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Grape seed extract has superior beneficial effects than vitamin E on oxidative stress and apoptosis in the hippocampus of streptozotocin induced diabetic rats

Goksin Nilufer Yonguc [a], Yavuz Dodurga [b,*], Esat Adiguzel [c], Gulsah Gundogdu [d], Vural Kucukatay [d], Seda Ozbal [e], Ismail Yilmaz [f], Ulker Cankurt [e], Yusuf Yilmaz [g], Ilgaz Akdogan [c]

[a] Izmir University School of Medicine, Department of Anatomy, Izmir, Turkey
[b] Pamukkale University School of Medicine, Department of Medical Biology, Denizli, Turkey
[c] Pamukkale University School of Medicine, Department of Anatomy, Denizli, Turkey
[d] Pamukkale University School of Medicine, Department of Physiology, Denizli, Turkey
[e] Dokuz Eylul University School of Medicine, Department of Histology and Embryology, Izmir, Turkey
[f] Izmir Bozyaka Training and Research Hospital, Department of Pharmacology, Izmir, Turkey
[g] Mehmet Akif Ersoy University Faculty of Engineering and Architecture, Department of Food Engineering, Burdur, Turkey

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ABSTRACT

We aimed to investigate the effects of grape seed extract (GSE) and vitamin E (Vit E) on oxidative stress and apoptosis in the hippocampus of streptozotocin-induced diabetic rats. In Control, Diabetic, and Diabetic treated with GSE (Diabetic + GSE) and vitamin E (Diabetic + Vit E) groups, oxidative stress index (OSI), TUNEL staining and Bcl-2, Bcl-XL, Bax, caspase-3, -9, and -8, Cyt-c, TNF-α, and NF-κB gene expressions were evaluated. OSI was significantly increased in the plasma and hippocampus of the Diabetic compared to Control group and decreased in Diabetic + GSE and Diabetic + Vit E groups compared to Diabetic. TUNEL positive neurons significantly increased in the hippocampus of the Diabetic group compared to Control and decreased in Diabetic + GSE (more prominently) and Diabetic + Vit E groups compared to Diabetic. In the hippocampus of the Diabetic group, Bcl-2 and Bcl-XL gene expressions were significantly decreased; Bax, caspase-3, -9, and -8, Cyt-c, TNF-α, and NF-κB gene expressions were significantly increased compared to Control. In Diabetic + GSE and Diabetic + Vit E groups, Bcl-2 gene expressions were significantly increased; Bcl-XL gene expressions did not differ compared to the Diabetic group. The expression of Bax, caspase-3, -9, and -8, Cyt-c, TNF-α, and NF-κB genes in the Diabetic + GSE group and the expression of caspase-3 and -9, TNF-α, and NF-κB genes in the Diabetic + Vit E group were significantly decreased compared to Diabetic. In conclusion, GSE (more prominently) and vitamin E decreased oxidative stress and neuronal apoptosis occurring in the hippocampus of diabetic rats.

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1. Introduction

Diabetes mellitus is an important metabolic disorder that causes functional and structural changes in the central nervous systems. Moderate cerebral atrophy and increased subcortical and brain stem lesions have been reported in uncontrolled diabetic patients. Previous studies have stated that memory, learning, and cognitive impairments are more common in diabetics than in nondiabetics (Reagan, 2007; Muriach et al., 2006; Li et al., 2002). The hippocampus is a critical integration center for cognitive functions such as learning and memory in the mammalian brain. Since the hippocampus is susceptible to hyperglycemic damage, it is considered to be a specific target for diabetes-related changes within the central nervous system (Alvarez et al., 2009).

Oxidative stress increased by hyperglycemia leads to numerous cellular reactions in the central nervous system. At the cellular level, mechanisms of high-glucose (HG)-induced cell death are sustained by oxidative stress in many cell types. Excessive formation of reactive oxygen-free radicals (ROS) as well as insufficient antioxidant capacity may damage cellular components. HG-induced free radical attack increases neuron death by causing damage to lipids, proteins and DNA (Bournival et al., 2012). At least, programmed cell death (apoptosis)

Abbreviations: GSE, grape seed extract; Vit E, vitamin E; OSI, oxidative stress index; TUNEL, Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP nick end labeling; ROS, reactive oxygen-free radicals; Cyt-c, cytochrome c; NF-κB, nuclear factor kappaB; TNF-α, tumor necrosis factor-α; TGF-1b, tumor growth factor-1b; STZ, streptozotocin; FRAP, Ferric Reducing Ability of Plasma; TOS, total oxidant status; TAS, total antioxidant status.

* Corresponding author at: Pamukkale University Medical Faculty, Medical Biology and Genetics, Denizli, Turkey.
E-mail address: yavuzdodurga@gmail.com (Y. Dodurga).

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may be a possible mechanism of glucose neurotoxicity (Piotrowski, 1999; Sharifi et al., 2007; Hernández-Fonseca et al., 2009; Bournival et al., 2012). Apoptosis occurs with gene regulation and is characterized by the induction of the expression of a number of transcription factors and genes. Increased Bax expression, alteration in Bcl-2 expression, increased cytochrome c (Cyt-c) release and increased caspase-3, -9, and -8 expressions have been shown to play a role in HG-mediated toxicity which induces apoptosis of neurons (Sharifi et al., 2007; Jafari Anarkooli et al., 2008; Hao et al., 2012; Fu et al., 2012). Additionally, induction of the apoptosis by increased activation of pro-inflammatory cytokines such as nuclear factor kappaB (NF-κB) (Alvarez-Nölting et al., 2012; Muriach et al., 2006; Jing et al., 2013) and tumor necrosis factor-α (TNF-α) (Marfella et al., 2006; Jing et al., 2013) in streptozotocin (STZ) diabetic rats has been previously reported. Consistent with the abovementioned mechanisms, increased numbers of TUNEL positive cells have been observed in various brain regions of diabetic animals (Li et al., 2002; Hernández-Fonseca et al., 2009; Huang et al., 2012; Liu et al., 2013).

