The Protective Effects of DA-9801 (*Dioscorea* Extract) on the Peripheral Nerves in Streptozotocin-Induced Diabetic Rats

Kyung Ae Lee, Heung Yong Jin, Hong Sun BaeK and Tae Sun Park

Division of Endocrinology and Metabolism, Department of Internal Medicine, Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute of Chonbuk National University Hospital, Jeonju, 561–712, South Korea

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Summary It has been reported that DA-9801, an extract mixture of *Dioscorea japonica* Thunb and *Dioscorea nipponica* Makino, produces a neurotrophic activity. Therefore, this study was conducted to examine the neuroprotective effects of DA-9801 in streptozotocin-induced diabetic rats. The experimental rats were divided into six groups: the control group, Group I (non-diabetic rats treated with DA-9801), Group II (diabetic, non-treated rats) and Groups III, IV, and V (diabetic rats treated with DA-9801 at doses of 10, 50 or 100 mg/kg/d). Following a 16-wk course of oral treatment with DA-9801, functional parameters (von Frey filament test, hot plate test), biochemical parameters (nerve growth factor (NGF), tumor necrosis factor (TNF)-α, interleukin (IL)-6) were measured. An immunohistochemical staining was done to assess the neuroprotective effects of DA-9081 in the skin, sciatic nerve, gastric mucosa and renal cortex. In Week 8, pain was evoked by either tactile or thermal stimuli, whose threshold was significantly higher in Group III, IV and V than Group II. Western blot analysis showed a more significant increase in NGF and decrease in TNF-α and IL-6 in Group III, IV and V than in Group II (p<0.05). Moreover, following the treatment with DA-9801, a loss of intraepidermal nerve fibers (IENFs) was inhibited to a significant level in the skin, myelinated axonal fibers of the sciatic nerve and small nerve fibers innervating the gastric mucosa or renal cortex (p<0.05). Our results demonstrated that DA-9801 is a beneficial agent that protects the peripheral nerves in diabetic rats.

Key Words DA-9801, diabetic peripheral neuropathy, nerve growth factor, neuroprotective effect

Diabetic peripheral neuropathy (DPN) affects up to 50% of diabetic patients and it is a major cause of morbidity, mortality and poor quality of life (1). Therefore, in patients with diagnosed with DPN, therapy can be instituted for the purposes of both reducing symptoms and preventing its progression (2). Hyperglycemic control is the central component. Nevertheless, it is difficult to achieve a hyperglycemic control in many patients. Furthermore, despite ongoing studies about several disease-modifying agents, there is only one anti-oxidant, α-lipoic acid, whose efficacy for patients with DPN has been supported in a meta-analysis (3). More studies are therefore needed to develop effective pathogenic treatments in patients with DPN. Previous reports have suggested that reduced availability of nerve growth factor (NGF) plays a key role in the pathogenesis of DPN (4, 5). Still, however, little is known about the efficacy of neurotrophic agents. This is because clinical trials have yielded no positive results although the efficacy of neurotrophic agents has been reported in some animal experimental studies (6, 7). However, it has been theoretically hypothesized that defective neurotrophic factors binding to specific receptors may cause DPN.

Traditionally, many species of *Dioscorea* have been used as folk medicine for various metabolic disorders including diabetes mellitus (8–11). Several recent studies demonstrated that saponins from *Dioscoreae* have a neurotrophic effect by inducing NGF (12, 13). In this regard, previous studies have shown that DA-9801, a mixture of extracts from *Dioscorea japonica* and *Dioscorea nipponica*, increased the serum levels of NGF and reduced the nociceptive pain (14, 15). To date, however, no studies have clarified the exact role of DA-9801 and the degree of its neurotrophic effect in DPN. Given the above background, we conducted this study to examine the neuroprotective effect of DA-9801 on the peripheral nerves of streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Materials. All the experiments and the relevant protocols were approved by the Institutional Rat Care and Use Committee of the Chonbuk National University Medical School (CBU 2011-0055). Six- to 8-wk-old male Sprague-Dawley (SD) rats weighing 160 to 180 g were purchased from Dae Han Biolink Co. Ltd. (Eum-sung, Chungbuk, Korea). Animals were housed under optimal conditions with a 12-h light-and-dark cycle. The room temperature was maintained at 23±1°C and humidity at 53±2%. The animals had free access to...
food and water.

