Effects of grape seed extract on oxidative stress and antioxidant defense markers in streptozotocin-induced diabetic rats

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Background/aim: To evaluate the effects of grape seed extract (GSE) supplementation on oxidative stress and antioxidant markers in streptozotocin (STZ)-induced diabetic rats.

Materials and methods: Thirty-six male rats were divided into the following four groups: control, GSE-supplemented control, diabetic, and GSE-supplemented diabetic. Beginning on day 7 after STZ injection, the rats were administered GSE (100 mg kg^{-1} day^{-1}) in drinking water for 6 weeks. At the end of week 6, rats were sacrificed by cardiac puncture. Plasma nitric oxide (NO) levels and xanthine oxidase (XO), adenosine deaminase (ADA), and glutathione peroxidase (GPx) activities were analyzed.

Results: Both XO and ADA activities increased and NO levels decreased in diabetic rats ($P < 0.05$). GSE supplementation normalized all of these changes. Antioxidant enzyme activities decreased in diabetic rats compared to the controls ($P < 0.05$). GSE supplementation increased antioxidant enzyme activities in both diabetic and healthy rats ($P < 0.05$).

Conclusion: These findings suggest that 6 weeks of oral GSE supplementation may prevent oxidative stress and improve antioxidant status in diabetic rats.

Key words: Experimental diabetes, grape seed extract, oxidative stress, antioxidant enzyme activity, adenosine deaminase, nitric oxide

1. Introduction

The function of oxidative stress in diabetes has been widely discussed since the early 1980s (1). Oxidative stress causes abnormalities in insulin secretion (2) and is involved in the maturation and progression of diabetes and its complications (3,4). Advanced oxidative stress in diabetes is due to multiple factors, such as glucose autoxidation. Other factors, such as cellular oxidation/reduction imbalances, reduction in antioxidant systems, and polyol pathways, also play a role (5). Hyperglycemia engenders free radicals and also impairs the endogenous antioxidant defense system in many ways during diabetes (4). To protect cells against damage caused by reactive oxygen species (ROS), organisms have developed several defense mechanisms. These include antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase and different antioxidant molecules including glutathione, flavonoids, vitamin A, vitamin E, vitamin C, and ubiquinone (6).

Grape seed contains polyphenol members of the family of proanthocyanidins (7). Proanthocyanidins are widely present in vegetables, fruits, seeds, nuts, flowers, and especially grape seed. The pharmacological, biological, and medicinal properties of proanthocyanidins have been widely reviewed (7-9). In addition to antioxidant activity, proanthocyanidins display vasodilatory, antiallergic, anticarcinogenic, antiinflammatory, antiviral, cardioprotective, immune-stimulating, and estrogenic activities, as well as being inhibitors of enzymes such as cyclooxygenase, phospholipase A2, and lipoxygenase (7,10). It has been discovered that grape seed extract (GSE) provides greater protection against free radicals and free radical-induced lipid peroxidation and DNA damage than vitamin E, vitamin C, and β-carotene (11).

There are limited studies (12-14) investigating the effects of GSE supplementation on diabetes-induced oxidative stress in rats. However, no study to date has investigated the effects of long-term GSE supplementation on oxidative stress and antioxidant defense systems in diabetic conditions in humans and experimental animals. Therefore, the aim of this study was to investigate the effects of GSE supplementation of 6 weeks in duration on oxidative stress and activities of circulatory enzymatic antioxidants in streptozotocin-induced diabetic rats.

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2. Materials and methods

2.1. Chemicals
Streptozotocin (STZ) was obtained from Sigma-Aldrich (USA). All other chemicals were purchased from Merck (Germany).

GSE (ActiVin) was kindly donated by San Joaquin Valley Concentrates (USA). GSE was extracted with a standardized water-ethanol mixture. GSE contains monomeric (less than 5%), dimeric (54%), trimeric (13%), and tetrameric proanthocyanidins (7%) and other flavonoids. Antioxidants like catechins also exist in the extract (15–17).

2.2. Animals
The study protocol was approved by the Ethics Committee of Experimental Medicine Research and Application Center, Selçuk University. The principles of laboratory animal care outlined in the National Institutes of Health guideline were followed in all these experiments. Thirty-six adult male Sprague Dawley rats (aged 16 weeks) weighing 200–300 g (236.1 ± 23.8 g) were used in the study. All animals were housed individually in polycarbonate cages (Tecniplast, Italy) with a 12-h light/dark cycle at 21 ± 2 °C and 50% humidity. The animals were given Purina rat chow and tap water ad libitum.

