Istanbul J Pharm 53 (2): 199-210
DOI: 10.26650/IstanbulJPharm.2023.1005935
Original Article

The combined ameliorative effects of α-lipoic acid, selenium, and vitamin E on the livers of STZ-diabetic mice

Ayse Karatug Kacar[1], Onur Ertik[2], Zeynep Mine Coskun Yazici[3], Sema Bolkent[4], Refiye Yanardag[2], Sehnaz Bolkent[3]

[1]Istanbul University, Faculty of Science, Department of Biology, Istanbul, Turkiye
[2]Istanbul University-Cerrahpasa, Faculty of Engineering, Department of Chemistry, Istanbul, Turkiye
[3]Demiroglu Bilim University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, Istanbul, Turkiye
[4]Istanbul University-Cerrahpasa, Faculty of Cerrahpasa Medicine, Department of Medical Biology, Istanbul, Turkiye

ORCID IDs of the authors: A.K.K. 0000-0001-6032-470X; O.E. 0000-0002-9686-8634; Z.M.C.Y. 0000-0003-4791-6537; S.B. 0000-0001-8463-5561; R.Y.0000-0003-4185-4363; S.B.0000-0002-1112-5162

Cite this article as: Karatug Kacar, A., Ertik, O., Coskun Yazici, Z.M., Bolkent, S., Yanardag, R., & Bolkent, S. (2023). The combined ameliorative effects of α-Lipoic Acid, Selenium, and Vitamin E on the livers of STZ-Diabetic mice. *Istanbul Journal of Pharmacy, 53*(2), 199-210. DOI: 10.26650/IstanbuJPharm.2023.1005935

ABSTRACT

Background and Aims: The aim of this study was to investigate the histological and biochemical effects of the antioxidant combination on liver tissue of streptozotocin (STZ)-induced diabetic mice.

Methods: Five groups of mice were given a citrate buffer (CB), the antioxidant solvents (AS), the antioxidant combination (A) (α-lipoic acid, selenium, and vitamin E), STZ (D), the antioxidant combination and STZ (A+D). The mice were sacrificed, and their liver tissues were taken out. The liver tissues were examined histologically and immune+ cell numbers of cannabinoid receptors (CB1R and CB2R) were detected. Xanthine oxidase (XO) activity, glutathione (GSH) and lipid peroxidation (LPO) levels, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), γ-glutamyl transferase (GGT), paraoxonase (PON), glucose-6-phosphate dehydrogenase (G6PD) activities, protein carbonyl content (PCC) and advanced oxidation protein product (AOPP), sialic acid, fucose, hexose and hydroxyproline (OH-proline) levels were biochemically determined.

Results: Certain degenerative changes were reduced in the A+D group compared histologically to the D group. There were no significant changes in the number of CB1R immune+ cells. The number of CB2R immune+ cells was significantly reduced in the D group compared to the CB group. The GSH level, CAT, SOD, GR, GPx, GST, PON, and G6PD activities were increased while XO and GGT activity, LPO, PCC, AOPP, hexose, fucose, sialic acid, and OH-proline level were biochemically decreased in the A+D group compared to the D group.

Conclusion: The use of the antioxidant combination had a positive effect on the livers of diabetic mice with histochemical and biochemical changes, while there was no effect on the regulation of cannabinoid receptors expressions.

Keywords: Diabetes, α-Lipoic Acid, Vitamin E, Selenium, Liver, Mouse

Address for Correspondence:
Ayse KARATUG KACAR, e-mail: akaratug@istanbul.edu.tr

Submitted: 07.10.2021
Revision Requested: 22.02.2022
Last Revision Received: 28.02.2022
Accepted: 13.04.2022
Published Online: 28.08.2023

INTRODUCTION

Diabetes mellitus was identified as one of the deadliest diseases according to a statement made by the World Health Organization (WHO)**.** Considering 415 million people had diabetes in 2015, it is now predicted that 642 million people may have diabetes in 2040 (IDF 2015). Diabetes includes conditions such as hyperglycemia, hyperinsulinemia, hyperlipidemia, and inflammation. These situations cause oxidative stress due to the formation of reactive oxygen species (ROS) (Holst, Vilsboll, & Deacon, 2009; Aydin et al. 2019). Insulin resistance leads to oxidative stress and liver abnormalities (Mohamed, Nazratun, Zariyantey, & Budin, 2016).

The liver is important in the regulation of liver glucose metabolism and is the major organ sensitive to hyperglycemia. Liver damage occurs in early-stage diabetes (Adeyemi, Ukwenya, Obuotor, & Adewole, 2014). Type 2 diabetes and liver dysfunction are related to each other (Wannamethee, Shaper, Lennon, & Whincup, 2005; Kunutsor, Apekey, & Walley, 2013; Ballestri et al. 2016). It is assumed that liver dysfunction can increase type 2 diabetes by increasing insulin resistance. It is also thought that type 2 diabetes may impair liver function by causing lipogenesis (Smith & Adams 2011). However, it is not known exactly whether these two different relationships exist (Taylor, 2008; Loria, Lonardo, & Anania, 2013; Taylor et al. 2018).

Pathological changes increase oxidative stress and decrease antioxidant activity in internal organs, such as the liver, in diabetes (Springer et al. 2013; Zimmet, Shi, El-Osta, & Ji, 2018). Researchers have shown that ROS are overproduced by decreasing antioxidant enzymes and increasing lipid peroxidation (LPO) in liver damage caused by diabetes (Lozano et al. 2016). Alpha lipoic acid (ALA) is reduced to dihydrolipoic acid (DHLA) and regenerates glutathione (GSH), which is a potent antioxidant. It has been shown that ALA directly scavenges ROS and protects against oxidative stress (Rochette, Ghibu, Muresan, & Vergely, 2015). ALA has been shown to affect glucose uptake via the insulin signal pathway (Konrad et al. 2001). It has also been noted that in type 2 diabetes, ALA is important to improve insulin resistance as well as impaired glucose metabolism (Lee et al. 2005).

