine and serum was obtained by centrifuging at 2000 g for 10 min. Serum ALT and lipid levels were measured. A portion of liver tissues were kept in 10% formaldehyde until histopathological examinations. The other portions of liver tissues were homogenized in 50 mM phosphate buffer (pH 7.4) and were centrifuged at 15000 g and +4°C for 15 min. Then antioxidant enzyme activities, MDA, GST, and SIRT1 levels were measured. Nuclear extracts from rat liver tissues were prepared using a nuclear extraction kit (Abcam, USA). Nrf2 and NF-$\kappa\beta$ levels were measured in nuclear extracts. The biochemical analyses were performed using a Hybrid Multi-Mode Microplate Reader (Biotek Synergy H1, USA).

Biochemical assays

Protein quantification was performed by the Bradford method [15]. Serum ALT activity was measured with the Alanine Aminotransferase Assay Kit (Bioassay Technology Laboratory, China). Triglyceride Colorimetric Assay Kit (Elabscience, USA) was used for Triglyceride measurement. Serum TC, HDL and LDL/VLDL levels were determined using HDL-LDL/VLDL Cholesterol Quantification Kit (Biovision, USA). MDA measurement was carried out using the method reported by Uchiyama and Mihara [16]. Tetra methoxy propane (20-200 nmol/L) was used as the standard. SOD activity was measured by using the method developed by Sun et al. [17]. CAT activity was measured by using the method of Aebi [18]. GPx activity was measured by using the procedure described by Paglia and Valentine [19]. Results of antioxidant enzymes were expressed as U/mg protein. Glutathione S-Transferase (GST) levels were measured with a Rat Glutathione S-Transferase ELISA kit (Bioassay Technology Laboratory, China). SIRT1 level was measured with a Rat Sirtuin-1 ELISA kit (Bioassay Technology Laboratory, China). The DNA binding activity of Nrf2 was determined with an Nrf2 Transcription Factor assay kit (Abcam, USA). NF-$\kappa\beta$ p65 ELISA kit (Elabscience, USA) was used to measure NF-$\kappa\beta$ level.

Histopathological examination

The liver tissue samples taken for histopathological examinations were fixed in 10% formaldehyde, embedded in paraffin, and cut into 5 µm thick slices. The hematoxylin-eosin (H-E) staining method was used for the light microscopic examinations of liver tissues. The microscopic damage for each criterion was defined as none (0), mild (1), moderate (2), and severe (3). The total score was calculated based on these parameters. The slides were examined and photographed by using a Leica DFC 280 light microscope and the Leica Q Win Image Analysis System.

Statistical analysis

For the analysis of the biochemical measurements data, the IBM SPSS Statistics 22.0 for Windows package program was used. The results are expressed as arithmetic means and standard deviations. The normality of the data distribution was tested using the Shapiro-Wilk test. Analysis of variance (ANOVA) was used in the intergroup comparisons among parametric tests, and pairwise comparisons were made using the LSD test. The level of statistical significance was accepted as $p<0.05$.

The statistical analyses on the histological examinations were conducted by using the SPSS and MedCalc programs. The mean values of all groups were compared using the non-parametric Kruskal-Wallis test. Mann-Whitney U test was used to conduct pairwise comparisons to determine whether or not the intergroup differences were significant. $p<0.0001$ was accepted as statistically significant.

Results

PEITC decreases serum ALT level

Serum ALT level was significantly enhanced with HFD+STZ induction compared to control. It was signif-

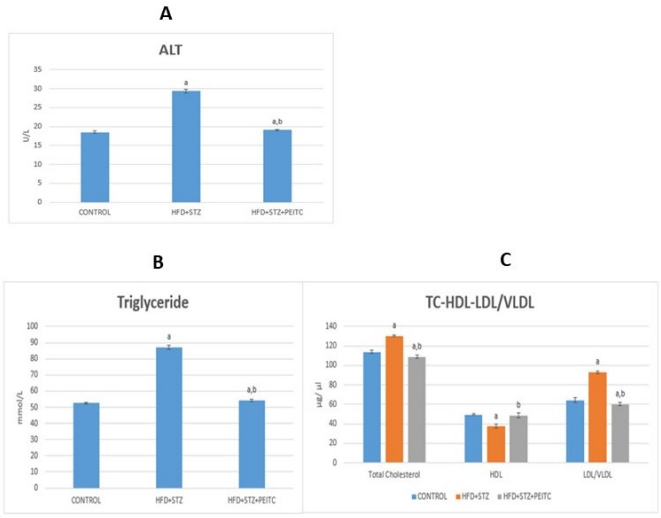

Figure 1. A) Effects of PEITC on serum ALT in all groups B) Effects of PEITC on levels of serum TG C) Effects of PEITC on levels of serum TC, HDL and LDL/VLDL levels. Data are presented as mean ± S.D. [a]: $p < 0.05$ versus control; [b]: $p < 0.05$ versus HFD+STZ.