Streptozotocin (STZ) (Sigma Chemical, St. Louis, MO) was dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5) and it was intraperitoneally injected into the experimental rats (60 mg/kg body weight) to induce diabetes. Forty-eight hours after STZ injection, the experimental rats with blood glucose levels of ≥20 mmol/L were confirmed to have diabetes. After subjecting rats to an overnight fast, blood samples were drawn from the tail vein and blood glucose levels were measured using Precision Xtra Plus® (Abbot Laboratories, Medisense Products, Bedford, MA). In the same manner, age-matched control rats received an equal volume of the vehicle, sodium citrate buffer (pH 4.5). DA-9801 (Dong-A Pharmaceutical Corp., Youngin, Korea) is a rhizome mixture of *D. japonica* and *D. nipponica* (ratio of 1 to 3), and it was extracted with 50% ethanol at room temperature for 48 h, and concentrated under vacuum (14).

**Experimental design.** Following a 4-wk intraperitoneal injection of STZ and sodium citrate buffer, diabetic rats and their age-matched controls were randomly assigned to six groups (*n* = 4–6 per group) according to the treatment agents and their dose: (1) The control group: Non-diabetic, age-matched controls (*n* = 4); (2) Group I: Non-diabetic, age-matched controls treated with DA-9801 at a dose of 100 mg/kg/d (*n* = 4); (3) Group II: Non-treated diabetic rats (*n* = 6); (4) Group III: Diabetic rats treated with DA-9801 at a dose of 10 mg/kg/d (*n* = 6); (5) Group IV: Diabetic rats treated with DA-9801 at a dose of 50 mg/kg/d (*n* = 6); (6) Group V: Diabetic rats treated with DA-9801 at a dose of 100 mg/kg/d (*n* = 6).

In the current experiment, the experimental rats were orally given DA-9801.

**Behavioral assessment by tactile and thermal response.** In Week 8 and 16, we performed a behavioral assessment. Tactile stimulation was performed for the experimental rats using a flexible von Frey filament (Stoelting Co., Wood Dale, IL). After adaptation to the testing condition for at least 20 min, rats were placed individually in a plastic cage with a 1-cm sized perforated mesh. Von Frey filaments, with calibrated bending forces (g), were applied perpendicularly to the plantar surface of the hind paw. Thus, attempts were made to deliver tactile stimuli to the experimental rats at varying intensities, which was done based on the method proposed by Chaplan et al. (16). The stimulation was performed five times at 5-s intervals. Then, when the experimental animals had an immediate withdrawal at least once after they were stimulated five times, they were determined to have a positive response. To measure thermal response, the experimental rats were placed on a hot-plate (Ugo Basile, Schwenksville, PA) at a mean temperature at 55 ± 1°C. When the experimental rats had latency to the first sign of paw licking response to avoid the heat, they were considered to have a threshold for thermal sensation.

The measurement of levels of NGF, tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the sciatic nerve and spinal cord. In Week 17, all the experimental rats were killed under deep anesthesia. This was followed by the rapid removal of sciatic nerves and lumbar segments of the spinal cord. Then, they were prepared for a Western blot analysis. The sciatic nerve and spinal cord were degraded mechanically using 100–200 μL of Triton lysis buffer. After centrifugation at 13,200 rpm for 15 min at 4°C, the supernatant was transferred to a fresh tube (1.5 mL capacity). This was followed by the quantification of protein concentration based on the Bradford method (17). Samples were loaded into a 10% SDS polyacrylamide gel. In addition, non-specific binding was blocked by placing the membrane in a dilute protein solution of non-fat dry milk (5% skim milk) with 1% Tween-20 in PBS for 3 h at room temperature. The blots were incubated with primary antibodies such as NGF, TNF-α and IL-6 (1 : 1,000; Abcam, Cambridge, UK) overnight at 4°C. The membranes were washed in 1 TBST by shaking three times for 10 min. Incubation with HRP-conjugated secondary antibody donkey anti-goat IgG, HRP conjugate (1 : 2,000; Santa Cruz Biotechnology, Santa Cruz, CA) was performed on a rocker for 1 h at 4°C. Protein bands were detected using ECL-plus kit (Amersham Pharmacia Biotech, Little Chalfont, England). Quantitative image analysis was conducted using LAS 3000 Fuji Film and film densitometry was performed using Multi Gauge version 3.0 (Fuji Film, Tokyo, Japan).