The glutathione peroxidases (GPx), iodothyronine deiodinases, and thioredoxin reductases that catalyze metabolic reactions are proteins containing selenium (Se), which is an essential nutrient (Lu & Holmgren 2009). The decreased Se level is associated with a weakened immune system and increased mortality (Rayman 2012). Conversely, additional Se intake when there is already sufficient Se may impair the function of the liver, heart, and related organs. (Zeng et al. 2012; Jablonska et al. 2016). The dose of Se intake is important. A study revealed that those who took Se supplements had a higher risk of diabetes (Stranges et al. 2007). In another study, it was shown that Se is effective in the regulation of glucose metabolism in the liver when given to diabetic rats (Vinceti, Filippini, & Rothman, 2018).

Vitamin E (Vit E), a lipid-soluble, is the most powerful micronutrient antioxidant (Peh, Tan, Liao, & Wong, 2016). It has promising positive results in the treatment of nonalcoholic fatty liver disease. Vit E ameliorated hepatocellular ballooning is a finding of nonalcoholic steatohepatitis in children (Lavine et al. 2011). Furthermore, it is suggested that inflammation and hepatic steatosis decreases in the treatment of nondiabetic adult patients with nonalcoholic steatohepatitis (Sanyal et al. 2010). Also, the risk of occurrence of nonalcoholic fatty liver disease, cirrhosis, and liver cancer in patients with type 2 diabetes is high (Younossi et al. 2018).

The endocannabinoid system contains two specific coupled G-protein receptors (CB1R and CB2R) and their ligands are called endocannabinoids. Bazwinsky-Wutschke, Zipprich, & Dehghani, (2019) asserted that the endocannabinoid system partially controls glucose metabolism in the liver. The system controls lipid and energy metabolism as well as glucose metabolism (Di Marzo 2008). Studies have shown that the endocannabinoid system is associated with diabetes (Gruden, Barutta, Kunos, & Pacher, 2016; Jourdan, Godlewski, & Kunos, 2016). The cannabinoid receptor agonists/antagonists may have potential roles in the treatment of many metabolic diseases, such as diabetes, by attenuating the autoimmune response and regulating insulin secretion, glucose, and energy metabolisms (Zhang et al., 2016; Heppenstall, Bunce, & Smith, 2012; Li, Kaminski, & Fischer, 2001).

In the present study, we investigated the effects of the ALA + Se + Vit E combination on the liver of STZ-diabetic mice and in addition, the role of the antioxidant combination on the endocannabinoid system of streptozotocin (STZ), both morphologically and biochemically.

MATERIALS AND METHODS

Animals and antioxidant treatments

This experimental study was carried out at the Institute's Animal Care and Use Committee of Istanbul University, in 2008-2009. The project was approved by the local ethics committee for animal experiments, at Istanbul University. Balb/c mice (2-2.5 months) provided by the Institute's Animal Care and Use Committee of Istanbul University, were used in the study. They were maintained under standard animal housing conditions in a controlled temperature clean room on a 12 h light/dark cycle and were given water and standard chow *ad libitum*. Five groups were randomly created for the experimental setup. The first group, a control group, was given 0.01 M citrate buffer (CB) group (pH 4.5) by intraperitoneal injections for five consecutive days (n=8), the second group, a control group, was given the antioxidant solvent (AS group) in distilled water including NaOH for ALA, distilled water for Se and flower oil for Vit E using the gavage technique (n=8), the third group, a control group, was given antioxidant combination (A) ALA (50 mg/kg), Se (0.25 mg/kg), and Vit E (100 mg/kg) for five consecutive days using the gavage technique (n=8), the fourth group, the experimental group, was given STZ (40 mg/kg) (D) group for five consecutive days by intraperitoneal injections (n=10), and the fifth group, the antioxidants+diabetes (A+D) group was given the antioxidant combination before diabetes (n=10). After 30 days, the mice were sacrificed by cervical dislocation. The liver tissues were quickly removed for histological and biochemical analyses.

Histological assay

The liver tissues were fixed for histological investigation in Bouin's solution and cleared in xylene. It was embedded in paraffin, after dehydration in ethanol series. The tissues embedded in paraffin were cut into 5 µm sections. these were then adhered to the microscope slide. Hematoxylin-Eosine and Masson's trichrome were used for staining sections.

Immunohistochemical assays

A 10% neutral buffered formalin solution was used to fix the tissue samples. 4 µm thick sections were taken. The deparaffinized sections were heated in a 0.01 mol/L CB (pH 6.0) in a microwave oven for antigen retrieval for 15 min. The immunoreactivities of antibodies were analyzed with a Histostain Plus Broad-Spectrum Kit (Invitrogen, Carlsbad, CA, USA). Endogenous peroxidase activity was blocked for 15 min with 3% hydrogen peroxide in methanol followed by the incubation of the sections with a 1:100 dilution of CB1R (rabbit polyclonal, Cayman Chemicals, Ann Arbor, MC, USA) and 1:200 dilution of CB2R (rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) primary antibodies overnight at 4 °C. After incubation, the sections were washed off with phosphate-buffered saline, and were then incubated with a biotinylated secondary antibody at room temperature. They were incubated with horseradish peroxidase (HRP)-labeled streptavidin. Visualization of the reaction was carried out using 3-amino-9-ethylcarbazole (AEC, Invitrogen, Carlsbad, CA, USA) as the chromogen. Mayer's hematoxylin was used for counterstaining for 40 seconds at room temperature. The slides were incubated in the absence of CB1R and CB2R primary antibodies for negative control. The liver tissue slides were examined with a Nikon Eclipse 80i light microscope equipped with a digital camera. This was analyzed with the NIS-Elements-D 3.1 microscope imaging software program. For the expressions of CB1R and CB2R, ten randomly selected areas in each slide (n=5) were examined.