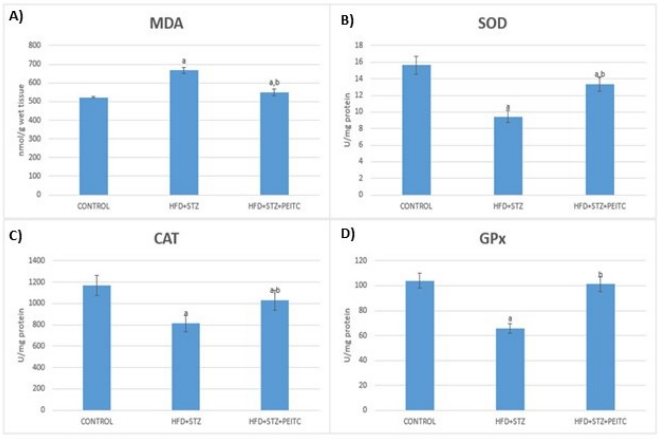

Figure 2. Effects of PEITC on oxidative stress parameters in all groups A) MDA levels B) SOD activity C) CAT activity D) GPx activity. Data are presented as mean ± S.D. [a]: $p < 0.05$ versus control; [b]: $p < 0.05$ versus HFD+STZ.

icantly reduced with PEITC pretreatment compared to HFD+STZ -induced rats. ($p<0.05$) (Figure 1A).

PEITC affects levels of serum lipid profile

It was observed that the amount of serum TG, TC and LDL/VLDL increased and the amount of HDL decreased in rats treated with HFD+STZ ($p<0.05$). It was found that the amount of serum TG (Figure 1B), TC and LDL/VLDL decreased and the amount of HDL (Figure 1C) increased in the HFD+STZ+PEITC group ($p<0.05$).

PEITC alleviates HFD+STZ -induced oxidative injury

Liver MDA levels were markedly raised with HFD+STZ-administration. The PEITC pre-administration signif-

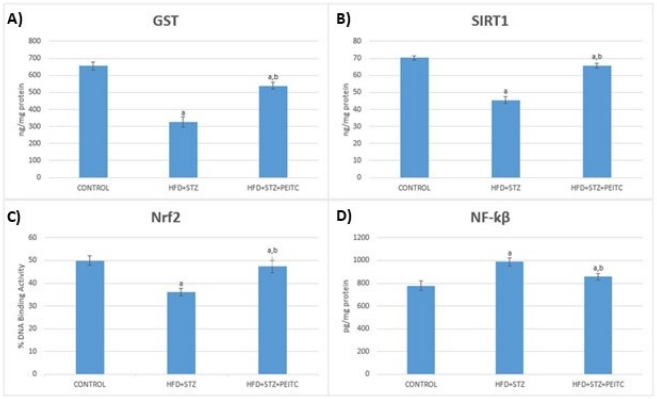

Figure 3. Effects of PEITC on the Nrf2 activity, levels of GST, SIRT 1, and NF-$\kappa\beta$ in all groups. A) GST levels B) SIRT1 levels C) Activity of Nrf2 D) NF-$\kappa\beta$ levels. Data are presented as mean ± S.D. [a]: $p < 0.05$ versus control; [b]: $p < 0.05$ versus HFD+STZ.

