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Original Research

Grape seed extract effects on serum amylase levels and immunohistochemical alterations in Streptozotocin-induced diabetic rats

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Abstract: In this study, serum amylase activity and structural changes of the pancreatic tissue in rats under the effects of grape seed extract were investigated. Thirty-two female Wistar albino rats were divided into 4 groups. First one was the control group. The second group was the streptozotocin (STZ)-induced diabetes mellitus (DM) group (45 mg/kg), while the third group was the grape seed extract (GSE) group, where the GSE was administrated intragastrically for 20 days (at 0.6 ml/rat). Lastly, the fourth group was the diabetes mellitus+GSE (DM+GSE) group. Blood samples were taken and analyzed for amylase activity. Caspase 3 expressions were inspected with immunohistochemistry. Amylase levels in the diabetic group were found to be the lowest (794.00±44.85 U/L, $p<0.001$), while the GSE group had the highest value (1623.63±80.04 U/L, $p<0.001$) Number of apoptotic cells was increased in Langerhans islets of the diabetic group. In the control and GSE groups, the apoptotic cells were found to be almost entirely absent. Increased number of apoptotic cells was found in the DM group, while decreased number of apoptotic cells was found in the DM+GSE group. Furthermore, atrophy in Langerhans islets, hyperemia in capillary veins, hydropic degeneration and necrosis in islet cells were determined in the diabetic group. Only mild hydropic degeneration in islet cells of Langerhans was observed in the DM+GSE group. Histopathologically beneficial changes in the pancreases were detected when grape seed extract was given to diabetic rats. As a conclusion, GSE was determined to have positive effects on the function and structure of the pancreas, improving enzyme activities and the structure of the Langerhans islets.

Key words: Amylase; Diabetes mellitus; Grape seed extract; Immunohistochemical alterations; Rat.

Introduction

Diabetes mellitus; which is characterized by insulin secretion deficiency or resistance to the metabolic effects of insulin in target tissues, courses with high morbidity and mortality rates when not treated (1,2). Diabetes is clinically characterized by polydipsia, polyuria, polyphagia, weight loss and disease-specific retinopathy and neuropathy (3). In addition to these complications, various disorders of the connective tissue occur, and the healing process of the wounds is delayed. With the decrease of the collagen amount in the bones and the cartilage, bone development decreases and thickening occurs in basal laminae of vessels (4).

Amylase is most commonly present in the pancreas and salivary glands, while the amount in the bloodstream is normally low and stable. Amylase level increases in acute pancreatitis cases. Hyperamylasemia usually refers to pancreatic diseases. However, in many other pathological and non-pathological conditions, there may be an increase in amylase activity as well (5).

The plants have long been the main source of medicines, and today's medicines are derived from a direct or indirect pathway. Many active substances derived from plant-derived groups of different chemical compounds can be used in the treatment of diabetes (6). Some traditional plants display antioxidant activity; which may be due to vitamins, phenols or tannins (7). Amongst those, flavonoids have been shown to exhibit particularly strong antioxidant activity (8-10). Grape seed extract is very rich in procyanidins from polyphenolic compounds. Proanthocyanidins cause inhibition of free oxygen radicals depending on concentration. Amongst various pharmacological, medical and therapeutic effects of proanthocyanidins to date, vasodilator, anticarcinogenic, anti-allergic, anti-inflammatory, antifungal, anti-arthritic, antibacterial, cardioprotective, immunostimulant and antiviral effects are particularly significant (11). Grape seed extract was shown to contain phenolic acids (para-coumaric, cinnamic, caffeic, genticidal, ferulic and vanillic acid), trihydroxy stilbenes (resveratrol and polydatin) and flavonoids (catechin, epicatechin, and quercetin) (12).

The pharmacologically significant proanthocyanins are the ones in the group known as oligopantanthocyanidins. Proanthocyanidins are condensed compounds that have biological, pharmacological and therapeutic effects against free oxygen radicals and oxidative stress (13). The inhibition of oxidative damage caused by substrate oxidation or free radicals at low concentrations, and the prevention of autooxidation along with the ability to bind free radicals by hydrogen bonding, are indicative of proanthocyanidins as a potent antioxidant (14).

Resveratrol is an antioxidant molecule found in high amounts in black grape clusters. In red wine, grapes,

and some other fruits, resveratrol is a form of trans-resveratrol that shows biological activity (15). Experimental studies have shown that resveratrol can induce anticarcinogenic effects by stimulating certain enzyme systems and immune system, and can inhibit cancer formation and prevent tumor metastasis (16). Present in high amounts in grape seeds, shells, and stems, resveratrol is an important bioactive substance that plays anticarcinogenic and antimutagenic roles in antioxidants and antimicrobials in the protection against cardiological diseases, inhibition of oxidation of LDL cholesterol, and inhibition of free radicals. Proanthocyanidins have 20 and 50 times stronger antioxidative effect than vitamins E and C, respectively (17).