Biochemical assays

In the previous study by Karatug & Bolkent (2013), fasting blood sugar levels of mice were presented (Karatug & Bolkent 2013). The liver tissue samples were washed off with saline and kept frozen until analyzed for biochemical analyses. The liver tissue samples were homogenized with 0.9% NaCl cold physiological saline solution by means of a glass homogenizer and made up to 10% (w/v) homogenate. After that, the homogenates were centrifuged at 10000 x g for 10 minutes. Liver tissue homogenates were used for enzyme analyses and protein carbonyl content (PCC), advanced oxidation protein product (AOPP), sialic acid, fucose, hexose, and hydroxyl proline levels.

Xanthine oxidase activity

Xanthine oxidase (XO) activities of liver tissues were performed according to Corte, & Stirpe (1968), with the help of uric acid at 240 nm. The results were given as U/mg protein.

GSH levels

GSH levels of liver tissues were evaluated by the method described by Beutler (1975). Metaphosphoric acid was applied for the precipitation of protein, and the development of product color was due to 5,5′-dithiobis-2-nitrobenzoic acid. The measurements were made spectrophotometrically at 405 nm. Results were expressed as nmol GSH/mg protein.

LPO levels

The lipid peroxidation (LPO) levels of the liver tissues were measured in accordance with the Ledwozyw et al. method (Ledwozyw, Michalak, Stepien, & Kadziołka, 1986). Malondialdehyde (MDA) is among the byproducts of LPO. The measurements were made spectrophotometrically at 532 nm. Results were given as nanomoles of MDA per milligram of protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) was assessed by the method described by Mylorie et al. (Mylorie, Colins, Umbles, & Kyle 1986). Phosphate buffer (pH 7.8), o-dianisidinedihydrochloride, and riboflavin were used for the reaction protocol. The reaction was performed using a fluorescent lamp and was initiated with the addition of riboflavin. The difference in absorbance between 0 and 8 minutes was recorded at 460 nm. The results were expressed as U/g protein.

Catalase activity

Catalase (CAT) activity was performed according to the Aebi (1984) method. The reduction of H_2O_2 to H_2O in the presence of CAT was assayed as the decrease of absorbance at 240 nm. The results were defined as U/mg protein.

Glutathione reductase activity

Glutathione reductase (GR) activity was determined according to the GR activity determined by Beutler (1971). GR activity was determined by calculating the amount of NADH oxidized during the reduction of oxidized glutathione with GR. GR activity was expressed as U/g protein.

Glutathione peroxidase activity

GPx activity was described by Paglia, & Valentine (1967). According to this method, GPx activity was measured by the conversion of H_2O_2 to H_2O in the presence of GSH, NADPH, and glutathione reductase (GR). The oxidation of NADPH to $NADP^+$ was considered in reaction media at 366 nm for the determination of GPx activity. GPx activity was expressed as U/g protein.

Glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity was performed according to the Habig, & Jakoby (1981) method. GST catalyzes the reaction between GSH and 1-chloro-2,4-dinitrobenzene. The resultant products formed were monitored spectrophotometrically at 340 nm. GST activity was expressed as U/mg protein.

Gamma glutamyl transferase activity

Gamma glutamyl transferase (GGT) activities were determined by the method described by Szasz (1969). This method is based on the determination of the amount of p-nitroaniline formed as a result of the reaction and the absorbance value at 405 nm in the spectrophotometer. GGT activity was expressed as U/g protein.

Paraoxonase activity

Paraoxonase activity (PON) of liver samples was assayed according to Furlong et al. (Furlong, Rchter, & Seidel 1988). The enzyme activity was determined using paraoxon ethyl as a substrate and the absorbance of yellowish-colored product p-nitrophenol was assayed at 405 nm. The results were expressed as U/g protein.

Glucose-6-phosphate dehydrogenase activity
Glucose-6-phosphate dehydrogenase (G6PD) activity in liver tissues was determined according to the Beutler (1984) method, by spectrophotometric measurement of NADPH formed during the reaction in the presence of Mg^{2+} ions at 340 nm (Beutler, 1984). The results were expressed as U/g protein.

Protein carbonyl content
Liver tissue protein carbonyl (PCC) contents were determined spectrophotometrically by the method of Levine et al. (1990). The results were expressed as nmol carbonyl/mg protein.

Advanced oxidation protein product levels
Advanced oxidation protein product (AOPP) level was determined according to the Witko-Sarsat et al. (1996) method. The liver tissue samples were treated with phosphate buffer at pH 7.4 and potassium iodide. Finally, glacial acetic acid was added, and the absorbance of the product was recorded at 340 nm. The results were expressed as nmol/mg protein.

Sialic acid levels
Sialic acid was estimated by the method of Lorentz et al. (Lorentz, Weiss, & Kraas, 1986). Sialic acid, which undergoes periodic acid oxidation, reacts with ß-formyl pyruvic acid, and thiobarbituric acid. It enters a colored compound with maximum absorbance at 546 nm. This compound formed is unstable, so it is attracted to the cyclohexanone phase. Results were expressed as µmol sialic acid/g protein.

Fucose levels
The amount of fucose in the liver tissue was determined according to the method of Dische, & Shettles (1948). The method is based on the color reaction of carbohydrates with thiol groups in the presence of sulfuric acid. Results were expressed as µg fucose/mg protein.

Hexose levels
Quantification of hexose in liver tissue was determined according to the method of Winzler (1955). The method is based on the color reaction of carbohydrates with orcinol in the presence of concentrated sulfuric acid, spectrophotometrically, in order to determine hexose compounds in tissues. Results were expressed as µg glucose/mg protein.

