Effects of Grape Seed Proanthocyanidin Extracts on Peripheral Nerves in Streptozocin-Induced Diabetic Rats

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(Received February 5, 2008)

Summary Diabetic peripheral neuropathy (DPN) is one of the most common diabetic chronic complications. The aim of this study was to clarify whether grape seed proanthocyanidins extracts (GSPE) are therapeutic agents against DPN. In this study, we used streptozocin (STZ) to induce diabetic rats. GSPEs (250 mg/kg body weight/d) were administrated to diabetic rats for 24 wk. Motor nerve conductive velocity (MNCV) and mechanical hyperalgesia were determined in the rats. Serum glucose, glycated hemoglobin, advanced glycation end products (AGEs), and tissue malondialdehyde (MDA) and superoxide dismutase (SOD) were determined. Light and electron microscopy were used to observe the changes of nerval ultrastructure. GSPE significantly increased the MNCV, mechanical hyperalgesia and SOD of diabetic rats (p<0.05) and reduced the AGES and MDA of diabetic rats (p<0.05). After being treated by GSPE, the severe segmental demyelination was decreased and Schwann cells were improved. In conclusion, GSPE plays an important role against DPN. With the decreasing of AGES and MDA, it can ameliorate oxidation-associated nerval damage. This study may provide a new recognition of natural medicine for the treatment of DPN.

Key Words grape seed proanthocyanidin extracts, diabetic peripheral neuropathy, oxidative stress, motor nerve conductive velocity, mechanical allodynia

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin, and is the most common cause of neuropathy in the western world. The prevalence of peripheral neuropathy in diabetes approaches 70%. Diabetic peripheral neuropathy (DPN) is estimated to be present in 50% of people living with diabetes mellitus (1). DPN is also the major reason for loss of protective limb mechanical sensations, traumatic ulcerations and therefore amputations (2).

Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses (3), and increased oxidative stress is now a widely accepted participant in the development and progression of diabetic neuropathy (4). Hyperglycemia is thought to promote oxidative stress through both enzymatic and non-enzymatic pathways of glucose metabolism that are complicated in the development of neuropathy.

The Diabetes Control and Complications Trial (DCCT) demonstrated that although intensive therapy of maintaining blood glucose concentrations close to the normal range is effective in reducing clinical complications significantly, even optimal control of blood glucose could not prevent them, suggesting that alternative treatment strategies are needed (5). Antioxidant therapy with aqueous or liquid-soluble free radical scavengers or metal chelators could improve nerve conduction velocity in experimental DPN (6, 7). It was shown that proanthocyanidins, belonging to polyphenols and widely distributed in the plant kingdom, were effective radical scavengers, especially of hydrophilic peroxy radicals in an aqueous system (8, 9). The superoxide anion scavenging activity of grape seed proanthocyanidin extracts (GSPE) was stronger than that of vitamin C, vitamin E or any other antioxidant (10). It has been reported to have protective effects on various forms of cardiac disorders (11), reduce hypoxic-ischemic brain injury (12), prevent diabetic nephropathy from progressing (13), and protect gastric mucosa (14).

However, the effect of GSPE on the peripheral nerves of diabetic rats has not been elaborated in previous reports. To test the hypothesis that GSPE may prevent the development of functional and structural neuropathy in STZ-induced diabetic rats, we have studied the differences in mechanical allodynia, electrophysiology, nerve morphology, and the oxidative and antioxidant levels among normal control and diabetic rats with and without 250 mg/kg GSPE treatment and finally demonstrated that GSPE treatment could apparently alleviate...
the peripheral neuropathy functionally and structurally without affecting blood glucose levels.

MATERIALS AND METHODS

Materials. The GSPEs we used in this experiment are commercial products. They were provided by Jianfeng Inc. (Tianjin, China), containing 56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins and small amounts of monomeric and high-molecular-weight oligomeric proanthocyanidins, and flavanols. The components of GSPE were analyzed using high-performance liquid chromatography with gas chromatography-mass spectrometry detection. Lot No: G050412. Streptozocin (STZ) was obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). SOD and MDA kits were purchased from KEYGEN Biotech. Co. Ltd., China (Nanjing, China).

Methods.

Induction of experimental diabetes: Ten-week-old 200–220 g male Wistar rats (*n* = 60) were purchased from the Laboratory Animal Center of Shandong University (Shandong, China). The animals were housed in cages and received normal rat chow and tap water ad libitum in a constant environment (room temperature 22±1.5°C, room humidity 55±5%) with a 12-h light, 12-h dark cycle. The animals were kept under observation for 1 wk prior to the start of the experiments. All procedures were approved by the animal ethics committee of Shandong University.