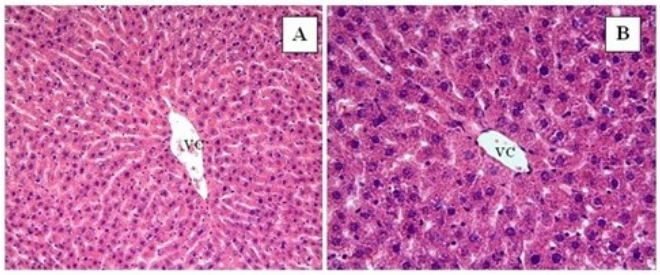

Figure 4. (A, B). Control group. Normal histological appearance of liver tissue. A:H-E; X20, B: H-E; X40.

icantly reduced MDA levels as compared to animals administrated with HFD+STZ ($p<0.05$) (Figure 2A). HFD+STZ administration caused reduced enzymatic activities of SOD, GPx, and CAT. Pretreatment of PEITC significantly increased the activities of SOD, CAT, and GPx comparison to the HFD+STZ group. ($p<0.05$) (Figure 2B, 2C, 2D).

PEITC increases levels of GST, and SIRT1 and activity of Nrf2

The level of GST was reduced in the HFD+STZ-group rats significantly as compared to control rats. PEITC administration led to a significantly increased level of GST compared to HFD+STZ-given rats ($p<0.05$) (Figure 3A). SIRT1 level was reduced in HFD+STZ-induced rats significantly as compared to control rats. PEITC led to a significantly increased level of SIRT1 compared to HFD+STZ -induced rats ($p<0.05$) (Figure 3B). Nrf2 activity was significantly diminished after HFD+STZ-induced in comparison to the control group ($p<0.05$). PEITC treatment showed a significant elevation in Nrf2 activity compared to HFD+STZ group ($p<0.05$) (Figure 3C).

PEITC attenuates NF-$\kappa\beta$ level changes

The NF-$\kappa\beta$ levels were significantly elevated in the HFD+STZ group in comparison to the control group and

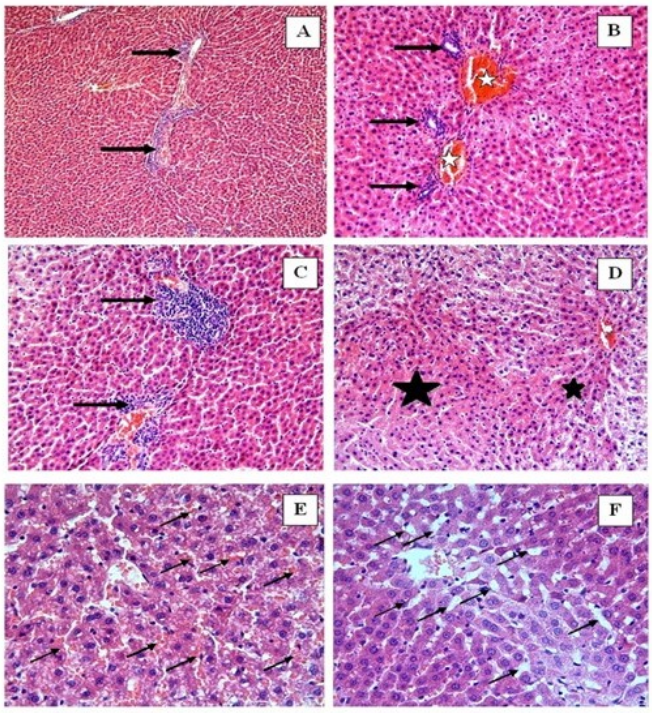

Figure 5. (A, B, C, D, E, F). Mononuclear cell infiltration (arrows) (Figure 5A, 5B, 5C), vascular congestion (white star) (Figure 5B, 5D), hepatocytes with eosinophilic cytoplasm and pyknotic nuclei (black star) (Figure 5D), hemorrhage (black thin arrows) (Figure 5E), vacuolization (Figure 5E) and sinusoidal dilatation (black arrows) (Figure 5F) were observed in HFD+STZ-induced group. A: H-E; X10, B, C, D: H-E; X20, E, F: H-E; X40.

pretreatment with PEITC led to a significant decrease in NF-$\kappa\beta$ levels ($p<0,05$) (Figure 3D).