Hydroxyproline levels
Liver hydroxyproline (OH-proline) levels were assayed by the method of Reedy, & Enwemeka (1996). Results were expressed as µg OH-proline/g protein.

Protein levels
The Lowry et al. method was used to determine the total protein levels of liver tissue samples (Lowry, Rosebrough, Farr, & Randall, 1951). The protein content of the tissue was applied with copper ions in an alkaline media and then Folin reactive was added. Finally, the blue-colored product absorbance was measured at 500 nm.

Statistical analysis
For histological analysis, the data were analyzed with one-way analysis of variance (ANOVA) followed by post hoc tests (Tukey's test) for differences among groups using a computer package (GraphPad Prism, version 6.0), and for immunohistochemical analysis, using SPSS software (version 21.0, SPSS, USA). Biochemical results were evaluated using an unpaired t-test and variance analysis (ANOVA). Analysis was performed with the NCSS statistical computer package. Findings were expressed as the mean ± standard error of the mean (SEM) for each group in the histological and immunohistochemical assays and as the mean ± standard deviation of the mean (SD) for each group in the biochemical assays. The differences were considered significant when the P value was <0.05.

RESULTS

Histological and immunohistochemical assays
The histological results are presented in Figure 1. Degenerative changes such as pyknotic nuclei, necrotic cell, hyperemia, and sinusoidal dilation increased in the D group compared to the CB group ($p<0.01$), but all of them decreased in the A+D group compared to the D group ($p<0.001$).

The immunohistological results are presented in Figures 2 and 3. There was no significant change in the number of CB1R immune$^+$ cells among all groups ($p>0.05$). A non-significant reduction in the number of CB1R immune$^+$ cells was observed in the D group as compared to the CB group ($p>0.05$) (Figure 2). There was a significant change in the number of CB2R immune+ cells among all groups ($p<0.05$) (Figure 3). The number of CB2R immune$^+$ cells was significantly reduced in the AS, A, D, and A+D groups as compared to the CB group ($p<0.05$ for all). CB1R and CB2R peptides were observed both in the nucleus and cytoplasm.

Biochemical assays
Fasting blood glucose levels were shown in our previous study. According to these results, fasting blood glucose levels in the diabetic group increased significantly compared to the control group ($p<0.001$) (Karatug & Bolkent, 2013).

XO activity, GSH, and LPO levels of liver tissue are presented in Table 1. XO activity and LPO level were increased in the D group as compared to the CB group ($p<0.05$; $p<0.01$ respectively). GSH level was significantly reduced in the D group compared to the CB group ($P < 0.005$). In the A+D group, the diabetic group treatment with antioxidants resulted in a significant increase in GSH level and noticeable decreases in XO activity and LPO level compared to the D group ($p<0.0001$; $p<0.005$; $p<0.0001$ respectively).

SOD, CAT, and GR results are presented in Table 2. Significant decreases were observed in SOD, CAT, and GR activities of the D group compared to the CB group ($p<0.05$; $p<0.0001$; $p<0.0001$ respectively). These decreases were negated by dietary antioxidants for SOD, CAT, and GR enzyme activities ($p<0.05$; $p<0.05$; $p<0.01$ respectively).

GPx, GST, and GGT activities in the liver tissue are shown in Table 3. In the D group, the activities of GPx and GST were decreased while GGT activity was increased compared to the CB group ($p<0.005$; $p<0.005$; $p<0.01$, respectively). Administration of antioxidants to the diabetic mice significantly increased GPx and GST activities in

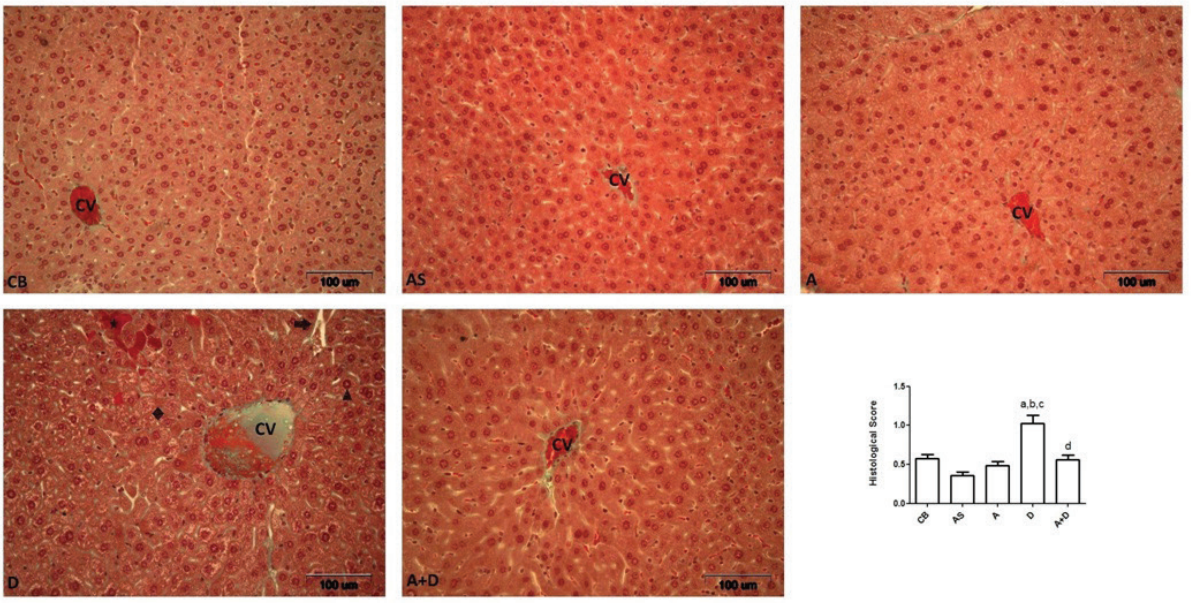

Figure 1. Histological appearance of the liver tissues of given citrate buffer (CB), antioxidants solvent (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+ Diabetes (A+D) groups. Necrotic areas (◆), hyperemia (★), pyknotic nucleus (▲), and sinusoidal dilatation (→) can be seen in diabetic mouse liver. CV: central vein. Masson's trichrome. [a]P<0.01 versus CB group, [b]P<0.001 versus AS group, [c]P<0.001 versus A group, [d]P<0.001 versus D group.