For protection from HG-related neurodegeneration, increasing antioxidant levels (such as vitamins A, E, and C and glutathione) has been reported to be as important as preventing the production of excessive free radicals (Zaltzberg et al., 1999). Vitamin E has been reported to reduce the generation of reactive oxygen-free radicals, damage oxidative substances, and maintain membrane fluidity in diabetic rat brains (Hong et al., 2004). Vitamin E prevents lipid peroxidation, elevates glutathione levels in the hippocampus, and ameliorates learning and memory performance in STZ diabetic rats (Tuzcu and Baydas, 2006). The neuroprotective action of vitamin E involves reduction of oxidative stress as well as partial inhibition of apoptosis (Demiralay et al., 2008). In fact, the inhibitory effect of vitamin E on caspase-3 and some cytokines has been alleged (such as TNF-a, NF-κB, IL-1b, TGF-1b) in diabetic rats (Kuhad and Chopra, 2009a, 2009b).

Grape seed extract (GSE) is another powerful free radical scavenger (Balu et al., 2006; Devi et al., 2006; Lu et al., 2010). Grape seeds contain flavonoids (catechin, epicatechin, procyanidins and anthocyanins) and phenolic acids (gallic acid and ellagic acid) (Yilmaz and Toledo, 2006). GSE has been shown to provide a superior anti-oxidant effect compared to vitamin E at equal doses by weight (Bagchi et al., 1997; Ariga, 2004). Recent studies have reported that GSE has a potential therapeutic effect via reducing oxidative stress on diabetes and its related complications (Sato et al., 2001; Pinent et al., 2004; Cui et al., 2008; Chis et al., 2009; Kiyici et al., 2010; Mansouri et al., 2011; Ding et al., 2013). However, it has also been alleged that it has limited effects in this context (Belviranli et al., 2012). Besides some of its positive effects on the hippocampus (Xu et al., 2008) and cerebral cortex (Lu et al., 2010) of STZ-induced diabetic rats, we have not encountered any study about its effects on diabetes-related cerebral neurodegeneration. Yet, it has been argued in recent years that it has neuroprotective effects on many individual diseases. It has been shown that grape seed extract suppresses lipid peroxidation and reduces brain injury in hypoxic–ischemic rat models (Feng et al., 2007). It protects from MeHg-induced (Yang* et al., 2012b) and O-ethyl-S,S-dipropyl phosphorodithioate (MOCAP)-induced neurotoxicity (Ray et al., 2000; Bagchi et al., 2001) and from Alzheimer's disease (Ksiezak-Reding et al., 2012), inhibits glutamate excitotoxicity (Ahn et al., 2011), and enhances neurogenesis in the dentate gyrus (Yoo et al., 2011). Although previous studies have already presented antiapoptotic effects of GSE in various tissues, including reduction of apoptotic cell death and suppression of proapoptotic proteins (Sato et al., 2001; Ozkan et al., 2012; Bayatli et al., 2013; Cedó et al., 2013; Chen et al., 2013; Katiyar and Athar, 2013), there are limited studies about its effects on neurons (Ray et al., 2000; Bagchi et al., 2001; Balu et al., 2006; Feng et al., 2007; Yang et al., 2012a, Yang et al., 2012b). Moreover, limited information is available about the effects of GSE on hippocampal neuronal loss in STZ-induced diabetic rats.

We attempted in this study to assess the anti-oxidative, anti-inflammatory, and anti-apoptotic effects of GSE that was obtained from red grapes of Calkarasi (*Vitis vinifera* L. cv.) from the vicinity of Denizli and vitamin E on neuronal loss occurring in the hippocampus of STZ-induced diabetic rats.

2. Materials and methods

2.1. Drugs

STZ and α-tocopherol (vitamin E) were obtained from Sigma (Sigma, St. Louis, USA). GSE was obtained from the wastes of grape molasses (pekmez) production (Department of Food Engineering, Pamukkale University, Denizli, Turkey). Manually cleaned seeds were ground with an electric coffee mill (MKM 6000, Type KM 13, Bosch Inc., Istanbul, Turkey). Powdered grape seeds were mixed with aqueous ethanol (70%, v/v) at a ratio of 1:10 (w/v) and the mixture was subjected to 15 min of sonication in an ultrasonic bath. Then, it was stirred on a mechanical shaker at room temperature for 30 min. After this step, the mixture was centrifuged at 4 °C for 20 min at 8500 rpm/min. Supernatants were collected with Pasteur pipettes and transferred into 30 mL amber glass bottles. Aqueous ethanol (70%, v/v) was added to the remaining residue at a ratio of 1:10 (w/v) and the extraction procedure was repeated. After the volume was adjusted in volumetric flasks, pooled supernatants were stored in a freezer at −20 °C in amber glass vials before freeze drying. Before lyophilization, ethanol in supernatants was first evaporated by a rotary evaporator (Büchi, Rotavapor, Germany) under vacuum at 40 °C (~10 min). Viscous extracts were frozen at −70 °C, and then lyophilized (Thermo M230, Germany). Freeze-dried powder was collected carefully with a micro-spatula, and amber vials of lyophilized GSE were stored at −24 °C until analyses. Experiments were followed gravimetrically and the extraction efficiency was calculated at the end of the process. Antioxidant activity was evaluated by using the Ferric Reducing Ability of Plasma (FRAP) method. Catechin, epicatechin and gallic acid contents of GSE were determined chromatographically (Yilmaz and Toledo, 2004). Analyses are presented in Tables 1–3.