Caspase activation is a marker of cell damage in diseases. Only the active caspase-3, which occurs in apoptotic cells, can be determined by the caspase-3 method. For this reason, it is necessary to know whether the caspase-3 exerts its action, or whether the agent causing apoptosis in the studied tissue breaks caspase-3. However, once this is known, apoptotic cells can be detected by this method. Using the same method, antibodies of different caspases can be used to evaluate the activity of these caspases by immunohistochemistry.

In this study, the effects of the grape seed extract on the pancreas, serum amylase levels and structural changes in rats with STZ-induced diabetes were investigated.

Materials and Methods

Test subject groups

The animal subject groups of the work were obtained from the Yuzuncu Yil University Experimental Animal Unit. Thirty-two female Wistar albino rats, aged approximately 7-8 weeks, were used for this purpose. Body weight of the rats at the beginning was between 0.30 and 0.35 kg. The subjects were randomly selected and divided into 4 groups. Group, I was the control group (n=8), Group II was the diabetes mellitus (DM) group (n=8), Group III was the grape seed extract (GSE) group (n=8), and Group IV was the diabetes mellitus + grape seed extract (DM+GSE) group (n=8). GSE extract was provided commercially (Grape Seed Extract 100 mg-SOLGAR). The rats were housed in cages under the continuous provision of food and fresh water, and their chambers were set at a temperature of 22 ± 2 ° C with dark/light cycle set for 12 hours each during the 20-day experiment period. All experiments were performed in accordance with protocols approved by the Van Yuzuncu Yil University Animal Researches Local Ethic Committee (Turkey).

The groups were formed as follows:

1) The control group (C): Pre-test blood sugar levels of the rats along with their weights were measured. Intra-peritoneal (i.p.) injection of 45 mg/kg single dose of saline was performed.

2) The group with diabetes and no grape seed extract (DM): Pre-experiment blood sugar levels and weights of rats were measured. To induce diabetes in rats, they were given a single injection of 45 mg/kg of STZ. STZ was dissolved in cold citrate buffer at pH 4.5 (Sigma, USA) and administered intra-peritoneally (i.p.) (18). After 72 hours, glucose levels in the blood samples taken from the tail vein were determined by means of Clevers Chek-TD-4222 biosensor sugar meter and strips. Subjects with blood glucose levels above 250 mg/dl were considered diabetic and were included in the study.

3) The group given the grape seed extract (GSE): Blood sugar levels were measured before the experimentation. Grape Seed Extract 100 mg was kept in carboxy methyl cellulose (CMC) (0.01 g/ml) and was given intragastrically every day for 20 days. (0.6 ml/rat) (19).

4) Diabetes mellitus and grape seed extract group (DM+GSE): This group consists of the rats in group 2 which were diagnosed with DM with (blood glucose level above 250 mg/dl). These were subjected to 100 mg grape seed extract which was maintained in CMC (0.01 g/ml) and administered intragastrically and orally every day for 20 days (0.6 ml/rat) (19).

Serum Amylase Analyzes

At the end of the experiment, the blood samples were taken from the left ventricles of rats under i.p. anesthesia induced with ketamine HCl (0.1 ml/100 g body weight). The samples were placed into glass tubes, which were then centrifuged at 3000 rpm for 5 minutes. Amylase assays were performed on the sera obtained from a Roche Modular P 800 auto-analyzer.

Immunohistochemical Analysis

Immunohistochemistry was performed to investigate Caspase 3 levels. Commercial antibodies were visualized on 4- to 5-µm-thick paraffin sections using an indirect streptavidin/biotin immunoperoxidase kit (HRP; Thermo Scientific, USA). All steps were carried out following the procedure described by Taylor et al. (20). Accordingly, tissue sections were placed on adhesive slides, de-paraffinized for 5 minutes in each of three xylene series, and rehydrated in a graded alcohol series and distilled water. Antigen retrieval was accomplished by boiling sections on glass slides in citrate buffer (pH 6.0; Thermo Scientific, USA) for 20 minutes. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in absolute methanol for 7 minutes at room temperature. Sections were rinsed three times with phosphate-buffered saline (pH 7.4) for 5 minutes between each step of the test. Sections were incubated in protein blocking (ultra v block) for 5 minutes to prevent non-spesific binding. Thereafter, tissue sections were incubated with the primary antibody (Caspase 3) for 60 minutes in a humidified chamber at room temperature. Sections were treated with a biotin-labeled secondary antibody for 15 minutes and with the streptavidin-peroxidase enzyme for 15 minutes at room temperature. Finally, sections were incubated in aminoethyl carbazole chromogen (Thermo Scientific, USA) for 5-10 minutes to induce a color reaction. Mayer's hematoxylin was applied as a counterstain for 30 seconds. Thereafter, sections were mounted with water-based mounting medium (Thermo Scientific, USA). As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted. Sections were immediately analyzed. Immunostaining was evaluated using a binocular microscope.