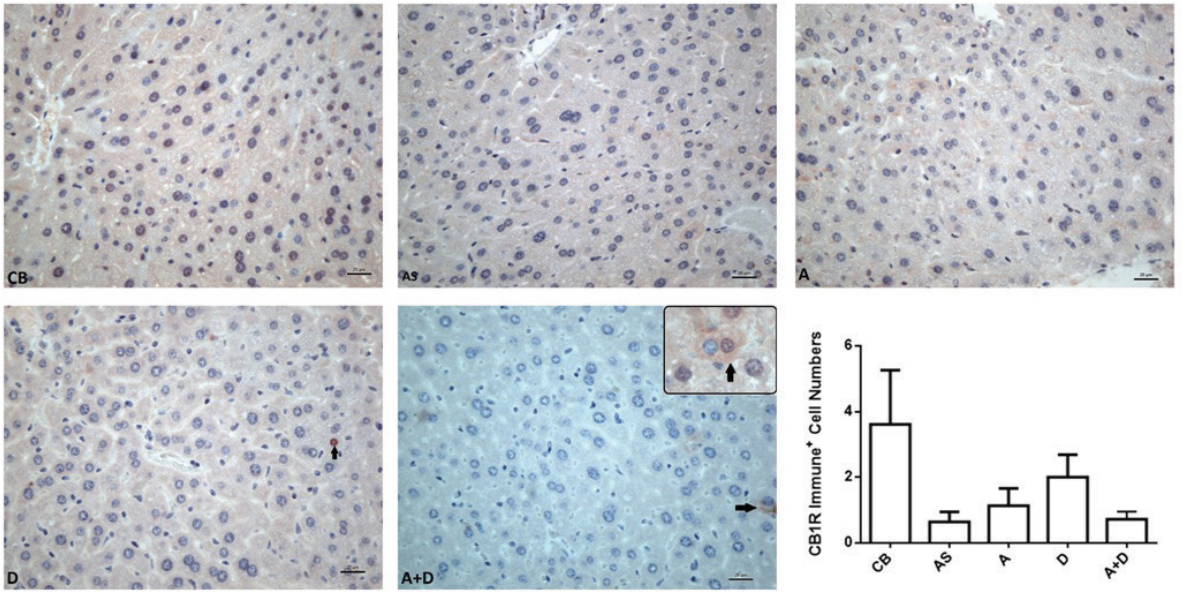

Figure 2. Immune+ cells (arrow) and the number of immune+ cells for cannabinoid 1 receptor (CB1R) in the liver of Citrate buffer (CB), the solvents of antioxidants (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+Diabetes (A+D) groups. Streptavidin-Biotin-Peroxidase technique, counterstain Hematoxylin. Scale bar = 20 µm.

the liver, while significantly decreased GGT activity compared to the D group ($p<0.05$; $p<0.005$; $p<0.0001$ respectively).

PON and G6PD activities, PCC, and AOPP levels are presented in Table 4. In the D group, the enzyme activities of PON and G6PD activities were reduced ($p<0.0001$; $p<0.0001$), PCC and AOPP levels were increased ($p<0.001$; $p<0.05$). Antioxidants supplementation administered to the diabetics resulted in an important increase in PON and G6PD activities ($p<0.05$; $p<0.0001$) whereas a reduction in PCC and AOPP levels was observed ($p<0.0001$; $p<0.05$).

Liver tissue glycoprotein levels are shown in Table 5. The glycoproteins containing sialic acid, fucose, hexose, and OH-proline levels were significantly increased in the D groups compared to the CB group ($p<0.01$; $p<0.05$; $p<0.05$; $p<0.01$ respectively).