2.2. Animals

Twenty-four male Sprague Dawley rats, weighing 250–300 g were purchased from the Animal Research Laboratory of Pamukkale University, Denizli, Turkey. The animals were housed in groups of three rats per cage during experiment, and had free access to rat chow and tap water. The cages had a plastic base and a stainless steel covering and were placed in an animal room. The environmental conditions of the animal room were controlled (humidity: 50 ± 5%, temperature: 22 ± 2 °C, 12-hour dark–light cycle). The study protocol was approved by the Pamukkale University Animal Care and Use Committee.

2.3. Experimental procedure

At the beginning of the experiment body weights and blood glucose levels of all the rats were measured (Table 4). Then, the animals were divided into two groups: control (n = 6) and diabetic (n = 18). Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight), freshly dissolved in phosphate buffered saline (0.1 M, pH 4.5), while control rats were injected with vehicle buffer only. Three days after the induction of diabetes, fasting blood glucose levels were measured from tail vein blood samples (Table 4). Animals

Table 1
Extraction properties of the freeze-dried grape seed extracts and the efficiency of the extraction procedure.

Property	Value (%)
Total phenolic content (gallic acid equivalent)	45.1
Total flavonoid content (catechin equivalent)	27.1
Extraction efficiency	1.12

Table 2
Phenolic content (on a dry matter basis) and antioxidant activity (FRAP method) of grape seeds obtained from the wastes of grape molasses production.

Property	Value
Total phenolic content (mg GAE/g)	54.6 ± 6.4
Antioxidant activity (mmol Trolox equivalent/g)	36.2 ± 5.5

with blood glucose level above 300 mg/dL were declared diabetic. Then, the diabetic animals were randomly assigned into three groups: diabetic (Diabetic; n = 6), GSE treated diabetic (Diabetic + GSE; n = 6) and vitamin E treated diabetic (Diabetic + Vit E; n = 6). GSE (100 mg/kg) and vitamin E (100 mg/kg) were administered orally using gavage needles everyday for six weeks in GSE treated and vitamin E treated groups respectively. Doses of GSE and vitamin E and duration of treatment were determined according to pilot studies as well as available published data reporting protective effects of GSE and vitamin E in diabetic rats (Tuzcu and Baydas, 2006; Kiyici et al., 2010). The body weights and blood glucose levels of animals were reassessed just prior to sacrificing the animals (Table 4).

At the end of the treatment period the rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The rats were decapitated and their brains were removed following intracardiac blood sampling. The plasma samples were stored at −20 °C for the measurement of parameters representing the total oxidant–antioxidant status. The left and right cerebral hemispheres of all the rats were separated. The left hemispheres were used for the detection of cell death. The right hemispheres were dissected, and hippocampi were separated. All the right hippocampi were further cut into two pieces. One piece was used for the measurement of parameters representing the total oxidant–antioxidant status, and the other was used for quantification of apoptosis related gene expression. All tissues from the right hemispheres were stored at −20 °C until they were used.

For detection of cell death, the left hemispheres of brain tissues were fixed in 10% formalin solution, processed by routine histological methods and embedded in paraffin blocks. Three consecutive coronal sections of 5 μm thickness were taken through the hippocampus (CA1, CA2, CA3 regions) that corresponded approximately to Plates 22 and 23 in the rat atlas of Paxinos and Watson (2002).

2.4. Measurement of total oxidant status

The hippocampal tissue samples were homogenized and the supernatants of the homogenates were prepared by centrifugation at 12,000 rpm for 15 min at +4 °C. The tissue protein concentration was measured using the Lowry method. The total oxidant status (TOS) of the plasma and tissue samples was determined using a novel automated colorimetric measurement method described by Erel (2005). In this method, the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L), and per milligram protein (μmol H_2O_2 Eq/mg protein), for plasma and tissue samples respectively.

2.5. Measurement of total antioxidant status

The total antioxidant status (TAS) of the plasma and tissue samples was determined using an automated colorimetric measurement method described by Erel (2004). In this method the assay results were expressed as mmol Trolox Eq/L and mmol Trolox Eq/mg protein, for plasma and tissue samples respectively. The precision value of this assay was lower than 3%.

Table 3
Gallic acid, catechin and epicatechin contents of freeze-dried extracts of grape seeds.

Substance	Concentration (mg/100 g GSE)
Gallic acid	53.5 ± 5.6
Catechin	65.9 ± 9.5
Epicatechin	45.4 ± 8.7

2.6. Determination of oxidative stress index

The measured ratio of TOS to TAS is referred to as the oxidative stress index (OSI). For calculation of OSI, the resulting unit of TAS was converted to mmol/L, and thereby, the OSI value was calculated using the following formulas: OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/L) / TAS (mmol Trolox Eq/L) × 100 for plasma and OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/mg protein) / TAS (mmol Trolox Eq/mg protein) × 100 for tissue samples (Demirbag et al., 2007).

2.7. In situ cell death detection

To detect DNA fragmentation, TUNEL (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany) was applied to the paraffin sections. The sections were stained with diaminobenzidine solution for 10 min at room temperature, counterstained with hematoxylin and analyzed by using a light microscope.

For quantitative measurement of the number of cells that underwent apoptosis, 1000 cells were randomly counted in cells showing apoptotic morphology in the hippocampus, and apoptotic cell percentages were calculated.