Twenty rats selected randomly were divided into 2 groups: group 1, control rats (vehicle, C1, *n* = 10); group 2, treated control rats (GSPE, 250 mg/kg body weight, C2, *n* = 10), which received a single tail vein injection of 0.1 mol/L citrate buffer only. The other forty rats received a single dose of STZ (55 mg·kg⁻¹·i.p., injected into tail veins) freshly dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5) after a 12-h overnight fast. Only rats with blood glucose higher than 16.7 mmol/L after 5 d were considered as being diabetic in the fasting state, by using a One Touch II Glucose Analyzer (Johnson & Johnson, USA). Ten rats with blood glucose levels of less than 16.7 mmol/L were excluded from the study. All studies were carried out 1 wk after STZ had been injected. The diabetic rats were randomly divided into 2 groups: group 3, untreated diabetic rats (STZ induced then vehicle, DM1, *n* = 15); group 4, treated diabetic rats (GSPE, 250 mg/kg body weight/d, DM2, *n* = 15). The GSPE was given in normal saline solution by intragastric administration for 24 wk. Each group of rats was observed from week 1 to week 24 without any administration of hypoglycemic therapy.

Measurement of mechanical allodynia and MNCV: Mechanical algesia was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using von Frey hairs (BME-403, Institute of Biomedical Engineering Chinese Academy of Medical Sciences, China: 1.19, 3.8, 5.8, 7.6, 10.12, 17.3, 52.0, 73.0 g).

The rat was placed in a hanging cage with a metal mesh floor and acclimated for at least 10 min. A von Frey hair was manually applied to the plantar surface of the hind paw one by one from 1.19 g with the pressure increasing until the filament bent to 90° for less than 4 s and the pressure at which a paw withdrawal occurred was recorded. For each filament, the procedure was repeated 10 times and the pressure necessary to elicit 50% brisk foot withdrawal in response to this mechanical stimulus was interpreted as mechanical allodynia.

Nerve conductive velocity was detected through a BL-310 biomechanical system (BL-310, Taimeng Co. Ltd., China). Animals were anesthetized with 10% chloral hydrate i.p. The left sciatic/tibial nerve was dissected rapidly and near nerve temperature was maintained at 37°C using liquid paraffin wax. The left sciatic/tibial nerve was placed in an insulated box and stimulated at the proximal end, and the action potentials were recorded at the distal end. The nerve was stimulated by square wave pulses (duration: 0.1 ms, intensity: 2 V). The average of 10 potential traces were measured and the nerve length between stimulation and recording electrode was recorded.

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MNCV (m/s) = \frac{(Distance \ between \ stimulating \ and \ recording \ electrode)/(Tibial \ M \ latency − Sciatic \ M \ latency)}{37\degree C}
\]

Estimation of plasma glucose level, HbA1c, AGEs, neurotic MDA and SOD: Prior to sacrifice after 24 wk of diabetes, animals were weighed. FBG was determined using a commercially available kit (Adamco Ltd., USA). Serum HbA1c level was determined with a commercially available kit (BIO-RAD Ltd., USA). Serum advanced glycation end-product (AGE)-specific fluorescence determinations were performed by measuring emission at 430 nm on excitation at 370 nm using a fluorescence spectrophotometer (HITACHI 850, Japan). Antioxidant activity and lipid peroxidation were estimated by measuring SOD activities and MDA levels (TBA method) in the sciatic nerves with commercially available kits.

Light and electron microscopy: The sciatic nerves were excised, and part of the nerve was fixed in 10% formaldehyde, embedded in paraffin and cut into 4 μm-thick sections for light microscopy with Loyez Stain and H.E. stain under a light microscope at a magnification of ×400. Moreover, part of the nerve was fixed in 3% glutaraldehyde. Ultrathin sections cut from the embedded blocks were stained with uranyl acetate and lead citrate, and were examined with a HITACHI H-800 electron microscope.

Statistical analysis. Results are shown as mean±SD. Statistical analysis was performed using SPSS13.0 software. Statistical significance of the differences among experimental groups was calculated by unpaired Student’s t-test and one way ANOVA. *p* value <0.05 was considered statistically significant.

RESULTS

Effects of GSPE on mechanical allodynia and MNCV

In the present study, mechanical allodynia was determined by measuring the hind paw withdrawal threshold in response to von Frey hairs. Diabetic rats showed a
significant decrease in the pressure required to elicit paw withdrawal as compared with normal controls ($p<0.01$), while the withdrawal threshold of rats treated with GSPE was much better ($p<0.05$). Figure 1 shows the effect of GSPE treatment on mechanical allodynia. Sciatic-tibial motor nerve conductive velocities (MNCVs) were significantly reduced ($p<0.01$) in untreated diabetic rats compared with normal controls, while treatment with 250 mg/kg GSPE apparently prevented this slowing ($p<0.01$). Table 2 shows the effect of GSPE treatment on MNCV.