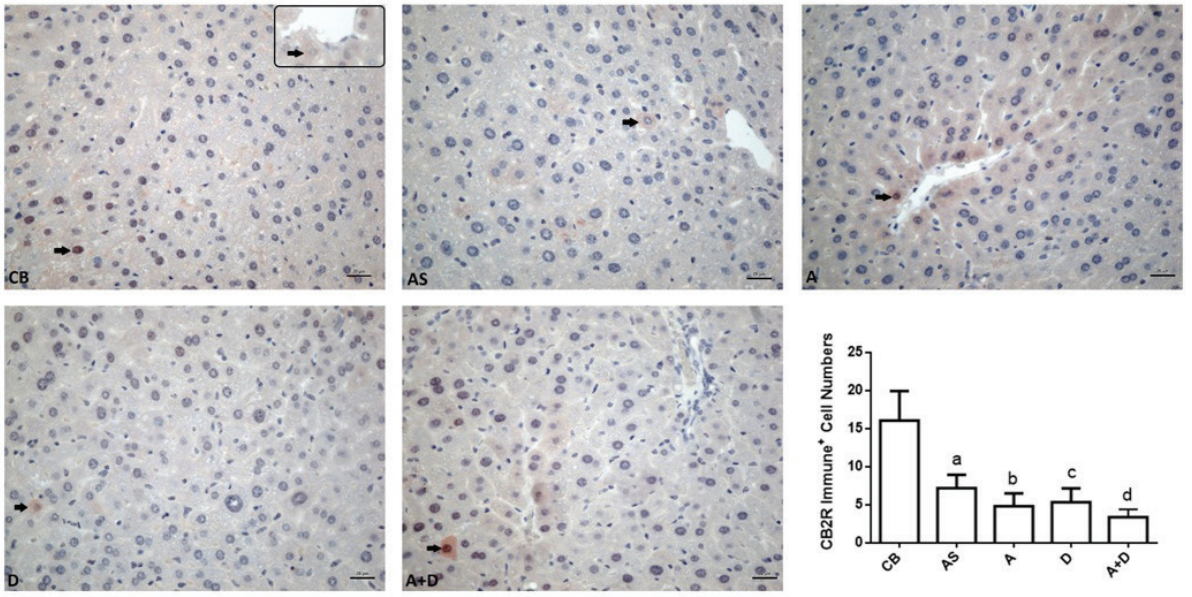

Figure 3. Immune+ cells (arrow) and the number of immune+ cells for cannabinoid 2 receptor (CB2R) in the liver of Citrate buffer (CB), the solvents of antioxidants (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+ Diabetes (A+D) groups. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin. Scale bar = 20 µm. [a]P<0.05 versus CB group, [b]P<0.05 versus CB group, [c]P<0.05 versus CB group, [d]P<0.05 versus CB group.

Table 1. Liver tissue xanthine oxidase (XO) activity, glutathione (GSH) and lipid peroxidation (LPO) levels of all groups.

Group	XO (U/g protein)*	GSH (nmol GSH/mg protein)*	LPO (mmol MDA/mg protein)*
Citrate Buffer (CB)	0.45 ± 0.08	10.60 ± 3.05	4.03 ± 1.56
Antioxidants (A)	0.32 ± 0.13	10.03 ± 1.18	3.70 ± 1.32
Solvents of antioxidants (AS)	0.26 ± 0.04	11.58 ± 1.83	4.92 ± 0.85
STZ-induced diabetes (D)	0.87 ± 0.31[a]	4.49 ± 1.73[c]	8.05 ± 1.55[e]
Antioxidants+Diabetes (A+D)	0.22 ± 0.06[b]	12.53 ± 1.60[d]	2.80 ± 0.61[d]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.05 versus CB group; [b]p<0.005 versus D group; [c]p<0.005 versus CB group; [d]p<0.0001 versus D group; [e]p<0.01 versus CB group

Table 2. Liver tissue superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities of all groups.

Group	SOD (U/mg protein)*	CAT (U/mg protein)*	GR (U/g protein)*
Citrate Buffer (CB)	14.35 ± 5.71	76.92 ± 12.38	643.28 ± 78.12
Antioxidants (A)	13.39 ± 2.15	43.94 ± 14.65	374.20 ± 56.69
Solvents of antioxidants (AS)	15.24 ± 3.14	46.17 ± 14.32	576.62 ± 28.99
STZ-induced diabetes (D)	4.97 ± 1.94[a]	28.99 ± 3.15[c]	174.62 ± 45.86[c]
Antioxidants+Diabetes (A+D)	8.17 ± 1.40[b]	32.21 ± 10.40[b]	343.97 ± 88.17[d]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.05 versus CB group; [b]p<0.05 versus D group; [c]p<0.0001 versus CB group; [d]p<0.01 versus D group

Table 3. Liver tissue glutathione peroxidase (GPx), glutathione-S-transferase (GST) and γ-glutamil transferase (GGT) activities of all groups.

Group	GPx (U/mg protein)*	GST (U/g protein)*	GGT (U/g protein)*
Citrate Buffer (CB)	144.75 ± 32.72	274.09 ± 37.48	11.13 ± 2.33
Antioxidants (A)	135.49 ± 20.80	251.61 ± 30.12	9.10 ± 1.81
Solvents of antioxidants (AS)	159.94 ± 22.04	300.69 ± 35.57	13.40 ± 1.64
STZ-induced diabetes (D)	74.81 ± 10.41[a]	210.95 ± 19.87[a]	30.02 ± 7.88[d]
Antioxidants+Diabetes (A+D)	97.59 ±12.37[b]	256.70 ± 25.46[c]	5.54 ± 1.68[e]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.005 versus CB group; [b]p<0.05 versus D group; [c]p<0.005 versus D group; [d]p<0.01 versus CB group; [e]p<0.0001 versus D group

Table 4. Liver tissue paraoxonase (PON), glucose-6-phosphate dehydrogenase (G-6-PD) activites and protein carbonyl content (PCC) and advanced oxidation protein product (AOPP) levels of all groups.

Group	PON (U/g protein)*	G-6-PD (U/g protein)*	PCC (nmol PCC/mg protein)*	AOPP (nmol/g protein)*
Citrate Buffer (CB)	11.24 ± 2.08	13.50 ± 4.55	4.26 ± 1.26	2.09 ± 0.95
Antioxidants (A)	11.31 ± 3.55	14.21 ± 3.57	4.06 ± 1.38	1.60 ± 0.48
Solvents of antioxidants (AS)	6.17 ± 0.98	15.62 ± 2.32	5.22 ± 0.79	2.06 ± 1.40
STZ-induced diabetes (D)	5.84 ± 0.38[a]	4.62 ± 1.72[a]	9.61 ± 1.30[d]	3.85 ± 0.75[e]
Antioxidants+Diabetes (A+D)	11.54 ± 4.08[b]	12.11 ± 2.27[c]	3.39 ± 1.24[c]	1.84 ± 1.10[b]
P_{Anova}	0.02	0.0001	0.0001	0.05

*Mean ± SD; [a]p<0.0001 versus CB group; [b]p<0.05 versus D group; [c]p<0.0001 versus D group; [d]p<0.001 versus CB group; [e]p<0.05 versus CB group

Table 5. Liver tissue sialic acid, fucose, hexose and OH proline levels of all groups.