2.3. Induction of experimental diabetes
A total of 36 rats were divided into two main groups: nondiabetic (n = 19) and diabetic (n = 17). Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ at a dose of 50 mg/kg body weight, freshly dissolved in physiological saline. It has been demonstrated that STZ destroys pancreatic beta cells and is a model of type 1 diabetes (18). Seven days after i.p. administration of STZ, diabetes was confirmed by measuring blood glucose levels with a glucometer (Optium Plus, Abbott Diabetes Care, UK) in the blood samples taken from tail. Rats with a blood glucose level of 300 mg/dL or higher were considered to be diabetic. Diabetes was observed in 13 of the 17 rats.

2.4. Groups and supplementation procedure
Rats were divided into four groups as follows:

Control (C, n = 9): Tap water was given ad libitum during the study period.

GSE-supplemented control (GC, n = 10): GSE was given in drinking water at 100 mg kg^{-1} day^{-1} for 6 weeks.

Diabetic (D, n = 6): Tap water was given ad libitum during the study period.

GSE-supplemented diabetic (GD, n = 7): After the induction of diabetes was confirmed, GSE was given in drinking water at 100 mg kg^{-1} day^{-1} for 6 weeks.

GSE supplementation started 7 days after STZ-injection and this was considered the first day of supplementation. The 100 mg kg^{-1} dosage was chosen because previous studies (13,19) demonstrated antioxidant effects of this

dosage in rats. Daily water consumption of rats was approximately 10–12 mL 100 g body weight^{-1} (20). GSE was dissolved in a volume of tap water less than the rats' daily volume consumption. First, rats were given tap water containing GSE, and then water bottles were filled with tap water again. Therefore, rats were given tap water ad libitum and this procedure was repeated every day during the 6 weeks. Changes in body weight were recorded at the end of the experiment.

At the end of week 6, blood samples for the analysis were drawn by cardiac puncture under anesthesia and then rats were sacrificed by cervical dislocation.

2.5. Biochemical analysis
Blood samples taken from the rats were transferred into EDTA-containing tubes and centrifuged at $1000 \times g$ for 10 min at 4 °C. Plasma samples were aliquoted and stored at –80 °C until biochemical analysis.

Plasma xanthine oxidase (XO) activity was measured spectrophotometrically according to the method of Prajda and Weber (21). This assay measures the formation of uric acid from xanthine through the increase in absorbance at 293 nm. Plasma XO activity was expressed as U mL^{-1}.

Plasma adenosine deaminase (ADA) activity was estimated spectrophotometrically according to the method of Giusti (22), which is based on the direct measurement of the formation of ammonia, produced when ADA acts in excess of adenosine. Plasma ADA activity was expressed as U L^{-1}.

Nitric oxide (NO) is quickly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), respectively. The analysis of plasma nitrite and nitrate levels was based on the Griess reaction (23). Total nitrite ($NO_2^- + NO_3^-$) was measured by spectrophotometer at 545 nm. Plasma NO level was expressed as mmol L^{-1}.

Plasma glutathione peroxidase (GPx) activity was measured using a kinetic colorimetric assay from Cayman Chemical Company (USA). The assay kit measures GPx activity by a coupled reaction with glutathione reductase. The decrease rate of the absorbance of NADPH at 340 nm is proportional to GPx activity. GPx activity was expressed as nmol min^{-1} mL^{-1}.

2.6. Statistical analysis
Statistical analysis was performed using SPSS 15.0 (SPSS Inc., USA). Data were expressed as mean ± SD. Statistical significance of differences among the groups was calculated by one-way analysis of variance (ANOVA) and post hoc Tukey test. $P < 0.05$ was considered statistically significant.

3. Results
Body weight changes of the groups are shown in Figure 1. At the end of the study, although body weight of the control groups increased (+35.6 ± 21.1 and +45.2 ± 9.3 g in C and GC, respectively), diabetic groups presented weight
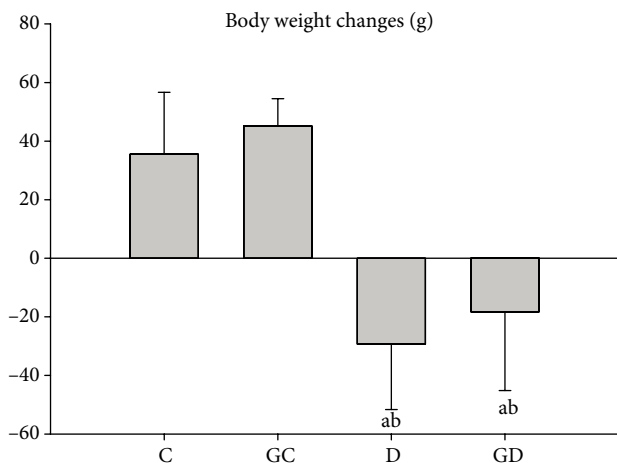
Figure 1. Body weight changes of the groups. C: Control, GC: GSE-supplemented control, D: diabetic, GD: GSE-supplemented diabetic. [a]P < 0.05 compared to C, [b]P < 0.05 compared to GC.

