Some Positive Effects of Pine Oil on Brain Tissue in Streptozotocin-Induced Diabetic Rats

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Summary: Pine oil has antiseptic, expectorant and antioxidant properties and has been used for treatment of rheumatism, respiratory and urinary system and skin diseases. We aimed to determine protective effects of pine oil (PO) on the lipid-soluble vitamins, cholesterol, GSH, total protein, MDA, fatty acid levels of brain tissue of the streptozotocin-induced diabetic rats. Rats were randomly divided into three groups: Control (C), streptozotocin (STZ), streptozotocin+pine oil (PO) groups. Streptozotocin was injected intraperitoneally single dose (65 mg/kg) to the STZ and PO groups for inducing of diabetes. To the PO group 1 mg/kg dose pine oil was intraperitoneally injected every next day. While the GSH and total protein were significantly decreased in the Streptozotocin (STZ) group, their levels were protected in PO group. MDA level was significantly increased in STZ group, its level significantly decreased in the PO group. Our results showed that PO has a positive effect on the GSH, total protein, and MDA levels in the brain tissue of diabetic rats. The PO and STZ administrations were affected by levels of some important fatty acids. The decrease in the MDA level and observed protecting effects can be attributed to PO extract, because it contains some important phytochemical constituents.

Keywords: Diabetes, Pine oil, MDA, Glutathione, Cholesterol, Brain

Introduction

Diabetes mellitus is a very important metabolic disease, and it is associated with many functional and structural changes in the tissues. Also, diabetes has the long-term complications on the some important organs, such as brain, liver, kidneys, eyes etc [1]. Diabetes can affect the central nervous system and it can cause structural and functional changes on this system. This situation can cause some pathological changes, metabolic imbalances and deterioration in mental concepts in the central nervous system [2]. It was expressed that these changes are associated to the production of reactive oxygen species (ROS) in the human and experimental animal diabetes. Previous studies have been shown that ROS play an important role in the formation of diabetes-specific complications, such as retinopathy, nephropathy and neuropathy [3-6].

Pine species are one of the most common medicinal plants in the Mediterranean and Irano-Turanian regions. Some drugs obtained from these species have been widely used as antiseptic, expectorant and treatment of urinary, respiratory system, skin diseases, rheumatism for many years in Turkey [7-9]. *Pinus* species have been used in the food and pharmaceutical industries due to high biological activity and great potential [10].

The aim of this study is to determine the possible positive effects of pine oil on the GSH, MDA, lipid soluble vitamin contents, total protein and fatty acid composition (saturated fatty acids and unsaturated fatty acid level) brain tissue of streptozotocin-induced diabetic rats.

Experimental

Chemicals

All the chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis Mo, USA).

Animals

All the experimental protocols were approved by the Ethical Committee of Firat University (Decision Number: 2011/81). Thirty healthy male *Wistar* albino rats, aged 8-10 weeks and weight in the range of 225-250 g, were obtained from Firat University Experimental Research Centre. The animals were housed in polycarbonate cages in a room with a 12 h day/night cycle, at the temperature of 24±3 °C and humidity of 45–65%. During the whole experimental period, animals were fed with a balanced commercial diet and water ad libitum.

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Experimental Design

The thirty rats were randomly divided into three groups each containing ten rats.

Group C (Control): Rats were injected with 0.5 mL dimethyl sulfoxide (DMSO) twice a week, the rats received tab water and were fed with standard pellet diet as ad libitum [11].

Group STZ (Streptozotocin): Rats were injected intraperitoneally with a single dose of 65 mg/kg streptozotocin dissolved in 0.1 M citrate buffer and were fed with standard pellet diet as ad libitum [12].

Group PO (Streptozotocin+Pine Oil): Rats were injected with a single dose of 65 mg/kg streptozotocin intraperitoneally, received 1 mg/kg pine oil every day (intraperitoneal injection), and this extract was added to drinking water of the rats (pine oil) at 1 g /500 mL dose (a diabetic rat was consumed about 75 mL from this water at one day, and this drinking water was replaced every day), and were fed with standard pellet diet as ad libitum [13].