Group	Sialic Acid (μmol sialic acid/g protein)*	Fucose (μg fucose/mg protein)*	Hexose (μg glucose/mg protein)*	OH-Proline (μg OH-Proline/ g protein)*
Citrate Buffer (CB)	23.95 ± 6.03	4.74 ± 0.74	12.43 ± 2.44	211.47 ± 32.18
Antioxidants (A)	21.34 ± 6.19	5.53 ± 1.25	12.60 ± 1.54	182.77 ± 54.32
Solvents of antioxidants (AS)	26.84 ± 7.24	6.02 ± 2.99	18.11 ± 2.43	233.62 ± 47.88
STZ-induced diabetes (D)	42.89 ± 5.83[a]	9.12 ± 2.76[c]	21.90 ± 5.86[c]	400.72 ± 62.10[a]
Antioxidants+Diabetes (A+D)	21.82 ± 3.85[b]	4.81 ± 1.51[d]	11.56 ± 1.74[d]	227.99 ± 35.43[e]
P_{Anova}	0.001	0.05	0.002	0.0001

*Mean ± SD; [a]p<0.01 versus CB group; [b]p<0.0001 versus D group; [c]p<0.05 versus CB group; [d]p<0.05 versus D group; [e]p<0.001 versus D group

In the A+D group which was given STZ, glycoprotein components (sialic acid, fucose, and hexose) and OH-proline levels were significantly decreased after the administration of the antioxidants (p<0.0001; p<0.05; p<0.05, p<0.001 respectively).

DISCUSSION

Diabetes mellitus is an important disease and affects many people all around the world annually and is characterized by hyperglycemia development. Fasting blood glucose levels increased significantly in diabetes and were partially prevented with administered ALA + Se + Vit E combination (Karatug & Bolkent 2013). Long-term administration of ALA to diabetic rats has been reported to cause low blood glucose levels in the literature (Lateef et al. 2005; Kojima et al. 2007). Vit E and Se acting in critical metabolic roles are essential substances for humans. As a result of the literature review, the application of Vit E and Se to diabetic rats had different effects on the blood glucose levels. While some studies show that administration of Vit E and Se has no significant effect on blood glucose level (Mukherjee et al. 1998; Gocmen et al. 2000; Barbosa et al. 2008), some studies show that blood glucose level decreases significantly (Shirpoor, Ansari, Salami, Pakdel, & Rasmi, 2007; Karatug & Bolkent 2013).

The liver is an important organ for the metabolism of carbohydrates, protein, and fat. It works to make fewer toxic or harmless foreign substances and molecules such as drugs (Ozougwu 2017). Free radicals cause oxidative stress in diabetes-induced liver damage (Zhou et al. 2015; Asmat, Abad & Ismail, 2016; Masarone et al. 2018). In some studies, pyknotic nuclei and necrotic areas were shown in the liver tissue of diabetic rats, which were created by the administration of STZ, similar to our study (Al-Attar & Alsalmi 2019; Faddladdeen 2021). STZ shows toxic effects in pancreatic beta cells and causes hyperglycemia. In this case, oxidative stress increases with the decrease of the antioxidant defense system (Maritim, Sanders, & Watkins, 2003; Papaccio, Pisanti, Latronico, Ammendola, & Galdieri, 2000). In the present study, the occurrence of these negativities, which increased in the liver due to the diabetic condition, was prevented by the combined antioxidants given to mice before diabetes. In previous studies, the effect of these triple combined antioxidants was unknown. In this context, the effects of these combined antioxidants on the liver are shown for the first time in terms of diabetic status.

In one study, the levels of cannabinoid receptors expressions in different rat tissues, CB1R and CB2R mRNA levels showed very high expression in the liver as compared to peripheral organs. The expression of CB1R and CB2R mRNA levels depends on diurnal variations. Furthermore, the researchers suggested that the deficiency of insulin due to increased glucose concentration in the blood may cause a loss of CB1R and CB2R in the liver of STZ-induced rats with daily fluctuations (Bazwinsky-Wutschke, Zipprich, & Dehghani, 2017). Moura et al. (2019) reported that in forebrain glucose metabolism, the reduced or absent CB1R expression is sufficient to mimic hyperglycemia-induced impairment and/or insulinopenia. According to the study by Sena, Cipriano, Botelho, & Seiça, (2018) on diabetes, ALA prevents hepatic steatosis by decreasing inflammation and oxidative stress. Similarly, Daniel, Adeoye, Ojowu, & Olorunsogo, (2018) suggested that Vit E can lead to the protection of hepatic tissue from diabetes-induced oxidative stress. The findings of Jacobs et al. (2019) did not support the effect of Se supplementation on insulin action and/or secretion in diabetes. In contrast, a population study exhibited a positive association between Se level and diabetes (Moon et al. 2019). Collectively, our immunohistochemistry findings showed that the number of CB2R immune^{+} cells significantly decreased in the diabetic liver, whereas CB1R did not. The treatment of the three antioxidant agents ALA, Se, and Vit E had no effect on either the CB1R or the CB2R in the diabetic liver. Salinthone et al., (2011) suggested that lipoic acid activates some G-protein-coupled receptors but not others. According to our findings, the combination of ALA, Se, and Vit E did not behave as a ligand for the cannabinoid receptors, one of the G-protein-coupled receptors in liver tissue.

ALA modulates the redox potential since ALA has the ability to match the redox status between different subcellular compartments as well as extracellularly. ALA is a potential antioxidant for humans (Rochette et al. 2013). Se and Vit E are also potential antioxidants for animals (Liebler 1993; Tinggi 2008).