Table 1. The amylase levels in control, DM, GSE and DM+GSE group of rats (n=8).

Parameters	Control Group $X \pm S_x$	DM Group $X \pm S_x$	GSE Group $X \pm S_x$	DM+GSE Group $X \pm S_x$	P
Amylase (U/L)	1468.25±60.12[a]	794.00±44.85[c]	1623.63±80,04[a]	1077.50±50.50[b]	p<0.001

a,b,c: The difference between group averages with different letters on the same line is statistically significant.

Statistical analysis

The descriptive statistics for the features studied were expressed as mean, standard deviation. Kruskal-Wallis test was used to compare the groups in terms of this characteristic. The statistical significance level in the calculations was determined as 5% and the statistical package program SPSS (ver: 21) was used for the calculations.

Results

Serum amylase levels of the groups are given in Table 1. The highest means in amylase levels were detected in the GSE group (1623.63±80.04 U/L) and the control group (1468.25±60.12 U/L) (p>0.05). Amylase levels of the rats in the DM group were the lowest among all groups and differences between the groups were statistically significant (p<0.001).

The microscopic images of the caspase-3 activities in Langerhans islets are given in Figures 1 A through D respectively, and the apoptotic results are given in Table 2. Immunohistochemical methods indicated that the number of apoptotic cells was increased in pancreas Langerhans islets of the diabetic group A. A Large number of positive cells were found in pancreas Langerhans islets in the second group (Fig. 1B). As for the control and GSE groups, the apoptotic cells were hardly observed (Fig.1A-1C). Immunohistochemical findings also showed that the increased number of apoptotic cells in DM groups was decreased in DM+GSE group, and only a small number of positive cells were found in their Langerhans islets cells (Fig 1D).

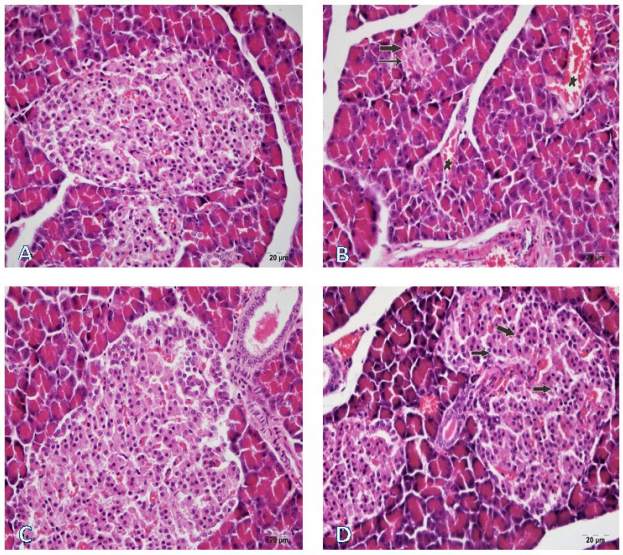

Figure 1. A: The control group, the normal histological structure of langerhans islands in the pancreas H & E, Bar: 20 µm. B: Diabetes mellitus group, atrophy in pancreatic langerhans, hyperaemia in capillar veins (asterics), hydropic degeneration (thick arrow) and necrosis (fine arrow) in islet cells, H & E, Bar: 20 µm. C: Grape seed extract group, the normal histological structure of the langerhans island in the pancreas H & E, Bar: 20 µm. **D:** Diabetes mellitus + Grape seed extract group, hyperaemia in capillar veins, mild hydropic degeneration (arrows) in islet cells of langerhans in pancreas, H & E, Bar: 20 µm

Discussion

Grape and grape products contain flavonoids, like monomeric flavonol, dimeric, trimeric and polymeric procyanidins, and phenolic acids like the gallic and epigallic acids. Proanthocyanidins, known as condensed tannins, are found mostly in the crust and core part of the grape. Tannins are structures that contain hydroxyl; these function by forming insoluble complexes with carbohydrates and proteins, and protecting connective tissues (21).