**Effects of GSPE on body weights, plasma glucose, HbA1c, AGEs, neurotic MDA and SOD**

Each group of rats was observed from 1st to 24th week without any administration of hypoglycemic agents. Baseline body weights and plasma glucose levels were similar in all 4 groups. Seventy-two hours after injection of STZ, 80% of the rats developed high levels of plasma glucose, whereas normal control rats had normal levels. The elevated plasma glucose in STZ-induced rats treated and untreated was maintained during the entire experimental period. The number remaining alive at the end of the study in the four groups was 10, 10, 8 and 12 respectively of normal control, normal treated, diabetes and GSPE-treated diabetes. Table 1 shows the results of comparisons of body weight findings among these four groups. The body weight of diabetic rats significantly decreased at the 8th, 16th, 20th and 24th week ($p<0.01$) compared with normal control rats. GSPE improved the body weight of diabetic rats at the 8th, 16th, 20th and 24th week ($p<0.01$). Table 2 shows the comparisons of FBG and HbA1c findings among the four groups. The FBG, HbA1c and AGEs of diabetic rats were higher than those of control rats ($p<0.01$; AGEs: $p<0.05$). GSPE significantly reduced HbA1c and AGEs of diabetic rats ($p<0.05$), but could not decrease the FBG of diabetic rats.

The MDA level in diabetic rats was significantly increased compared to the age-matched control rats ($p<0.01$). Treatment with GSPE could reverse this increase ($p<0.01$). In the 24th week, SOD activity in diabetic rats significantly decreased compared to the age-matched normal rats ($p<0.01$) and GSPE treatment inhibited this decrease of SOD activity ($p<0.01$). GSPE had no effect on the MDA level ($p>0.05$) or SOD activity ($p>0.05$) in the normal control group. Table 2 shows the effect of GSPE treatment on MDA levels and SOD activities.

**Histological and electron micrographical findings**

It could be seen with H.E. staining by light microscope that the axons from the transverse sections swelled and could not be clearly discerned in diabetic

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**Table 1. Effects of GSPE on body weight (g) of diabetic rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>0th week</th>
<th>8th week</th>
<th>16th week</th>
<th>20th week</th>
<th>24th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>199.00±11.79</td>
<td>293.60±16.41</td>
<td>342.50±28.39</td>
<td>396.30±55.19</td>
<td>449.10±29.97</td>
</tr>
<tr>
<td>C2</td>
<td>196.00±8.39</td>
<td>290.60±34.90</td>
<td>344.60±24.69</td>
<td>386.80±29.12</td>
<td>446.20±57.08</td>
</tr>
<tr>
<td>DM1</td>
<td>199.25±27.81</td>
<td>216.13±31.19**</td>
<td>203.00±44.06**</td>
<td>228.00±30.40**</td>
<td>210.13±30.05**</td>
</tr>
<tr>
<td>DM2</td>
<td>194.00±17.94</td>
<td>249.33±52.27*</td>
<td>276.33±76.66**</td>
<td>309.03±93.14*</td>
<td>337.13±119.19**##</td>
</tr>
</tbody>
</table>

C1: untreated control group; C2: GSPE-treated control group; DM1: diabetes untreated; DM2: GSPE-treated diabetes. *$p<0.05$, **$p<0.01$: vs. C1; $^#p<0.05$, **$p<0.01$: DM2 vs. DM1.

**Table 2. Effects of GSPE on FBG, HbA1c, MNCV, MDA and SOD activity of diabetic rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>FBG (mmol/L)</th>
<th>HbA1c (%)</th>
<th>AGEs (AU/mg)</th>
<th>MNCV (m/s)</th>
<th>MDA (nm/mL)</th>
<th>SOD activity (NU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>10</td>
<td>6.66±0.46</td>
<td>5.61±0.73</td>
<td>0.017±0.004</td>
<td>35.76±2.25</td>
<td>9.41±0.65</td>
<td>131.9±11.59</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
<td>7.00±0.90</td>
<td>5.57±0.71</td>
<td>0.026±0.026</td>
<td>35.04±2.03</td>
<td>8.3±0.9</td>
<td>134.3±6.49</td>
</tr>
<tr>
<td>DM1</td>
<td>8</td>
<td>21.35±3.59**</td>
<td>12.40±2.35**</td>
<td>0.100±0.056**</td>
<td>18.08±3.22**</td>
<td>20.35±1.74**</td>
<td>77.37±6.9**</td>
</tr>
<tr>
<td>DM2</td>
<td>12</td>
<td>19.04±3.24**</td>
<td>9.61±2.14**</td>
<td>0.041±0.028**</td>
<td>29.36±3.05**</td>
<td>12.1±1.61**##</td>
<td>122.44±2.9**##</td>
</tr>
</tbody>
</table>