**Morphometric assessment of the cutaneous nerve and sciatic nerve.** In Week 0 and 8, tissue samples of 3×3 mm in size were extracted from the dorsum of the foot via a skin biopsy. Thus, attempts were made to perform an immunohistochemical analysis of the intraepidermal nerve fibers. In Week 17, all the rats were sacrificed under deep anesthesia. This was followed by the final tissue sampling for the cutaneous tissue covering the feet and segments of the sciatic nerve from each rat. Sciatic nerve tissue samples were immersed in a fixative (2.5% glutaraldehyde in phosphate-buffered saline (PBS)) and incubated overnight at 4°C. These samples were then embedded in JB-4 solution (Polysciences, Inc., Eppelheim, Germany), and transverse sections with a thickness of 1.5 μm were stained with toluidine blue. Immunohistochemical staining was done as previously described (18). Skin tissue specimens were fixed with periodate-lysine-paraformaldehyde (PLP) (2% paraformaldehyde, 0.075 M lysine, 0.05 M phosphate buffer pH 7.4, and 0.01 M sodium m-periodate) solution for 24 h. After thorough rinsing in PBS containing 20% glycerol–0.1 M phosphate buffer for 48 h at 4°C, the tissue specimens were cryoprotected with Tissue-Tec® (OCT compound) (Miles, Elkhart, IN). Sections of 40 μm in thickness were cut perpendicular to the dermis, and were prepared with a sliding cryostat (Leica CM 1510®, Leica Microsystems AG, Wetzlar, Germany). Then, the sections were immersed in PBS for 15 min at room temperature and transferred into microtubes containing Dako Protein Block Serum-Free® (Dako, Carpinteria,
CA) as a blocking buffer supplemented with 3% goat serum. After 30 min of blocking on a shaker table at room temperature, specimen sections were washed with PBS twice for 10 min and then incubated overnight with primary antibody, rabbit anti-protein-gene-product 9.5 (PGP 9.5) (Biogenesis, Poole, UK) at a dilution ratio of 1:100 at 4˚C. The antibodies were diluted in antibody diluent (Dako) supplemented with 1% goat serum. After complete washing, the specimens were incubated with the secondary antibody, goat anti-rabbit IgG-FITC (1:200, Vector Labs, Peterborough, UK), for 1 h at room temperature in a dark room. After washing with PBS, sections were placed on slides and then mounted with a fluorescent mounting media (Dako). Photomicrographs of the myelinated fiber and intraepidermal nerve fiber (IENF) were captured using a digital camera (Axiocam HRC®, Carl Zeiss, Goettingen, Germany) at a final magnification of \( \times 400 \) and \( \times 100 \), respectively. The myelinated fiber or axonal area in the sciatic nerve, represented by the outer or inner border of the myelin sheath, was measured using analySIS® image software (Soft Imaging Systems GmbH, Munster, Germany). This was followed by the calculation of the mean area of the myelinated nerve fiber. In addition, we also measured the thickness of myelin sheath and the diameter of axonal fiber. PGP 9.5-immunoreactive nerve fibers in the epidermis of each section were counted as described previously (19). Each cutaneous nerve fiber was counted as a single fiber if it had branching points inside the epidermis. The number of intraepidermal nerve fibers (IENFs) per length (mm) was considered as the amount of innervation. To avoid any possible bias during preparation and calculation, two independent observers were blinded to the experimental groups. Moreover, the slides were mixed with a set of normal ones prior to the test.

The morphometric assessment of the nerves innervating the gastric mucosa and renal cortex. The peripheral nerves innervating the gastric mucosa and renal cortex were also analyzed to examine the neuroprotective effects of DA-9801. In an attempt to compare the quantity of the nerves innervating the gastric mucosa, as well as the morphometric characteristics, among the six groups, we arbitrarily made a horizontal line connecting the points at a 100-μm distance on the luminal side. In renal cortex, however, only immunostained nerve fiber patterns were observed without quantitative comparison among experimental groups.