Resveratrol, which is found intensely in grape seeds, shells, and stems, is an important bioactive substance that plays anticarcinogenic and antimutagenic roles as an antioxidant, acts as an antimicrobial agent in the protection against cardiological diseases, and in inhibits the oxidation of LDL cholesterol and free radicals (17).

Belviranlı et al. (22), conducted a study in rats to demonstrate the effects of grape seed extract on the antioxidant defense mechanisms of the liver tissue and oxidative stress change in diabetic rats. They found that the protective effect of the grape seed extract over liver tissue by affecting the nitric oxide level was limited. Free radical-induced tissue damage causes pancreatic β-cell dysfunction and prevents the use of glucose in peripheral tissues by reducing insulin secretion (23).

Oxidative reactions are found at the base of diabetic defects, as oxygen free radicals are formed in the oxidation process, causing oxidative damage in affected cells and tissues. Amylase excreted above 450 U/L in the blood test usually indicates damage to the pancreatic islets. In such a case, insulin secretion may be disturbed and diabetes symptoms may be observed (24). In our study, the amylase activity of the diabetic rats was the lowest, while in the grape seed extract group, the amy-

Table 2. Immunoperoxidase test results.

Individual	Control Group	DM Group	GSE Group	DM+GSE Group
1	-	+++	-	-
2	-	++	+	+
3	-	+++	-	+
4	-	++	-	-
5	+	++	-	+
6	-	+++	-	+
7	-	++	-	++
8	-	++	-	-

+++ = number of positive cells ≥ 40% ; ++ = number of positive cells 30-40%; + = 20% less positive cells.

lase activity was the highest ($p<0.001$).

In a different study that investigated whether Methidathion, an organophosphate insecticide, caused pancreatic damage in rats and whether vitamin E and C combination could prevent this damage, Methidathion+Vitamin E and C combination groups showed significant increase in amylase activity compared to the control and Methidathion groups, and it was concluded that vitamin C may cause increased amylase activity (25).

In our study, high levels of amylase in rats in the grape seed extract group were similar to those observed in the studies of Mollaoğlu et al., (25) and Nonaka et al. (26). The increase in amylase levels is thusly proven to be due to the strong antioxidant compounds in the grape seed extracts.

Resveratrol in grape seeds and shells has a role in protecting from diabetes and alleviating some diabetic complications (27). It has been suggested that resveratrol in diabetic rats partially reduces plasma glucose and triglyceride concentrations, reducing the effect of insulinemia, which improves metabolic parameters.

Chen et al. (28) report that insulin secretion increased after resveratrol injection in rats, and therefore plasma glucose levels decreased. This effect suggested that resveratrol resulted in adenosine-triphosphate mediated potassium channels and voltage-gated potassium channels and caused a blocking effect in pancreatic beta cells in return. In a study insulin-dependent and insulin-independent diabetes mellitus with streptozocin in males, it was shown that resveratrol increased the insulin levels in both the control and diabetic groups and that the plasma glucose levels decreased significantly after resveratrol administration determined.

A study using a single dose (60 mg/kg) intravenous streptozocin injection in diabetic rats reported that resveratrol was dose-dependent, and helped reducing plasma glucose and lipid concentrations and improving general symptoms of diabetes even with a single dose (29).

In a study of insulin-dependent and insulin-independent diabetes mellitus with streptozocin, it was proven that resveratrol increased the insulin level in both the control and diabetic groups and that the plasma glucose level decreased significantly after resveratrol administration. The study also shows that the long-term use of grape seed can reduce the blood glucose level and eliminate the adverse conditions of diabetes (19).

In another study, the grape extract was found to cause stimulation of insulin secretion from pancreatic beta cells in diabetic rats (30). Hvang et al. (31) indicated that grape seed extract has a potent effect of reducing blood glucose and HgA1c levels in 4-12 week old mice, and can be considered as a healthy food for reducing blood glucose levels.

Majeed et al. (32) gave diabetes-induced rats STZ 20 mg/250 gr grape extract. The extract was given to each rat for 30 days and the researchers found that the decrease in glucose level and liver enzyme activity showed an increase in glutathione level, which was caused by the antihyperglycemic effect of grape seed extract. The same researchers have come to the conclusion that compounds such as quercetin and resveratrol in the grape kernel increase the cellular antioxidant defense and prevent the adverse effects of radicals.