C1: untreated control group; C2: GSPE-treated control group; DM1: diabetes untreated; DM2: GSPE-treated diabetes. FBG: fasting blood glucose; HbA1c: glycated hemoglobin; MNCV: motor nerve conductive velocity; MDA: malondialdehyde; SOD: superoxide dismutase. *$p<0.05$, **$p<0.01$: vs. C1; $^#p<0.05$, **$p<0.01$: DM2 vs. DM1.
There was apparent segmental demyelination of nerves from diabetic rats using Loyez Stain (Figs. 2 and 3). The nerves from treated and untreated normal control groups showed normal ultramicrostructure under electron microscope.

Sciatic nerves from the untreated diabetic group showed obvious derangement of the myelin with disconnected layers and much lighter electron density. Axons were depressed by thickened tunica vaginalis with more glial, whereas microfilaments, microtubules and mitochondria were hardly seen. Proliferated Schwann cells crenulated with high electron density, many fewer organelles and impaired basement membranes. The nuclear chromatin massively aggregated to the edges of the irregular nuclei. Nerves from the GSPE-treated group had almost normal ultramicrostructures except that some myelin major dense lines and intraperiod lines had abnormal width forming bands displaying lighter electron density and resulted in breakage of myelin layers under the electron microscope (Fig. 4).

**DISCUSSION**

Hyperglycemia must be the initiator in the pathogenesis of diabetic chronic complications. Diabetic peripheral neuropathy, one of the most common chronic complications of diabetes, is a heterogeneous group of disorders with various pathologies. Diabetes is usually accompanied by excessive production of free radicals (3, 15), and it was recently demonstrated that hyperglycemia-induced mitochondrial ROS production could be a key event in the development of diabetic complications (16). Oxidative stress exerts its devastating effects by damaging DNA, proteins and cellular lipids directly. Biomarkers for oxidative damage such as 8-OhdG and MDA were demonstrated to increase in vitro and in vivo with diabetes (17). Oxidative stress also diminishes endogenous antioxidant enzyme defenses such as SOD activity (18), which is very important to the regulation of oxidative status in diabetes.

GSPE exhibited much stronger inhibition of superoxide anions and hydroxyl radicals, compared with vitamin C and vitamin E succinate under similar conditions (8). The antioxidant components of GSPE are very complex, containing procyanidolic oligomers (dimer, trimer and tetramer), polymers (including pentamers and more), some monomers and flavanols belonging to stilbenes. The antioxidant activity of proanthocyanidins
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contained in GSPE is related with their polymerizations; oligomers are proved to exhibit the best radical cation scavenging activity in the aqueous phase (19). Fujii et al. also proved that oligomers have more potent effects than others (monomer, polymer, and oligonol) against high glucose-induced oxidative stress (20). The oligomers in the GSPE we used in this experiment were as much as 74.6% or more, contributing to its excellent antioxidant effects. Our previous research has already demonstrated that GSPE can suppress ROS generation in vitro in human umbilical vein endothelial cells (HUVEC) (21). In this study, we observed a significant increase in MDA levels and decrease in SOD activities in diabetic rats and treatment with 250 mg/kg GSPE could reverse the MDA increase and impaired SOD activity in rats with diabetes. The excellent free radical scavenging activity of GSPE could be a possible reason for this reversal effect of MDA levels and SOD activities (8).

Our results indicate that rats with diabetes induced by STZ showed body weight reduction during the 24-wk experimental period. GSPE increased the body weight from the initial value. On the other hand, the levels of serum glucose and HbA1c in untreated diabetic rats were increased beyond those of untreated control rats. GSPE-treated diabetic rats showed decreased HbA1c levels, but fasting blood glucose did not show corresponding reductions. No obvious explanation is evident for this observation and further study is needed. Therefore, we suppose that the antioxidant ability of GSPE has a correlation with body weight gain.