Statistical analysis. All data were expressed as mean±SD (standard deviation). To compare between the experimental groups, we used one-way ANOVA with the Tukey-Kramer method. A p-value of <0.05 was considered statistically significant at a 95% confidence interval. Statistical analyses were done using SPSS 12.0 software (SPSS Inc., Chicago, IL).

RESULTS

Effect of DA-9801 on the body weight and blood glucose level

Two weeks after STZ injection, blood glucose levels were higher and body weight was lower in diabetic rats compared to normal rats. Following a 16-wk course of DA-9801 treatment, there was no significant effect in the body weight of either the both normal or diabetic groups (Fig. 1A). In Week 16, blood glucose levels were significantly lower in Groups III, IV and V as compared with Group II (20.9±0.3, 20.8±0.5, 20.7±0.5 vs. 24.8±1.1, p<0.001) (Fig. 1B). However, the hypoglycemic effect of DA-9801 did not reach a therapeutic significance because there was a persistent hyperglycemia of >20 mmol/L in the in Groups III, IV and V. Furthermore, there was no dose-dependent hypoglycemic effect of DA-9801.

Fig. 1. Body weight and blood glucose levels in the experimental groups. (A) Body weight was increased gradually in normal rats. In the diabetic rats, however, body weight failed to increase, with no respect to the DA-9801 treatment. (B) In Week 16, following the treatment with DA-9801, blood glucose levels were significantly lower in Groups III, IV, and V as compared with Group II. Data are presented as mean±SD. # indicates statistical significance within diabetic groups (\( # p<0.001 \) vs. DM).
Effect of DA-9801 on the tactile response and thermal response

In Week 8, when stimulated with von Frey filaments, the paw withdrawal threshold was significantly lower by 59% in Group II as compared with the control group. But this hyperalgesic response was significantly prevented in Groups III, IV and V as compared with Group II (Fig. 2A). The latency to withdrawal of rat paw on a hot plate was significantly lower by 34% in Group II as compared with the control group. But this hypersensitive response was significantly prevented in Groups III, IV and V as compared with Group II (Fig. 2B). In Week 16, this pattern of response to von Frey filaments was reversed and more blunted response was observed in Group II. The threshold of response to von Frey filaments was lower at a statistically significant level in Groups III, IV and V as compared with Group II (Fig. 2C). The latency of thermal withdrawal was also higher in Group II as compared with control group and Groups III, IV, and V. and this reverse of blunted response was similar to that observed in the von Frey test (Fig. 2D). But there were no dose-dependent effects of DA-9801 on the tactile or thermal response.

Effect of DA-9801 on the NGF, TNF-α and IL-6 levels on the sciatic nerve and spinal cord

NGF levels in the sciatic nerve and lumbar segment of the spinal cord were significantly lower in Group II as compared with the control group. But there was no significant difference in the NGF level between Groups III, IV and V and the control group (Fig. 3A and B). In particular, the NGF levels were maintained at a significantly higher degree in Groups IV and V as compared with Group III. The levels of TNF-α and IL-6 were significantly higher in Group II as compared with the control group. However, the levels of such pro-inflammatory cytokines as TNF-α and IL-6 were significantly lower in Groups III, IV and V as compared with Group II (Fig. 3C–F). Furthermore, IL-6 levels in the sciatic nerve were decreased to a significantly higher degree in Group V as compared with Groups III and IV.

Morphometric and quantitative comparison of the peripheral nerves among the six groups

Following a comparison of the quantity of the cutaneous peripheral nerves among the six groups, it was found that the IENF density was significantly lower in Group II as compared with the control group and Group I. The shortening and degeneration of small peripheral
Fig. 3. Levels of NGF, TNF-α, and IL-6 in the sciatic nerve and spinal cord in the experimental groups. NGF levels in the sciatic nerve (A) and lumbar segments of the spinal cord (B) were significantly lower in Group II as compared with the control group. But there was no significant difference in NGF level between Groups III, IV and V and the control group and Group I. The levels of TNF-α (C, D) and IL-6 (E, F) were significantly higher in diabetic rats as compared with their non-diabetic counterparts. The levels of TNF-α and IL-6 were decreased following the treatment with DA-9801 in diabetic rats. Data are presented as mean±SD. *p<0.05 vs. Normal, **p<0.01 vs. Normal, #p<0.05, ##p<0.01 vs. DM, $p<0.05$ vs. DM+DA-9801 10 mg, $$$p<0.05$ vs. DM+DA-9801 50 mg. * and ** indicate statistical significance within the diabetic groups. $ and $$$ indicate statistical significance within the treated diabetic groups.