2.8. RNA isolation and analyses of apoptosis related gene expression

The hippocampi from the right hemispheres were divided into small pieces in trizol solution by a lancet on ice. Total RNA was extracted from the tissues using an RNA isolation reagent (Tri reagent) procedure (Sigma, St. Louis, MO, USA). An EasyScript Plus cDNA Synthesis kit was used for the synthesis of complementary DNA (cDNA) from the tissues.

2.9. Relative quantification of apoptosis related gene expression

Real-time quantitative RT-PCR analyses of all genes were performed in 96 well microplates using a StepOne Plus RT-PCR instrument and software. Rat primers were used for gene expression analyses. Beta actin and the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene were chosen as a standard to control the variability in amplification. RT-PCR was performed by using an EvaGreen qPCR master mix-ROX kit according to the instructions of the manufacturer. The experiments were repeated twice using duplicates in each group.

2.10. Statistical analyses

The data from the measurement of body weight, blood glucose level, TOS, TAS and OSI were expressed as means ± S.E.M. Statistical comparisons between the groups were carried out using the Kruskal–Wallis test. Differences between the two groups were analyzed with the Mann–Whitney U-test. p values <0.05 were considered statistically significant.

Data from apoptosis related gene expressions were analyzed by the Delta–Delta CT method, and quantitated by LightCycler Quantification Software. All the statistical analyses were carried out using SPSS 15.0 (Statistical Package for Social Sciences, SPSS Inc.).

3. Results

3.1. Body weights and glucose levels

The mean blood glucose levels and body weights in the four groups of rats are summarized in Table 4. At the onset of the study, all animals in four groups had equivalent blood glucose levels. Three days after STZ injection, all diabetic animals had significantly elevated blood glucose

Table 4
Means of blood glucose levels and body weights of the groups.

Groups	Blood glucose levels (mg/dL)			Body weights (g)		Signs of diabetes
	Onset of study	Three days after STZ injection	End of study	Onset of study	End of study	Polyuria, polydipsia**
Control	117.8 ± 2.4	114.8 ± 3*	117.5 ± 2.3*	267.6 ± 4.5	310.3 ± 4.7*	−, −
Diabetic	120.6 ± 1.7	448 ± 3.3	446.6 ± 6.6	270.3 ± 2	223 ± 3.9	+, +
Diabetic + GSE	120.3 ± 1.3	439.1 ± 4.9	432.5 ± 6.3	269 ± 5.2	234.8 ± 3.9	+, +
Diabetic + Vit E	116.8 ± 1.2	446 ± 6.8	428.6 ± 6.1	278.3 ± 5.6	241.6 ± 7.1	+, +

Values are expressed as means ± S.E.M.; n = 6 all groups.
* Significant at p = 0.002 compared to STZ, STZ + GSE, and STZ + Vit E groups.
** Polyuria and polydipsia were based on frequency of cage cleaning and number of bottles consumed per day.

levels. At the end of the experiment, glucose levels were significantly elevated in all STZ injected diabetic animals compared to controls ($p < 0.05$). These high levels did not significantly alter throughout the experimental period in any of the diabetic groups (Diabetic, Diabetic + GSE and Diabetic + Vit E). At the end of the experiment, significant body weight loss was observed in the all groups of diabetic animals compared to those in the Control group ($p < 0.05$) (Table 4).

3.2. Total antioxidant status (TAS) and total oxidant status (TOS)

TAS and TOS levels of the plasma and hippocampus are shown in Figs. 1 and 2, respectively. TAS levels of the plasma and the hippocampus were significantly decreased in the Diabetic group compared to the Control group ($p < 0.05$), and increased by GSE and vitamin E treatments in the Diabetic + GSE and Diabetic + Vit E groups compared to the Diabetic group ($p < 0.05$). Furthermore, there were no statistically significant differences between the mean TAS levels of the plasma and the hippocampus of Diabetic + GSE and Diabetic + Vit E groups ($p > 0.05$) (Fig. 1).

As seen in Fig. 2, TOS levels of the plasma and hippocampus in the Diabetic group were higher than those in the Control group, but the differences were not statistically significant ($p > 0.05$). Although the TOS levels of the plasma and hippocampus tended to decrease in the Diabetic + GSE and Diabetic + Vit E groups this decrease was not statistically significant either ($p > 0.05$).

3.3. Oxidative stress index (OSI)

The mean OSI levels of the plasma and hippocampus were increased in the Diabetic group compared to the Control group ($p < 0.05$), and were significantly reduced by GSE and vitamin E treatments in Diabetic + GSE and Diabetic + Vit E groups compared to the Diabetic group ($p < 0.05$, Fig. 3). Additionally, the differences between the mean OSI ratios in the plasma and hippocampus of Diabetic + GSE and Diabetic + Vit E groups were not statistically significant ($p > 0.05$).

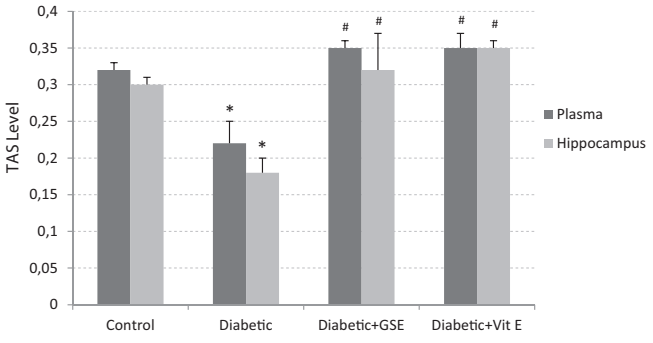


Fig. 1. Effects of vitamin E and GSE treatments on plasma and hippocampus TAS levels of control, diabetic and GSE treated and vitamin E treated diabetic rats. TAS: μmol H_2O_2 Eq/L for plasma and mmol Trolox Eq/mg protein for hippocampus. Values are expressed as means ± S.E.M.; n = 6 all groups. *$p < 0.05$ difference from control and #$p < 0.05$ difference from diabetic group.