loss compared to both controls (–29.3 ± 22.3 and –18.3 ± 26.9 g in D and GD, respectively) (P < 0.05). There was no difference in mean weight loss between diabetic and GSE-supplemented diabetic groups. Blood glucose levels of the rats are shown in the Table. GSE supplementation did not affect blood glucose levels in diabetic rats (P > 0.05).

Both XO and ADA activities in diabetic rats were higher than those of the other groups (P < 0.05). There were no significant differences among the C, GC, and GD groups: XO activities were 0.22 ± 0.07, 0.18 ± 0.04, 0.39 ± 0.06, and 0.23 ± 0.06 in the C, GC, D, and GD groups, respectively, and ADA activities were 14.17 ± 2.25, 15.18 ± 4.31, 27.55 ± 2.92, and 17.37 ± 4.41 in the C, GC, D, and GD groups, respectively (Figures 2 and 3, respectively).

Plasma NO levels increased in the GC group compared to the control and they were the highest in this group (P < 0.05). Plasma NO levels decreased in diabetic rats compared to the GC group (P < 0.05), but there was no difference between the control and diabetic group. NO levels in the GD groups were higher than those of the diabetic group (P < 0.05) but were not different from those of the control and GC groups (NO levels were 75.00 ± 8.71, 94.22 ± 7.77, 65.60 ± 3.40, and 84.10 ± 8.35 in the C, GC, D, and GD groups, respectively) (Figure 4).

GPx activities increased in the GC group compared to the control (P < 0.05). Antioxidant enzyme activities significantly decreased in diabetic groups compared to the controls, and GSE supplementation ameliorated this decrease in antioxidant enzyme activities (P < 0.05) (GPx activities were 99.19 ± 7.28, 110.32 ± 3.43, 82.84 ± 5.40, and 94.98 ± 2.99 in the C, GC, D, and GD groups, respectively) (Figure 5).

Table. Blood glucose levels of the diabetic groups before and after GSE supplementation.

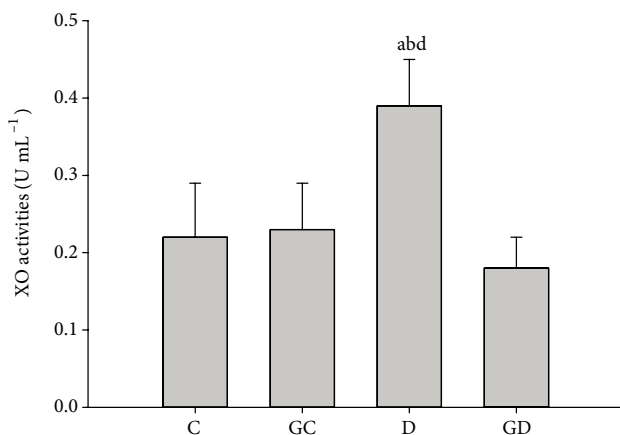| Groups	n	Before supplementation (mg/dL)	After supplementation (mg/dL)
D	6	380.7 ± 38.7	464.3 ± 31.3
GD	7	385.6 ± 63.5	467.0 ± 36.5

D: Diabetic, GD: GSE-supplemented diabetic.

Figure 2. Effect of GSE supplementation on plasma XO activities. C: Control, GC: GSE-supplemented control, D: diabetic, GD: GSE-supplemented diabetic. [a]P < 0.05 compared to C, [b]P < 0.05 compared to GC, [d]P < 0.05 compared to GD.
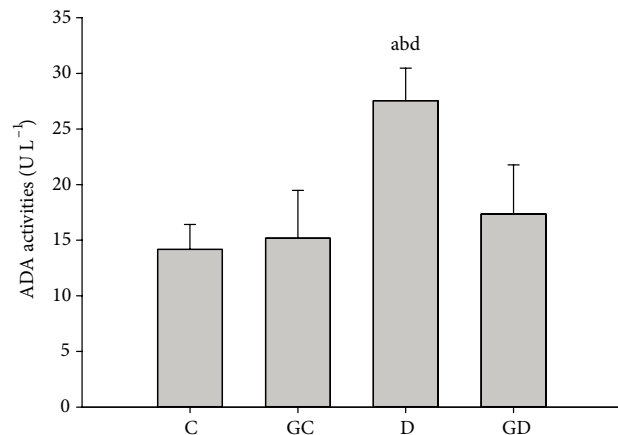