All treatments were continued for 2 months. Each experimental rat was decapitated after one week of the last injection of DMSO and the last intake of the pine oil. At the end of the experimental period, animals were fasted overnight and sacrificed by cervical decapitation. Brain tissues were washed in ice-cold saline to remove blood. The tissues were homogenized in Tris-HCl and ethylenediaminetetraacetic acid (EDTA) buffer at pH=7.4 and then centrifuged at 10000 rpm for 25 min at +4 °C. The supernatants were used for lipid peroxidation, GSH and total protein, and the pellets were used for fatty acids, vitamin and sterol analysis.

Induction of Diabetes

Overnight fasting animals were made diabetic by a single intraperitoneal injection of freshly prepared streptozotocin (STZ) (65 mg/kg) dissolved in 0.1 M citrate buffer (pH=4.5) [14]. Control rats were injected with the same volume of isotonic saline. Plasma glucose was determined at the end of 3 days, and the rats with glucose levels greater than 250 mg/dL were used in the present study. The blood glucose levels were measured using reagent strips (Contour, Bayer, Leverkusen, Germany).

Determination of Malondialdehyde (MDA) Level

MDA level in tissue homogenate was estimated using thiobarbituric acid reactive substances by the method of Ohkawa *et al.,* [15]. To 1.0 mL tissue homogenate, 0.5 mL of 8.1% sodium dodecyl sulfate (SDS), 1.0 mL of (20% acetic acid/NaOH, pH=3.5), 1.0 mL of 10% trichloroacetic acid (TCA), 50 µL of 2% butylated hydroxytoluene (BHT) and 1.0 mL of 0.8% thiobarbituric acid (TBA) were added. The mixture was heated in a water bath at 95 °C for 60 min. After cooling, 4.0 mL of n-butanol/pyridine mixture was added and shaken vigorously. After centrifugation at 4250 rpm for 15 min, the organic layer was taken and its absorbance at 532 nm was measured. 1,1,3,3-tetramethoxypropane was used as standard. The results were calculated as nmol MDA/g tissue.

Determination of Reduced Glutathione (GSH)

Reduced glutathione (GSH) was determined by the method of Ellman [16]. Briefly 1.0 mL of tissue homogenate was treated with 1.0 mL of 10% TCA. The mixtures were centrifuged in 5000 rpm for 10 min, and the supernatants were taken. After deproteinization, the supernatants were allowed to react with 1.0 mL of Ellman's reagent (30 mM, 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium citrate). The absorbance of the yellow product was read at 412 nm in a spectrophotometer. Pure GSH was used as the standard for establishing the calibration curve [17].

Lipid Extraction

Lipids of tissue samples were extracted with hexane–isopropanol (3:2, v/v) according to the method of Hara and Radin [18]. 1.0 g tissue sample was homogenized with 10.0 mL hexane-isopropyl alcohol mixture. Fatty acids in the lipid extracts were converted into methyl esters including 2% sulfuric acid (v/v) in methanol [19]. The fatty acid methyl esters were extracted with 5.0 mL n-hexane. Analysis of fatty acid methyl ester was performed in a Shimadzu GC-17A instrument gas chromatography equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm Permabond fused-silica capillary column (Machery–Nagel, Germany). The oven temperature was programmed between 145 and 215 °C, at the heating rate of 4 °C/min. Injector and FID temperatures were 240 and 280 °C, respectively. The nitrogen carrier gas flow was 1.0 mL/min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10

software version 2.01 was used to process the data. The results were expressed as percent amount/tissue.