3.4. In situ cell death detection

An increased number of neurons with fragmented DNA labeled by the TUNEL assay, were found in the hippocampus (CA1, CA2, CA3) of the Diabetic group rats, compared to the Control group ($p < 0.05$). On the other hand, GSE and vitamin E treatments significantly reduced the number of TUNEL-positive neurons in Diabetic + GSE and Diabetic + Vit E groups compared to the Diabetic group ($p < 0.05$ for all). Furthermore, the number of TUNEL-positive neurons of the Diabetic + GSE group was significantly lower than those of the Diabetic + Vit E group ($p < 0.05$) (Figs. 4 and 5).

3.5. Apoptosis related gene expression analyses with RT-PCR

Analyses of the gene expression changes in all groups were performed using a special analysis called "Volcano Plot". Bcl-2 and Bcl-XL gene expressions were significantly decreased in the Diabetic group compared to the Control group ($p = 0.002$ for both gene comparisons). The expression of the Bcl-2 gene was significantly increased in the Diabetic + GSE group and Diabetic + Vit E group compared to the Diabetic group ($p = 0.002$ and $p = 0.04$, respectively). On the other hand, there were no significant changes of the expression of the Bcl-XL gene among the Diabetic, Diabetic + GSE and Diabetic + Vit E groups ($p > 0.05$).

The expression of the Bax gene was significantly increased in the Diabetic group compared to the Control group ($p = 0.004$) and Bax gene expression was significantly decreased in the Diabetic + GSE group compared to the Diabetic group ($p = 0.004$). There was no significant change in the Bax gene expression level in the Diabetic + Vit E group ($p > 0.05$, Diabetic + Vit E vs Diabetic group).

Caspase-3, -9 and -8 levels were significantly increased in the Diabetic group ($p = 0.002$, $p = 0.04$ and $p = 0.002$ respectively, Diabetic group vs Control group), and were significantly decreased in the Diabetic + GSE group ($p = 0.002$, $p = 0.004$ and $p = 0.009$ respectively, Diabetic + GSE group vs Diabetic group). While caspase-3 and -9 gene expression levels were decreased in the Diabetic + Vit E group ($p = 0.004$, $p = 0.002$ respectively, Diabetic + Vit E group vs Diabetic group),

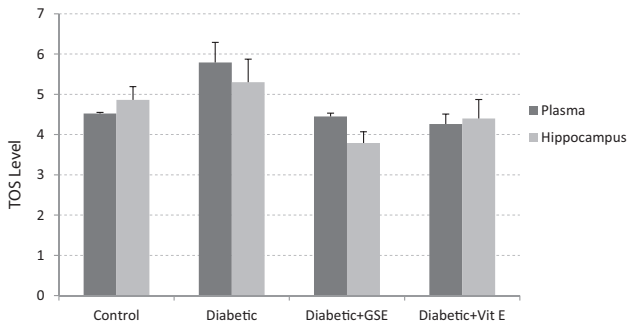


Fig. 2. Effects of vitamin E and GSE treatments on plasma and hippocampus TOS levels of control, diabetic and GSE treated and vitamin E treated diabetic rats. TOS: μmol H_2O_2 Eq/L for plasma and μmol H_2O_2 Eq/mg protein for hippocampus. Values are expressed as means ± S.E.M.; n = 6 all groups.

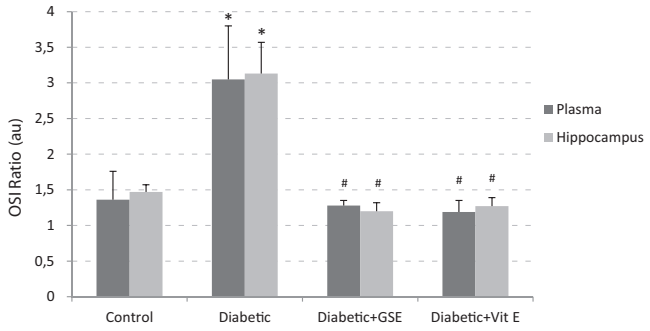


Fig. 3. Effects of vitamin E and GSE treatments on plasma and hippocampus OSI ratio of control, diabetic and GSE treated and vitamin E treated diabetic rats. OSI: au for both plasma and hippocampus. Values are expressed as means ± S.E.M.; n = 6 all groups. *p < 0.05 difference from control and #p < 0.05 difference from diabetic group.

there was no significant difference in increased levels of caspase-8 gene expression (p > 0.05, Diabetic + Vit E vs Diabetic group).

Cyt-c expression levels were significantly increased in the Diabetic group compared to the Control group (p = 0.004). Cyt-c expression was significantly decreased in the Diabetic + GSE group (p = 0.004, Diabetic + GSE vs Diabetic group). There was no significant difference in Cyt-c expressions between Diabetic + Vit E and Diabetic groups (p > 0.05).

TNF-α and NF-κB gene expressions were significantly increased in the Diabetic group (p = 0.004 and p = 0.002 respectively, Diabetic group vs Control group). In Diabetic + GSE and Diabetic + Vit E groups, TNF-α (p = 0.01 and p = 0.004, Diabetic + GSE and Diabetic + Vit E groups vs Diabetic group, respectively) and NF-κB (p = 0.02 and p = 0.004, Diabetic + GSE and Diabetic + Vit E groups vs Diabetic group, respectively) expressions were significantly decreased.