Figure 3. Effect of GSE supplementation on plasma ADA activities. C: Control, GC: GSE-supplemented control, D: diabetic, GD: GSE-supplemented diabetic. [a]P < 0.05 compared to C, [b]P < 0.05 compared to GC, [d]P < 0.05 compared to GD.
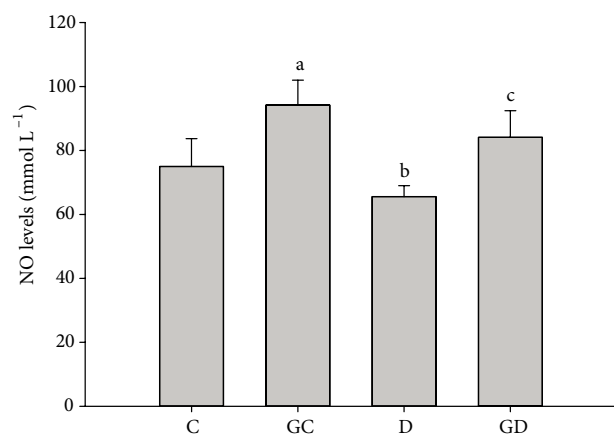
Figure 4. Effect of GSE supplementation on plasma NO levels. C: Control, GC: GSE-supplemented control, D: diabetic, GD: GSE-supplemented diabetic. [a]P < 0.05 compared to C, [b]P < 0.05 compared to GC, [c]P < 0.05 compared to D.
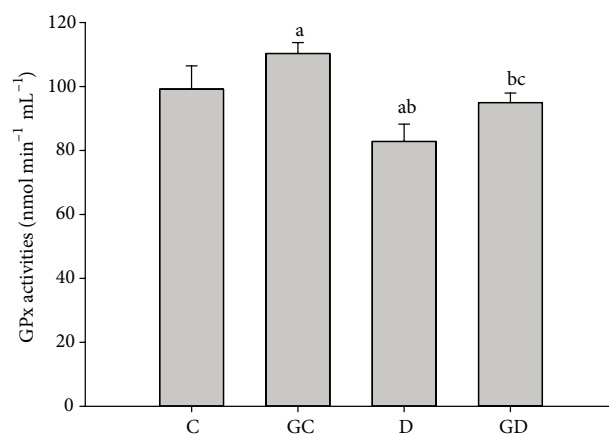

Figure 5. Effect of GSE supplementation on plasma GPx activities. C: Control, GC: GSE-supplemented control, D: diabetic, GD: GSE-supplemented diabetic. [a]P < 0.05 compared to C, [b]P < 0.05 compared to GC, [c]P < 0.05 compared to D.

4. Discussion

Diabetes is usually accompanied by excessive production of free radicals (3), and it was shown that hyperglycemia-induced ROS production could be a key event in the expansion of diabetic complications (24). The sensitivity of β-cells to oxidative stress has been attributed to their low levels of antioxidants (25).

Our results demonstrated that body weight decreased in diabetic rats during the 6-week experimental period. These results suggest that these animals suffered growth retardation due to the hindrance of glucose uptake (26) but oral GSE supplementation did not affect body weight in diabetic rats. Although in recent studies (14,26,27) it has been suggested that oral GSE supplementation increased body weight changes in diabetic rats compared to the untreated diabetic controls, in these studies the GSE dosage was larger (250 mg kg^{-1} day^{-1}) and the supplementation period was longer (24 weeks) than in our study. According to their results, previous authors assumed that GSE could show an anti-nonenzymatic glycation effect, having a correlation with body weight gain (26).