Lipid Soluble Vitamins and Phytosterols Analyses

Lipid-soluble vitamins and phytosterols were extracted from the lipid fraction according to the method of Sanchez-Machado *et al.,* [20] with minor modifications. 5.0 mL of n-hexane/isopropyl alcohol mixture treated with 5.0 mL of KOH solution (0.5 M in methanol) were added and immediately vortexed for 20 s. The tubes were placed in a water bath at 80 °C for 15 min, and cooled in iced water. 1.0 mL of distilled water and 5.0 mL of hexane were added, and the mixture was rapidly vortexed for 1 min, then centrifuged at 5000 rpm for 5 min. The supernatant phases were transferred to another test tube and dried under nitrogen. The residue was redissolved in 1.0 mL of the mobile phase (68:28:4, v/v/v, methanol/acetonitrile/water). Finally, an aliquot of 20 µL was injected into the HPLC column. Before injection, the extracts were maintained at −20 °C away from light.

Chromatographic Conditions

Chromatographic analysis was performed using an analytical scale (15 cm × 0.45 cm) Supelco LC 18 DB column with a particle size 5 µm (Sigma, USA). HPLC conditions are given as follows: mobile phase 60:38:2 (v/v/v): acetonitrile/methanol/water; a flow rate of 1 mL/min; column temperature 30 °C. The detection was operated using two channels of a diode-array spectrophotometer, and cholesterol and lipid-soluble vitamins were identified by retention and spectral data [21, 22].

Determination of Total Protein

Total protein was estimated by the method of Lowry *et al.,* [23]. 10 µL of tissue homogenate was added to a 4.0 mL of alkaline copper reagent, and the mix was kept at room temperature. Then 0.5 mL of Folin's-Ciocalteau reagent was added to the mixture and the developing colour was read after 30 min at 750 nm. A standard curve was obtained using bovine serum albumin. The level of protein was expressed as mg albumin/g of tissue.

Statistical Analysis

All the data were statistically evaluated with SPSS/15.0 software. The statistical significance of the data was determined using one-way analysis of variance (ANOVA), and the group means were compared by Duncan's multiple range test (DMRT). $P<0.05$ was considered to indicate statistical significance.

Results and Discussion

The lipid-soluble vitamins, GSH, total protein and MDA levels of brain tissue are summarized in Table-1. Compared to the C group, α-tocopherol, cholesterol and GSH levels were decreased in the STZ and PO groups ($P<0.05$). α-tocopherol and GSH levels were protected in the PO group when compared to the STZ group. Vitamin D_2 and vitamin K_2 levels were increased in the STZ and PO groups when compared to C group ($P<0.05$). While total protein level was not changed in the PO group ($P>0.05$), its level was decreased in the STZ group ($P<0.05$). Once MDA level was increased in the STZ group ($P<0.05$), its level was decreased in the PO group ($P<0.05$).

Table-1: The biochemical parameters in brain of *Wistar* rats.

Biochemical Parameters	Control (C)	Streptozotocin (STZ)	Streptozotocin + Pine Oil (PO)
α-tocopherol (µg/g)	109.26±2.59^a	60.48±0.24^c	71.71±0.78^b
Vitamin D_2 (µg/g)	0.26±0.02^b	1.65±0.03^a	1.65±0.02^a
Vitamin K_2 (µg/g)	2.80±0.04^c	18.49±0.10^b	19.96±0.19^a
Cholesterol (µmol/g)	7.18±0.23^a	6.33±0.02^b	6.50±0.12^b
GSH (µmol/g)	1.63±0.01^a	0.68±0.02^c	1.15±0.03^b
Total Protein (µg/g)	45.93±1.70^a	44.18±0.90^b	45.39±0.80^a
MDA (nmol/g)	12.35±0.16^b	14.05±0.21^a	11.32±0.13^c

Data include mean±SD values for ten rats in each group. There is not any statistically difference among the groups described with same letters. ($P<0.05$)

The fatty acid composition of brain tissue is given in Table-2. Compared to the C group, 16:0, 16:1, 18:0, 18:2 fatty acid levels were increased in the STZ and PO groups ($P<0.05$). Although 18:1 fatty acid level was increased in the STZ group ($P<0.05$), its level was not changed in the PO group ($P>0.05$). While 18:3 fatty acid level was not changed in the STZ group ($P>0.05$), its level was decreased in the PO group ($P<0.05$). 20:4 and 22:6 fatty acid levels were decreased in the STZ group ($P<0.05$), but these levels did not change in the PO group ($P>0.05$) compared to the C group.