4. Discussion

In the present study, we investigated the effects of GSE and vitamin E on oxidative stress and apoptosis in the hippocampus of STZ induced diabetic rats. Importantly, it was determined that both GSE and Vit E decreased oxidative stress and apoptosis which occurred in the hippocampus of STZ induced diabetic rats. Additionally, our study is the first to investigate the effects GSE that was obtained from red grapes of Calkarasi (*V. vinifera* L. cv.) from the vicinity of Denizli, on the neuronal loss that occurs in the hippocampus of STZ diabetic rats.

In many studies, GSE and vitamin E treatments exerted an antihyperglycemic property in diabetic rats (Pinent et al., 2004; Chis et al., 2009; Kuhad and Chopra, 2009b; Ding et al., 2013). However, in our study GSE and vitamin E treatments did not exert an antihyperglycemic effect. Consistent with our results, Belviranli et al. (2012) and Cui et al. (2008) have reported that GSE has no effect on increased blood glucose levels in STZ diabetic rats.

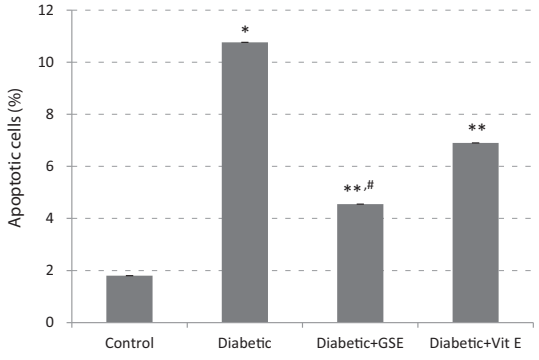


Fig. 4. Effects of GSE and vitamin E treatments on neuronal apoptosis of diabetic rat hippocampus. Values are expressed as means ± S.E.M.; n = 6 all groups. *p < 0.05 difference from Control, **p < 0.05 difference from Diabetic, and #p < 0.05 difference from Diabetic + Vit E group.

Similarly, it has been reported that vitamin E has no effects on blood glucose levels of type-2 diabetic patients although it improved HbA1c (Suksomboon et al., 2011). These contradicting results relating to GSE and vitamin E may have arisen partially due to different doses and administration times employed in the studies. Both GSE and vitamin E treatments did not ameliorate body weight loss in diabetic groups in our study. Although GSE and vitamin E treated diabetic groups had higher body weights than that of untreated diabetic rats, there was a significant decrease in the body weights of three diabetic groups in comparison to the control group of rats. Mansouri et al. (2011) and Rupérez et al. (2008) have also reported that GSE and vitamin E have no significant effect on increased body weights of STZ diabetic rats, although they improve it slightly. Contrary to our results, Cui et al. (2008) and Kuhad and Chopra (2009a) have reported that GSE and vitamin E significantly improve body weights of diabetic rats when they are administered for 8–24 weeks.

In normal conditions, there is a delicate balance between free radical formation and their removal. However, impairment of this balance in favor of oxidative stress in diabetics due to excessive elevation of blood glucose plays a prominent role in the development of diabetic complications. In our study, we tried to reveal the relationship between oxidative stress and antioxidants in the brain and there are limited studies in literature on this matter. Oxidative stress in diabetes is characterized by a reduction in the antioxidant capacity, which can increase the deleterious effects of free radicals (Chis et al., 2009). In general, measuring only one of the oxidant or antioxidant parameters does not provide proper information about the oxidative status. For this reason, in our study we measured both TOS and TAS of the hippocampal tissues and serum. Also, we used OSI as another indicator of oxidative stress. OSI is the ratio of TOS to TAS and it was proposed to reflect the oxidative status more accurately than TOS (Harma et al., 2005). Firstly, our results revealed that OSI was significantly higher and the TAS level was lower in the hippocampus and plasma of diabetic rats (Acar et al., 2012; Uzar et al., 2012). Secondly, GSE and vitamin E treatments decreased oxidative stress in the hippocampus and plasma of diabetic rats. Consistent with our results, grape seed proanthocyanidin extracts (Xu et al., 2008) and vitamin E (Hong et al., 2004; Tuzcu and Baydas, 2006; Kabay et al., 2009) have been shown to ameliorate increased oxidative stress in the brains of diabetic rats. Also, Sato et al. (2001) have stated that grape seed proanthocyanidin extracts have in vivo antioxidant activity and can be as important as vitamin E in preventing ischemia/reperfusion induced oxidative damage and apoptosis in cardiomyocytes by reducing the lipid oxidation and/or inhibiting the production of free radicals. Similarly, grape seed polyphenols have been shown to have antioxidant effects on exercise-induced oxidative stress in liver tissue (Belviranli et al., 2013). When oxidative stress was considered, there was no significant difference between the results of GSE and vitamin E treatments, in our study. This finding was inconsistent with the studies of Bagchi et al. (1997) and Ariga (2004), which suggest that GSE proanthocyanidins have superior antioxidant effects. Our results suggested that GSE proanthocyanidins are free radical scavengers at least as important as vitamin E and our study is one of the few studies (Xu et al., 2008) that have demonstrated how it suppresses the oxidative stress in the hippocampus of diabetic animals and enhances the antioxidative capacity.