In the present study, we observed that 6 weeks of oral GSE supplementation did not affect blood glucose levels in diabetic rats. In contrast to other studies (13,26,28,29), in this study, although it was not statistically significant, blood glucose levels increased in both the D and GD groups after supplementation. One possible explanation for this situation is related to the STZ dosage and duration of blood glucose measurement. In the present study, we confirmed diabetes 7 days after STZ administration. Our findings suggest that β-cell destruction continued during the study period, and therefore blood glucose levels were elevated at the end of the study independently from the effect of GSE. It is generally accepted that GSE supplementation

decreases blood glucose levels in diabetic rats (13,26,28). Procyanidins influence insulin effects by acting on specific parts of the insulin-signaling transduction pathway in STZ-induced diabetic rats (12). These researchers also claimed that GSE was an antihyperglycemic agent with insulin-mimetic properties by increasing glucose transporter-4 in the membrane. El-Alfy et al. (13) demonstrated that grape seed proanthocyanidins ameliorated pancreatic damage in alloxan-induced diabetic rats. Similarly, Al-Awwadi et al. (29) showed that red wine polyphenols had antidiabetic activity in STZ-induced diabetic rats. A recent study (28) showed that GSE supplementation decreased blood glucose concentrations and HbA1c levels in diabetic db/db mice. However, consistent with our findings, Zhang et al. (30) demonstrated that GSE supplementation did not influence blood glucose levels in mice; they claimed that the renoprotective effects of GSE were independent of antihyperglycemia. Therefore, we may also suggest that GSE shows antioxidant effects independent of antihyperglycemia. The differences between the results might be due to the supplementation period or GSE dosage.

Various studies (29–34) have shown that clinical and experimental diabetes are associated with increased formation of free radicals and with decreases in antioxidant potential. In the present study, plasma XO activity significantly increased in diabetic rats. Increased XO activity is related to oxidative damage induced by diabetes and its complications (35). Celik et al. (36) showed that XO activity was significantly increased in the brain tissue of STZ-induced diabetic rats and they suggested that an important source of increased ROS in the STZ-induced diabetic rat was XO. GSE supplementation decreased diabetes-induced overproduction of XO in the plasma of diabetic rats. This is the first study investigating the

effects of GSE supplementation on plasma XO activities in diabetic rats and these findings support the antioxidant potential of GSE.

Increased ADA activity was demonstrated in both blood and tissues of diabetic humans and animals (37–40). This increase in ADA activity could be due to the altered insulin-related T lymphocyte function that plays a central role in the immune response (39) or the induction of activity and/or expression of ADA in the damaged tissue (41). Recent studies (42,43) have shown that GSE supplementation reduces the concentration of free Ca^{2+} and elevated Ca^{2+}-ATPase activity in sciatic nerves of diabetic rats. In the present study, we found that GSE supplementation suppressed ADA activities in the plasma of the diabetic rats. ADA inhibition may exert a dual protective role by facilitating purine salvage for ATP synthesis and inhibiting lipid peroxidation.

NO can exert a variety of biological activities in various physiological and pathological conditions. It can be either proapoptotic or antiapoptotic, depending on the type of cell, the location, the timing, and the amount produced (44). It has been shown that NO levels increase in diabetic conditions (37,45,46). However, in the present study, plasma NO levels in the diabetic group did not differ from the control. According to these findings, the mechanisms of similar NO levels in diabetic rats and controls are unclear and more research is needed. It has been shown that GSE increases NO levels by activating the inducible NOS (47,48). In the present study, GSE supplementation significantly increased plasma NO levels in both supplemented groups compared to their own controls; there was no difference between the GSE-supplemented groups. These findings support the beneficial effects of

GSE on NO levels. In a previous study (49) we showed that GSE supplementation restores endothelial function and reduces the risk of vascular disease in diabetic rats. This might be related to the increased NO production induced by GSE supplementation.

A relationship between diabetes and oxidative stress has been confirmed together with reduced total antioxidant defense (50). Such a prooxidant environment was demonstrated in our study by the inhibition of the activity of antioxidant enzyme GPx. Decreased GPx activities were observed in the blood of diabetic patients (36,51,52) and in blood and tissues of diabetic animals (53,54). In our study, 6 weeks of GSE supplementation improved GPx activity in diabetic rats and our results are consistent with those of previous studies (27). The free radical scavenging activity of GSE could be a possible reason for this reversal effect of antioxidant enzyme activities (8).

In conclusion, in the present study, although GSE supplementation did not affect blood glucose levels, it induced a decrease in oxidative stress markers. These data suggest that GSE supplementation affects oxidative stress markers rather than blood glucose levels and more detailed histopathological and molecular research is needed to clarify the real mechanism of action of GSE. Additionally, 6 weeks of oral GSE supplementation exerted a protective effect on oxidative stress and antioxidant enzyme activities in STZ-induced diabetic rats. However, more detailed animal studies are needed to clarify the mechanism of action of GSE along with investigations in human subjects to support these data.

Acknowledgment

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