The phenolic compounds have natural antioxidant activity and have been used for treatment of diabetes, cancer and neurodegenerative diseases [24]. It has been suggested that active compounds, found in the plants, protect the tissues against some damages caused by free radicals. Many studies have been shown that these compounds have hypoglycemic, hypolipidemic and antioxidant properties [25].

Table-2: The fatty acid composition in brain of *Wistar* rats (%).

Fatty Acids	Control (C)	Streptozotocin (STZ)	Streptozotocin + Pine Oil (PO)
16:0	16.57±0.30^{c}	17.26±0.11^{b}	18.08±0.15^{a}
16:1	1.13±0.02^{b}	1.23±0.02^{a}	1.20±0.02^{a}
18:0	17.05±0.24^{c}	19.14±0.08^{a}	18.66±0.18^{b}
18:1	21.94±0.17^{b}	22.40±0.12^{a}	21.71±0.16^{b}
18:2	0.54±0.01^{c}	0.81±0.02^{a}	0.66±0.01^{b}
18:3	0.33±0.02^{a}	0.32±0.01^{a}	0.25±0.01^{b}
20:4	20.44±0.04^{a}	19.73±0.10^{b}	20.54±0.15^{a}
22:6	12.46±0.06^{a}	11.94±0.14^{b}	12.73±0.14^{a}
Others	9.54±0.72	7.14±0.25	6.17±0.18

Data include mean±SD values for ten rats in each group. There is not any statistically difference among the groups described with same letters. ($P<0.05$)

Lipid peroxidation (LPO) plays an important role in the pathology of some neurodegenerative diseases, such as Parkinson, Huntington, and Alzheimer [26]. Previous studies have been shown that antioxidant compounds inhibit the LPO, and these compounds strengthen the antioxidant system of brain [27, 28]. In the present study, while the MDA level was significantly increased, the GSH level was significantly decreased in the STZ group. As a result of administration of pine oil, the MDA level was decreased, the GSH level was protected in the PO group. The positive effects of pine oil on the oxidative stress are very important for the brain tissue against neurodegenerative diseases. Previous studies have been indicated that the antioxidant defense system activity and oxidative stress markers (MDA etc) were significantly decreased in the various tissues streptozotocin-induced diabetic rats [29-31].

There are various active compounds in the plant derived products, such as phenolic compounds, vitamins, sterols, flavonoids etc. These active compounds have the anti-inflammatory, antioxidant, anti-allergy, liver protective, anti-thrombotic, anti-viral, anticancer and hypoglycemic properties [32]. Kivrak *et al.,* [33] reported that there are very important phenolic compounds in the pine bark. In another study, it has been shown that pine leaves are rich in terms of catechin and proanthocyanidins [34]. Catechin hydrate protects the heart tissue in the high sucrose and high fat diet induced oxidative stress [35]. Previous studies have been shown that the pycnogenol, a compound isolated from pine bark, has protective effects on the kidney, erythrocytes, and brain tissues [36-38]. In the present study, it can be said that the MDA level was significantly decreased by the pine oil administration, thus this oil protected the brain tissue of diabetic rats. The brain is more open tissue against oxidative stress, free radical attack and LPO because this tissue contains high levels polyunsaturated fatty acids in its structure [39]. Therefore, the reduction of LPO level is very important for some pathological diseases.

Glutathione (GSH) is very important antioxidant molecule for brain, liver and kidney tissues. GSH can be effectively scavenged the reactive oxygen species (ROS), such as hydroxyl, peroxy nitrite. GSH deficiency can be lead to mitochondrial damage in the brain. GSH depletion has been associated to oxidative stress and the neuronal degeneration. In different neurodegenerative disorders, oxidative stress can be an important factor [40]. Scheff *et al.,* [37] showed that the pycnogenol administration was prevented the decrease of GSH and increase of GSSG in the traumatic brain damage created rats. In our study, while the GSH level was significantly decreased in the STZ group, its level was increased in the PO group as a result of pine oil administration. In the light of these results, the protection of GSH level is very important for oxidative stress and some neurodegenerative disorders in the brain tissue. We think that the pine oil and its antioxidant compounds were protected the GSH level and thus these compounds can be strengthening the antioxidant defense system in the brain tissue.