In recent studies, the number of TUNEL positive cells has been observed to increase more in the brains of diabetic animals than in those of the controls (Huang et al., 2012; Liu et al., 2013), but this increase failed to reach statistical significance in the study by Hernández-Fonseca et al. (2009). We found in our study that there was significantly more neuronal apoptosis in the hippocampus of diabetic rats than in those of the controls. Li et al. (2002) also stated that there was an increase in TUNEL (+) neurons in the hippocampus of diabetic rats. In our study, while both vitamin E and proanthocyanidins in GSE decreased the number of TUNEL (+) neurons in the hippocampus, GSE was more effective than vitamin E.

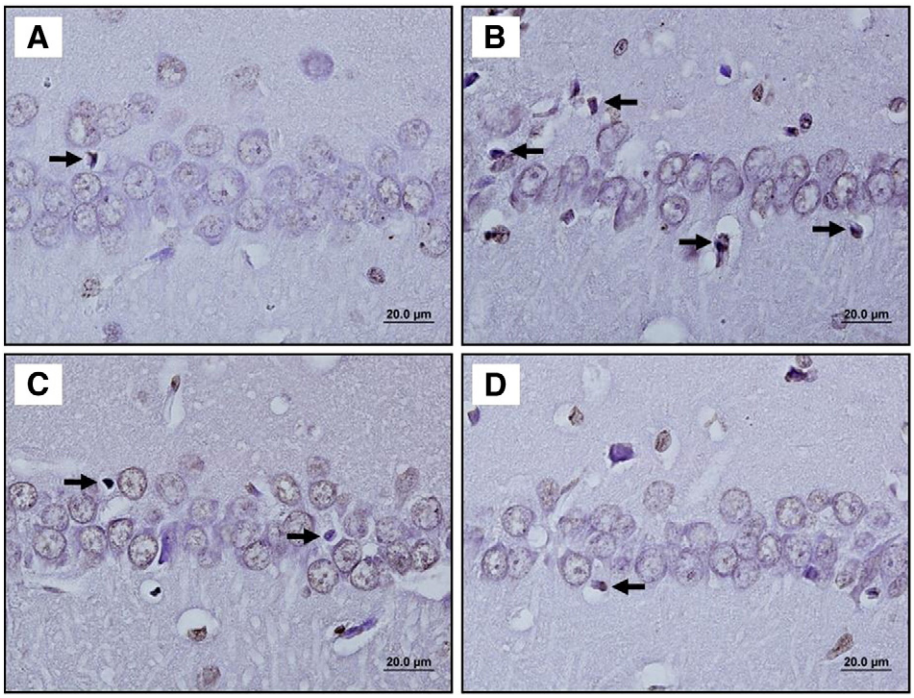


Fig. 5. Effects of diabetes on TUNEL reactivity of hippocampus. TUNEL-positive cells enhanced in the diabetic group (B). In vitamin E treatment group (C) and GSE treatment group (D) the number of apoptotic neurons was reduced significantly compared to control group (A). Arrows indicate TUNEL-positive cells.

Balu et al. (2006) showed that GSE had an inhibiting effect on age-related oxidative DNA damages in the spinal cord and in various brain regions such as the cerebral cortex, striatum and hippocampus. GSE also reduced a hypoxia–ischemia-induced increase in the proapoptotic protein c-jun in the brain cortex (Feng et al., 2007) and produced a neuroprotective effect against oxidative stress-induced apoptotic death in a transformed retinal ganglion cell line (Yang et al., 2012a). The present study is the first to show the neuroprotective effect of GSE on the hippocampal neuronal apoptosis caused by diabetes. This effect, which was more apparent in GSE than in vitamin E, may be occurring because GSE is a strong antioxidant and has greater anti-apoptotic and anti-inflammatory properties. It has been shown in various tissues, other than the brain, that vitamin E also inhibits apoptosis and reduces the number of TUNEL (+) cells in STZ-induced diabetic rats (Ustuner et al., 2010; Kaplanoglu et al., 2013). Apart from these studies, we demonstrated in the present study that vitamin E had a neuroprotective effect on hippocampal pyramidal neurons of STZ-induced diabetic rats.

Apoptosis is a programmed cell death process that involves two main protein families, caspase enzymes and Bcl-2 family, and is associated with induction of the expression of a number of pro- as well as antiapoptotic transcription factors and genes (Jafari Anarkooli et al., 2008). We found in this study that hippocampal Bcl-2 and Bcl-XL expressions decreased more and Bax expressions increased more in the hippocampus of diabetic rats than those of the controls. These results are in line with those of many similar studies, most of which were done in vitro (Hao et al., 2012; Fu et al., 2012; Bournival et al., 2012; Huang et al., 2012). In one of these in vitro studies, Fu et al. (2012) reported increased Bax expressions and decreased Bcl-2 expressions in different glucose concentrations. By contrast, Sharifi et al. (2007) argued that Bcl-2 expression did not significantly change in high glucose treated cell culture. Additionally, diabetes induced translocation of Bax to mitochondria and increased Cyt-c release into the cytoplasm have been demonstrated in recent studies (Ye et al., 2011; Hao et al., 2012). The release of Cyt-c from mitochondria into the cytoplasm may be responsible for apoptosis in the CA1 pyramidal neurons, which leads to a decrease in the number of hippocampal neurons (Ye et al., 2011). In our study, GSE reduced the increased Cyt-c expression in the hippocampus of diabetic rats, whereas vitamin E had no effect. While vitamin E had no effect on Bax expression in diabetic rats, it increased Bcl-2 expression. Gäreskog et al. (2006) stated, in an embryotoxicity study, that administration of vitamin E and folic acid combination normalized the Bcl-2 level (by increasing it) and Bax level (by decreasing it) in diabetic rats. However, the results of the aforementioned study do not reflect neuronal tissues in particular as in our study. In the present study, GSE proanthocyanidins significantly reduced Bax expression and increased Bcl-2 expression in diabetic rats. These changes in GSE treated and vitamin E treated groups may support their anti-apoptotic effect. Neither vitamin E nor GSE had any effect on Bcl-XL.