PEITC reduces histopathological damage induced by HFD+STZ

The pathologic analyses of the samples of the groups showed that the control group had a normal histological structure of liver cells (Figure 4A and 4B). In the HFD+STZ group, mononuclear cell infiltration (arrows) (Figure 5A, 5B, 5C), vascular congestion (white star) (Figure 5B, 5D), hepatocytes with eosinophilic cytoplasm and pyknotic nuclei (black star) (Figure 5D), hemorrhage (black thin arrows) (Figure 5E), vacuolization (Figure 5E) and sinusoidal dilatation (black arrows) (Figure 5F) were observed. In the HFD+STZ+PEITC group, however, histopathological damage in the liver tissue was observed to be significantly reduced. A small amount of mononuclear cell infiltration (white arrow) (Figure 6A, 6B), vascular congestion (black arrow) (Figure 6A, 6B), hepatocytes with eosinophilic cytoplasm and pyknotic nuclei (black arrows) (Figure 6C) were observed. The liver damage score of the groups were control group (0.49 ± 0.07^{a}), HFD+STZ group (2.39 ± 0.09^{b}), and HFD+STZ+PEITC group (1.86 ± 0.08^{c}), respectively (Mean ±SEM). The lowercase letters a, b, and c indicate the differences between the groups.

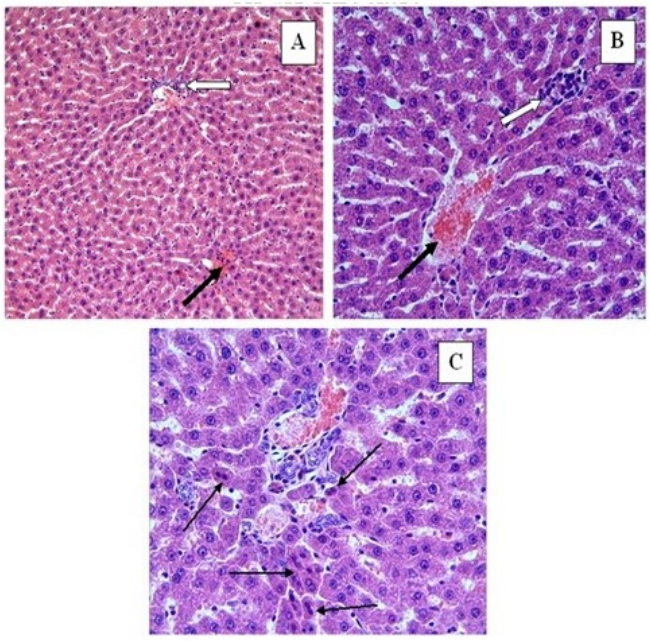

Figure 6. (A, B, C). Decrease of the histopathological damages in the HFD+STZ+PEITC group. We observed little mononuclear cell infiltration (white arrow) (Figure 6A, 6B), vascular congestion (black arrow) (Figure 6A, 6B), hepatocytes with eosinophilic cytoplasm and pyknotic nuclei (black arrows) (Figure 6C) were observed. A: H-E; X20, B, C: H-E; X40.

Discussion

Natural products are traditionally used in various regions of the world to therapy liver disorders. As mentioned above excessive oxidative stress and high level of lipids are a risk for liver diseases. We investigated the hepatoprotective and serum lipid-lowering effects of PEITC on rats with HFD and STZ-induced diabetes and these effects' relationship with the Nrf2 and SIRT1 pathways.

Hyperglycemia in DM increases causing damage to the liver [20]. The appearance of diabetes in the liver is related to biochemical and functional disorders, and oxidative stress [21]. Inflammation, necrosis, and fibrosis of liver disease have been reported to occur after the onset of diabetes [22]. Serum ALT level can be utilized as a marker to predict the liver damage in patients with diabetes. Increased ALT levels are related to decrease hepatic insulin sensitivity and estimate the progress of DM. ALT levels were higher than AST levels for patients with nonalcoholic fatty livers than patients with alcoholic fatty livers. Increased ALT levels may represent fatty changes in the liver independently of the presence of DM. Also, it has been reported that serum ALT levels are related to hepatic insulin resistance [23]. In our study, ALT levels in the HFD+STZ group increased and observed that PEITC reduced ALT level. Histopathological examinations of the liver revealed that the administration of HFD+STZ increased liver degeneration score in comparison to the control group. These findings were parallel to the results of Kurek et al. [24]. Moreover, according to the study by

Dwivedi et al., HFD+STZ administration has been shown to cause disruption of normal liver cells, and moderate inflammation [25]. These changes in the HFD+STZ group seem to have decreased significantly with the PEITC administration. There is an increasing interest in studies on the hypolipidemic effect of PEITC. A study by Gwon et al. revealed that PEITC ameliorated lipid metabolism, and suppressed inflammation in high-fat/cholesterol diet induced obesity [26]. Therefore, we measured triglycerides (TGs) and total cholesterol that reflect lipid disturbances in DM. We observed that serum lipid levels except HDL-C increased following HFD+STZ induction. Conversely, PEITC diminished TC, TG, LDL/VLDL-C levels and increased HDL-C levels. So, it was maintained that PEITC may be helpful to balance serum lipid profile and reduce fatty liver risk in DM.