Due to the antioxidant properties of all these substances, they can play a crucial role in the prevention of oxidative stress caused by diabetes. Several clinical and experimental studies have identified a relationship between diabetes, hyperglycemia, and oxidative stress. Also, oxidative stress is characterized by a change in the enzymatic or non-enzymatic antioxidative system and/or increased LPO levels. These systems prevent oxidative stress and reduce the concentration of ROS (Kanbagli, Balkan, Aykac Toker, & Uysal, 2002). GSH is a non-enzymatic antioxidant and prevents the harmful effects of free radicals. Diabetes studies showed that GSH level is generally low value in diabetic humans and animals (Gezginci-Oktayoglu, Sacan, Yanardag, Karatug, & Bolkent, 2011). In this study, the GSH level was reduced in the diabetic group and the administration of ALA, Se, and Vit E was reversed back to normal levels when compared to the control group. LPO is an important marker associated with oxidative stress. Due to the increase in free radical amount, polyunsaturated fatty acids in the cell membrane react with free radicals and as a result of this reaction, the amount of LPO increases in diabetic groups (Girotti, 1985). In our study, the LPO value increased in diabetic rats and treatment with the combination of ALA, Se, and Vit E decreased the LPO value. XO is the key enzyme in purine metabolism and can be an important biological source of ROS. For that reason, XO is an important marker for oxidative stress, and it has been shown that the activity of XO is increased experimental diabetes (Matsumoto, Koshiishi, Inoguchi, Nawata, & Utsumi, 2003). Increased XO activity means there is oxidative stress in the liver due to diabetes. The application of the antioxidant combination before the formation of diabetes protected the liver significantly against XO-derived ROS formation.

Free radicals may play an important role in the complications and causation of diabetes mellitus. Free radical production levels and oxidative stress increase in diabetic patients and animals. For that reason, antioxidant systems have a very critical role in organisms. CAT, SOD, GR, GPX, GST, and endogenous antioxidant enzymes in organisms, play a key role in reducing the harmful effects of reactive oxygen species and free radicals (Harini & Pugalendi 2010; Sacan et al. 2016). In this study, CAT, SOD, GR, GPX, and GST antioxidant enzymes activities were decreased in diabetic rats and the results were reversed when antioxidants were used. The results show that the antioxidant combination may have helped increase the activity of antioxidant enzymes by reducing free radical formation. The combination of ALA, Se, and Vit E might be a scavenger for free radicals and might reduce the activities of endogenous antioxidant enzymes in that way. GGT is a marker for oxidative stress, and it contributes to the extracellular catabolism of glutathione (Whitfield 2001). Higher GGT levels are associated with diabetes (Sabanayagam, Shankar, Li, Pollard, & Ducatman, 2009). GGT levels increased significantly in diabetic rats in this study. The enzyme level decreased with the administration of a combination of antioxidants.

The activity of the PON enzyme has been linked to many diseases such as inflammation, stroke, myocardial infarction, diabetes, and Alzheimer's. Decreased PON activity is responsible for inflammation in patients who have cancer and diabetes (Camps,

Marsillach, & Joven, 2009). The study has shown that PON1 activity is reduced in Type 1 and Type 2 diabetic patients (Durrington, Mackness, & Mackness, 2001). In our study, PON activity was decreased in the diabetic group and this activity might be associated with hyperglycemia and oxidative stress (Nair, Shah, Taggarsi, & Nayak, 2011; Jamuna Rani, Mythili, & Nagarajan, 2014). The first enzyme of the pentose phosphate pathway is G6PD. It provides NADPH production. NADPH levels impact the entire antioxidant system and make tissues very vulnerable to oxidative damage. In our study, PON and G6PD activities were found significantly decreased in diabetic rats and were reversed back when a combination of antioxidants was administered. Oxidized amino acid residues in diabetes cause protein damage and PCC levels are used as a biomarker for this protein damage. Several studies showed that PCC levels increased in the diabetic group when compared to the control group (Dayanand, Kumar Vegi, & Kutty, 2012). AOPP is a marker that shows oxidative stress-based protein damage. Some researchers have reported that there is a relationship between ATP levels and diabetes (Baskol, Gumus, Oner, Arda, & Karakucuk, 2008). Treatment of diabetic rats with the antioxidant combination reversed increased PCC and AOPP levels in diabetic liver tissues. As a result, it can be concluded that the use of combined antioxidants before diabetes occurs is very effective in protecting liver tissue from any damage that might occur and combined antioxidants can be used as a hypoglycemic agent.

Glycoproteins are the main structures of the matrix, and they have many functions as hormones and enzymes. The metabolism of glycoproteins is very critical in diabetes. The amount of glycoprotein increases in high blood glucose levels. The carbonyl groups of amino acids react slowly with glucose and form Schiff-base. In this study, we observed increased levels of sialic acid, fucose, and hexose in the liver tissue of STZ-induced hyperglycemic rats when compared to the control group. Collagen is one of the proteins which contains hydroxyproline. Some authors consider hydroxyproline as a marker of collagen content. Hydroxyproline levels increased in the liver tissues of diabetic rats. This indicates that diabetes has a negative effect on liver collagen. Antioxidant administration reverses this increase showing the restoration of collagen damage (Wangoo et al. 2000).

CONCLUSION

Triple antioxidant treatment reversed the negative effects of diabetes, both morphologically and biochemically. In contrast, the combined treatment of antioxidants does not affect the regulation of cannabinoid receptors expressions.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- A.K.K., S.B.; Data Acquisition- A.K.K., O.E., Z.M.C.Y.; Data Analysis/Interpretation- A.K.K., O.E., Z.M.C.Y., S.B., R.Y.; Drafting Manuscript- A.K.K.; Critical Revision of Manuscript- O.E., Z.M.C.Y., S.B., R.Y., S.B.; Final Approval and Accountability- O.E., Z.M.C.Y., S.B., R.Y., S.B.

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

Financial Disclosure: This study was supported by the Research Fund of Istanbul University. Project Numbers T-891 and BEK-2017-24663.

Ethical Approval: This article contains studies with animals performed. This experimental study was carried out at the Institute's Animal Care and Use Committee of Istanbul University, in 2007-2008. The project was approved by the local ethics committee for animal experiments, at Istanbul University.

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