Caspases are a class of cysteine proteases that play a pivotal role in the execution of apoptosis. Caspase enzymes work as a cascade; caspases 8 and 9 play an essential role in apoptotic pathways. In turn, these caspases activate caspase-3, which is the most important member of this family playing an effective role in apoptosis of central nervous system neurons (Sharifi et al., 2009). In our study, caspase-3, -9 and -8 expressions were observed to increase in diabetic rats. Consistent with our results, increased activity of these caspases, especially caspase-3, has been reported in the hippocampus and other neuronal tissues in both in vivo and in vitro models of diabetes (Piotrowski et al., 2001; Jafari Anarkooli et al., 2008; Sharifi et al., 2009; Bournival et al., 2012; Hao et al., 2012; Huang et al., 2012; Liu et al., 2013). In the present study, GSE proanthocyanidins significantly decreased caspase-3, -9 and -8 expressions in the hippocampus of diabetic rats. Its properties in decreasing the intracellular expression levels of Bax and Cyt-c, increasing the expression levels of Bcl-2, and decreasing caspase-3, -9 and -8 expressions suggest that the mitochondrial mechanism of apoptosis may play an important role on the neuroprotective effect of GSE. Vitamin E, on the other hand, has been shown to particularly inhibit caspase-3 in various experimental studies relating to neuronal tissues (Kölsch et al., 2001; Kuhad and Chopra, 2009a, 2009b). Similar findings have also been shown in the hippocampus of diabetic rats (Kuhad et al., 2009). In our study, vitamin E reduced the increase in caspase-3 and -9 expressions, but did not affect caspase-8 in STZ diabetic rats.

Hyperglycemia has been shown to cause inflammation in the brain. Evidence has accumulated indicating that inflammatory mediators

increase in the brain of diabetics (Cai et al., 2011). In recent years, pro-inflammatory cytokines such as TNF-α and interleukin-1 beta have been shown to increase in the hippocampus of STZ-induced diabetic rats (Kuhad and Chopra, 2009a; Kuhad et al., 2009; Jing et al., 2013). Moreover, Kuhad and Chopra (2009a) and Kuhad et al. (2009) showed that tocotrienol (vitamin E) treatment significantly, and dose dependently inhibited TNF-α levels, prevented formation of reactive oxygen species and induced NF-κB p56 subunit expression in the nuclear fraction of different brain areas of treated STZ diabetic rats. In line with these findings, our results relating to vitamin E support its anti-inflammatory effects. Proanthocyanidins from grape seeds and grape-seed procyanidins are known to enhance the production of anti-inflammatory cytokines and to have inflammation-reducing effects (Li et al., 2001; Manach et al., 2005) in obese patients with type-2 diabetes (Chacón et al., 2009) and in neuronal tissue (Wang et al., 2009). In the present study, GSE treatment decreased TNF-α expression in the hippocampus of STZ-induced diabetic rats. Additionally, NF-κB has been blamed for TNF-mediated apoptosis and has a pivotal role in cellular response to oxidative stress (Fitzgerald et al., 2007). Neuronal damage due to NF-κB activation in different neuronal tissues (Muriach et al., 2006; Jing et al., 2013), increased NF-κB positive cells in the hippocampus (Alvarez-Nölting et al., 2012), diabetes-induced hippocampal impairments and impaired cognitive function (Kuhad et al., 2009) due to activation of the NF-κB signaling pathway have been shown in experimental animal models of diabetes. Xu et al. (2008) reported that oral administration of grape seed proanthocyanidin extracts decreased NF-κB p65 expression in the hippocampus of diabetic rats and reduced the negative structural changes associated with it. Lu et al. (2010) found in a similar study that these grape seed proanthocyanidin-related positive effects were also seen in the cerebral cortices of STZ-induced diabetic rats. Grape seed proanthocyanidin extract have shown to protect human lens epithelial cells from H_2O_2-induced oxidative stress via reducing NF-κB and MAPK protein expression (Jia et al., 2011). Additionally, tocotrienol treatment significantly and dose dependently (except 25 mg/kg) prevented NF-κB p56 subunit expression in the nuclear fraction of different brain areas of STZ diabetic rats (Kuhad and Chopra, 2009a). In line with these studies, our study also demonstrated that while NF-κB expression was increasing in the hippocampus of diabetic animals, both GSE and vitamin E decreased NF-κB expression. These findings suggest that the activation of the NF-κB pathway by hyperglycemia induces apoptosis in neuronal cells and this mechanism may also play a role in the anti-apoptotic effect of GSE proanthocyanidins and vitamin E (Kuhad et al., 2009; Du et al., 1998).

In conclusion, the present study suggests that STZ-induced diabetes induces oxidative stress and increases TUNEL positive neurons, which are closely associated with the alterations of apoptosis related genes, in the hippocampus of rats. More importantly, both GSE proanthocyanidins (more prominently) and vitamin E treatments decrease hippocampal TUNEL positive neurons. Our results revealed that, GSE proanthocyanidins and vitamin E exert neuroprotective effects by reducing oxidative stress and the expression of apoptosis related genes. Due to their promising results in the field of neuroprotective treatment, performing larger detailed and comparative studies about the mechanisms of action of GSE and vitamin E will be useful to reveal more about the treatment of diabetic complications.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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