Diabetes induced by HFD and STZ causes oxidative stress, lipid peroxidation and antioxidant enzyme inactivation in the liver [27]. Oxidative stress causes dysfunction, injury, and death of cells. So, disrupted antioxidant status in the liver contributes notably to the pathogenesis of chronic liver diseases [28]. Enhancement in the hepatic oxidative stress could be the earliest abnormality in liver diseases such as NAFLD because lipotoxicity is associated with increased reactive oxygen species (ROS) production [29]. Our results demonstrate that SOD, GPx, and CAT activities reduced and MDA levels increased following administration with HFD+STZ. These results indicated that PEITC treatment attenuated hepatic injury induced by HFD+STZ that increased ROS production and exhausted the antioxidant system.

Nrf2 alleviates oxidative stress damage. So it is thought to be a potential target for the therapy of liver diseases [30]. In this research, we observed that PEITC pretreatment increased the reduced Nrf2 activity by HFD+STZ. Similar to our study, Naidu et al. showed that PEITC played a substantial role in the activation of Nrf2 due to its ability to interact with cysteine amino acids [31]. It was displayed that PEITC induces phase II detoxifying enzymes such as GSTs by increasing the efficiency of the Nrf2 transcription factor. GSTs are contributed to cell protection via the elimination of ROS and detoxification of electrophilic compounds [32]. In our study, an enhancement of GST level was observed by PEITC in the liver. A study has been shown that liver GST activity was decreased in DM model [33]. Also, the study of Seo KW et al. has indicated that treatment with PEITC caused an increase in liver GST expression and protected against hepatotoxicity [34]. Protective effects of Nrf2 have been described in many liver diseases such as acute hepatotoxicity, NAFLD, and viral hepatitis [35]. We suggest that the activation of Nrf2 signaling may be a hopeful approach to the treatment of liver diseases. We also investigated Sirtuin levels that have been proven to play a crucial role against oxidative damage [36]. It is stated that sirtuins deacetylate MnSOD and led to increasing scavenging of ROS [37]. Moreover, SIRT1 plays a beneficial role in the arrangement of hepatic inflammation that mediates oxidative stress and the early liver diseases [38]. It has been represented that SIRT1 regulates inflammatory responses by inhibiting NF-$\kappa\beta$ [39]. Also, it was shown that in oxidative liver damage induced by metabolic syndrome, Nrf2 levels decreased and NF-$\kappa\beta$ increased [40]. It was suggested that there is an inverse correlation between Nrf2 and NF-$\kappa\beta$. So, NF-$\kappa\beta$ inhibition through Nrf2 or SIRT1 activation may be a considerable target for ameliorating inflammation in liver diseases. In present study, we observed that the NF-$\kappa\beta$ levels in the nuclear fraction of the HFD+STZ group increased and the NF-$\kappa\beta$ levels of the PEITC given group decreased. Similarly, Wang et al. presented that NF-$\kappa\beta$ activity was inhibited by PEITC [41].

According our results, we can suggest that PEITC ameliorated HFD and STZ-induced liver damage and oxidative stress by activating the Nrf2 and SIRT1, as well as inhibiting the NF-$\kappa\beta$ inflammatory response. Therefore, we can propose that PEITC may be a useful possible agent against liver diseases.

Conclusion

Liver damage related to obesity and diabetes continues to rise as a serious problem. According to our data, PEITC can be beneficial against liver damage induced by HFD and STZ. It ameliorated hyperglycemia induced-oxidative stress and hepatic inflammation via Nrf2/SIRT1 activation and inhibition of NF-$\kappa\beta$. Additionally, we observed that PEITC ameliorated serum lipid profile. But, it is needed to research the related metabolic pathways and molecular docking analyses in future studies.

Ethics approval

The study was carried out with the permission of the Local Ethics Committee of the Animal Experiments of İnönü University (Decision Date: 16.04.2019 and Number: 2018/A-49).

Conflict of interest statement

The authors have no conflicts of interest to declare.

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