Oxidative stress is also known to be able to induce cell apoptosis. Glucose can undergo auto-oxidation or decomposition via numerous intermediates to yield reactive dicarbonyl glyoxal, methylglyoxal and 3-deoxyglucosone (22), which can then react with proteins to generate AGEs. In a previous study finding pathological changes in apoptosis in DRG neurons and glial cells in STZ-induced diabetic rats, acutely hyperglycemic rats and high glucose cultured DRG neurons supposed oxidative stress as a central event (23). ROS-induced programmed cell death of neurons may share similar cell death pathways with neurotrophic withdrawal (24). Our previous study already demonstrated that GSPE could prevent nonenzyme glycation in kidneys of diabetic rats (25); we now proved that GSPE could prevent nonenzyme glycation in nerves of diabetic rats, too, which resulted in altered function that may lead to cell death.

Fig. 3. Light micrographs of longitudinal sections of sciatic nerves in normal control group (A), normal control with GSPE treated group (B), diabetic control group (C) and 250 mg/kg GSPE-treated group (D). Loyez staining, ×400.
via NF-κB or ROS generation (26).

Most commonly diabetes is associated with asymmetrical sensory polyneuropathy (27). It seems clear in this experiment that diabetes results in a decrease in nerve conductive velocity as previous studies reported (6), and treatment with GSPE could reverse this decrease. The primary sensory neurons of the peripheral nervous system are located within DRG. Oxidative stress in DRG neurons might be responsible for axonopathy and impaired regenerative of capacities of axons. This axon degeneration is associated with pathogenesis of negative signs of DPN such as NCV decrease (28). In the present study, we found that axons of diabetic nerves were depressed by thickened tunica vaginalis with more glial and microfilaments, microtubules and mitochondria hardly seen. With GSPE treatment, this axonal

Fig. 4. Electron micrographs of myelined fibers and Schwann cells in normal control group (A), diabetic control group (C), normal control with GSPE-treated group (B) and 250 mg/kg GSPE-treated group (D). Ax: axon, MNF: myelined nerve fiber, SC: Schwann cell, Mi: mitochondria, BM: basement member, Go: Golgi apparatus, RER: rough endoplasmic reticulum, Ly: lysosome, Co: collagen.
degeneration was ameliorated, contributing to NCV improvement. Schwann cells, playing a crucial role in the synthesis and maintenance of the peripheral nerve myelin, are susceptible to hyperglycemia since they take up glucose through the insulin-independent glucose transporters (29). We could observe significant ultrastructure injury of Schwann cells by TEM which displayed itself as apparent demyelination under light microscope in diabetes. The protection of GSPE on Schwann cells alleviated demyelination contributing to the improvement of NCV as myelin integration was important in impulse transduction in nerve fibers. Although structure impairment is insufficient to explain NCV decrease in early diabetes (30), the demyelination and nerve fiber loss do play a crucial role in DPN progression in long-term diabetes.

Diabetes could lead to mechanical allodynia (31). There were 10 untreated diabetic rats (83%) showing decreased paw withdrawal pressure and the threshold of the other two remained normal in the 12th week. After repeated tests, we observed that after 24 wk living with diabetes only one untreated diabetic rat (12.5%) showed hypalgesia and one displayed normal mechanical pain threshold (12.5%); the other 6 still reacted with paw withdrawal, sucking their feet and vocalizing to light pressure, indicating hyperalgesia. This is interesting because hyperalgesia usually occurs acutely and tends to run a limiting course in human diabetes according to our previous knowledge. Whether it is caused by individual differences or some other mechanisms is not clear. The mechanism underlying neuropathic pain in diabetes is still unclear, but the following has been reported to be postulated in the etiology of diabetic neuropathic pain: alterations in the expression and function of voltage-gated sodium channels (32), increased TNF-α and NO levels (33), COX-2 activation in spinal cord (34) and excitotoxic glutamate release (35). In our study, GSPE treatment could ameliorate this allodynia, indicating oxidative stress is involved in diabetic neuropathic pain; the mechanism is still unclear and further studies are wanted.

CONCLUSION

During this 24-wk study, we demonstrated a protective effect of GSPE in experimental diabetic neuropathy. GSPE showed significant protection in diabetic neuropathy; it could apparently improve the decreased mechanical allodynia and sciatic-tibial nerve conduction velocity, and alleviate nerve impairment of diabetic rats without affecting body weights or plasma glucose levels. We also proved that GSPE didn’t have side effects on normal nerve structures or functions. The protective effect of GSPE in diabetic neuropathy may be attributed to its antioxidant activity.

Acknowledgments

We wish to thank the Center of Medical Biotechnology, Shandong Academy of Medical Sciences, the Department of Experimental Pathology and Physiology, Shandong Academy of Medical Sciences and the Center for New Drug Evaluation of Shandong University. We also thank Professor Wei-dong Zhang and Professor Hong Li for their assistance.

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