In this study, it was determined that there are significantly changes in the fatty acid composition, and the palmitic and stearic acid levels were significantly increased in the brain tissue of diabetic rats. In the type-I diabetic patients, stearic acid synthesis is very important when compared to other fatty acid synthesis [41]. Acetyl CoA carboxylase is catalyze to first phase of fatty acid biosynthesis and it synthesizes the palmitic acid. Acetyl CoA carboxylase and fatty acid synthase are important regulators of fatty acid biosynthesis [42, 43]. In the present study, when compared to STZ group values, while the stearic acid level was reduced, the palmitic acid level was increased as result of pine oil administration to diabetic rats. Some researchers have shown that the changes in the fatty acid composition of the brain tissue are depending on the diet [43-48]. In this study, the palmitoleic and oleic acid levels were significantly increased in the brain tissue. Pari and Venkateswaran [41] have indicated that the oleic acid level was significantly increased in the type-I and type-II diabetic patients.

Polyunsaturated fatty acids are necessary for the membrane structure, membrane viscosity and production of eicosanoids [41]. In mammalians, delta 5 desaturase and delta 6 desaturase enzymes synthesize the polyunsaturated fatty acids from linoleic, linolenic and oleic acids [49-51]. In our study, the linoleic, linolenic, arachidonic and docosahexaenoic acid levels were significantly changed in the brain tissue of diabetic rats. It has been observed that some important fatty acids were

accumulated or reduced in the diabetic and some pathological conditions due to inhibiting the activity of these enzymes. Also, it has been reported that some dramatically changes were occurred in the central nervous system functions, such as neuron size, learning and memory actions, responses of visual and odor warning, and nerve growth factor when the docosahexaenoic acid level is low in the brain tissue [52, 53].

Vitamin E is an antioxidant molecule and it has chain breaker property for antioxidant defense system of body. While α-tocopherol level was decreased in the STZ group, in contrast, its level was increased in the pine oil given PO group in the brain tissue of diabetic rats. Previous studies have been indicated that the LPO level was increased; α-tocopherol level was decreased in the STZ-induced diabetic rats [54, 55]. We can say that α-tocopherol level was reduced due to oxidative stress and consumption of endogenous antioxidant molecules in the STZ group, the α-tocopherol level was increased because pine oil and its content was supported the endogenous antioxidant molecules in the PO group. Kretzschmar *et al.,* [56] have reached the similar results in their study.

The brain is the richest tissue for cholesterol content. Diabetes mellitus may change the brain cholesterol metabolism via insulin level. These changes in cholesterol metabolism play an important role in the diabetes and neurological function disorders [57]. In our study, the cholesterol level was significantly decreased in the STZ and PO groups. Suzuki *et al.,* [57] have shown that the cholesterol synthesis was inhibited and the cholesterol content was reduced in the synaptosomal membranes of the brain tissue in the STZ-induced diabetic rats. Ozkan *et al.,* [58] have indicated that the cholesterol level was significantly decreased in the brain tissue of STZ-induced diabetic rats. The insulin deficiency can be caused the inhibiting of cholesterol biosynthesis in the brain tissue [57, 59]. Demir *et al.,* [60] have shown that the cholesterol level was significantly decreased in the pine oil given diabetic rats.

Conclusion

This study has been indicated that although the GSH level was significantly reduced in the STZ administration diabetes group, its level was protected in the pine oil given PO group. Also, the MDA level was significantly increased in the STZ group, and its level significantly decreased in the PO given group. The pine oil administration was changed the levels of some important fatty acids in fatty acid biosynthesis. The decrease of MDA level and increase of GSH level can be attributed to pine oil, because it contains some very important phytochemical compounds (sterol, flavonoids, phenolic acids and vitamins etc.).

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