The study of Sieman and Creasy (33) suggests that the hypoglycemic effect of resveratrol in grape seed extract is not due to the stimulation of insulin secretion and therefore does not cause an additional surplus on pancreatic beta cells and produces hypoglycemia by another mechanism. This hypoglycemic effect has been reported to be moderate, resulting from increased glucose uptake and increased glycogen synthesis. It has been reported that epicatechins in grape seeds induce hypoglycemia by stimulating regeneration of beta cells, catechins reducing intestinal glucose absorption, and epigallocatechin increasing hypoglycemia synthesis (34). Su et al. (29) have suggested that resveratrol stimulates glucose uptake, reduces oxidative stress, helps cells normalize the redox state, and mediates normal functioning of the insulin signaling mechanism.

Apoptosis, which is the programmed cell death process initiated by the physiological environment or by damage, is important for the continuation of homeostasis in the organism. With apoptosis, damaged cells in the liver, such as those found in other tissues, are removed without harming the environment. Apoptosis can be triggered by extracellular or intracellular signals. It can be interpreted that apoptotic indices for caspase-8 and caspase-9 antigens are induced in the tissues, induced intracisternally via apoptosis receptor-mediated extrinsic or mitochondria. Caspase-3 is the intersection of these two routes.

In our study, the activation of Caspase-3 in the pancreas was examined. Immunohistochemical methods showed that apoptotic cells were increased in the pancreatic tissues of the STZ administrated group. The microscopic images of the caspase-3 activities in pancreas islets were given in Figure 1 and the apoptotic indices were given in Table 1. Apoptosis in the control and grape seed extract groups were hardly observed. Apoptosis significantly increased in the diabetic group and decreased in the diabetic groups treated with grape seed extract. We deduce that grape seed extract inhibits apoptosis by protecting the cells against damage. The beneficial effects of resveratrol have resided in phenol rings, and it has been shown that it naturally acts as an antioxidant and thus reduces cellular oxidative stress and concomitant oxidative damage. It has been suggested that this effect of reducing oxidative stress in resveratrol may also reduce apoptosis at the same time. Similarly, the reduction of caspase-3 influence by resveratrol suggests that resveratrol may have positive effects on apoptosis pathway in the pancreas (35).

In this study, apoptotic cells were identified in the pancreas Langerhans islets with the help of caspase-3 activities. With this method, it has been found out that caspase-3 (+) cell count in the DM group was higher compared to the other groups. The increase in the number of caspase-3 stained cells in the pancreas Langerhans islets suggests that STZ causes apoptosis through caspase activation. It has formerly been reported that the increase in free radical formation in the hyperglycemia participates in the development of diabetic complications and that the newly formulated oxidative stress activates apoptotic pathway (36,37). It is also known that caspases, by initiating proteolytic cleavage cascade during the apoptosis, play a critical role in the development of apoptotic events. Caspase-3, one of the members of 14-member caspase family, is a key protease in the early

stages of apoptosis (38). An experimental STZ-induced diabetic study showed a rise of apoptotic cells in liver sections of rats (39). Oxidative stress is known to have an important role in the pathophysiology of chronic complications in diabetes (40).

Diabetes mellitus is a chronic metabolic disorder which is associated with hyperglycemia. It is caused by a derangement in the secretion or function of the endocrinal portion of the pancreas. Significantly low serum amylase levels were found in the diabetic rats as compared to those in the other groups and there was a statistically significant difference between the groups ($p < 0.001$). Wherever the blood glucose level was higher, the serum amylase activity was found to be significantly lower (41).

Immunohistochemical methods showed that apoptotic cells were increased in the pancreatic tissues of the STZ administrated group. In the control and grape seed extract groups, apoptotic cells were hardly observed. Apoptosis significantly increased in the diabetic group and decreased in the diabetic groups treated with grape seed extract. It shows that grape seed extract inhibits apoptosis by protecting the cells against damage. It has also been found out that caspase-3 (+) cell count in the DM group was higher compared with other groups. The increase in the number of caspase-3 stained cells in the pancreas Langerhans islets suggests that STZ causes apoptosis through caspase activation. In the diabetes group, atrophy in pancreatic Langerhans, hyperemia in capillary veins, hydropic degeneration and necrosis in islet cells were determined, but as for hyperemia in capillary veins, when grape seed extract was given to diabetic rats, only mild hydropic degeneration in islet cells of Langerhans in the pancreas was observed. Positive changes in pancreatic histopathologic appearance were detected when grape seed extract was given to diabetics.

All these findings suggest that the grape seed extract has a very positive effect on the function and structure of the pancreas, improving enzyme activities and the structure of the Langerhans islets.

Conflicts of Interest

The authors declare no conflict of interest.

Author's contribution

KI designed the research plan and organized the study. KI and SY collected samples and executed the experimental work. KI, HM, and NM contributed to the data analysis and the wrote the manuscript.

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