(A) NGF in sciatic nerve  (B) NGF in spinal cord

(C) TNF-α in sciatic nerve  (D) TNF-α in spinal cord

(E) IL-6 in sciatic nerve  (F) IL-6 in spinal cord
nerve fibers was seen in Group II (Fig. 4A). But this morphological change and the reduction of IENF density were blunted in Groups III, IV and V. In Week 8, there was no dose-dependent effect of DA-9801. In Week 16, however, the IENF density was maintained at a significantly higher level in Group V as compared with Groups III and IV (Fig. 4B, 4C). Following a comparison of the quantity of the sciatic nerves among the six groups, similar results were found for the IENF density to that in the cutaneous nerves. On the transverse sections of sciatic nerve samples, the area of endoneurium was significantly greater in Groups III, IV and V as compared with Group II. It was also shown that the quantity of nerve fibers with a degeneration of the myelin sheath was significantly lower in Groups III, IV and V as compared with Group II (Fig. 5A). The mean area of myelinated axonal fiber was significantly smaller in Group II as compared with the control group and Group I. This effect was not seen in Groups III, IV and V, where there was no dose-dependent effect (Fig. 5B). The diameter and thickness of myelin sheath were significantly greater in Groups III, IV and V as compared with Group II (Fig. 5C and D).

Morphometric comparison between the nerves innervating the gastric mucosa and renal cortex

The quantity of small nerve fibers with a positive immunohistochemistry, innervating the horizontal line connecting the points at a 100-μm distance on the luminal side, was significantly greater in Groups III, IV and V as compared with Group II. In addition, its degree was the highest in Group V (Fig. 6A). The degree of the shortening and degeneration of the small nerve fibers innervating the gastric mucosa was significantly higher in Group II as compared with Groups III, IV and V (Fig. 6B).

Fig. 4. Immunohistochemistry of cutaneous small nerve fibers of the dorsum of the hind foot and the IENF density in Weeks 8 and 16 in the experimental Groups. (A) In Groups III, IV and V, as compared with Group II, PGP 9.5-stained small nerve fibers were more preserved and less shortened and the IENF density (B, C) was higher. Data are presented as mean±SD. Bar indicates the 100 μm. a: The control group, b: Group I, c: Group II, d: Group III, e: Group IV and f: Group V. **p<0.01 vs. Normal, #p<0.01 vs. DM, **p<0.05 vs. DM+DA-9801 50 mg. # indicates statistical significance within the diabetic groups. $$$ indicates statistical significance within the treated diabetic groups.
Fig. 5. Immunohistochemistry of the sciatic nerve (A) and the area of myelinated nerve fiber (B), the diameter of myelin sheath (C) and the diameter of axon (D) in the experimental groups in Week 16. Myelinated axonal nerve fibers were less degenerated in Groups III, IV and V. In addition, the size of nerve fibers with or without myelin sheath was significantly greater in Groups III, IV and V as compared with Group II. Data are presented as mean ± SD. Bar indicates 20 μm. a: The control group, b: Group I, c: Group II, d: Group III, e: Group IV and f: Group V. *p<0.05 vs. Normal, **p<0.01 vs. Normal, #p<0.01 vs. DM. # indicates statistical significance within the diabetic groups.

Fig. 6. The mean number of nerve fibers innervating the gastric mucosa and PGP 9.5-positive ones. (A) The mean number of nerve fibers innervating the gastric mucosa was significantly smaller in Group II as compared with Groups III, IV and V. (B) The nerve fibers had a shorter length and a higher degree of degeneration to a significant extent in the diabetic rats as compared with their non-diabetic controls. In addition, the length and the degree of the degeneration of nerve fibers were significantly higher in Groups III, IV and V as compared with Group II. Data are presented as mean ± SD. Horizontal bar indicates 100 μm. a: The control group, b: Group I, c: Group II, d: Group III, e: Group IV and f: Group V. *p<0.05 vs. Normal, **p<0.01 vs. Normal, #p<0.01 vs. DM. ** indicates statistical significance between normal and non-treated diabetic groups. # indicates statistical significance within the diabetic groups. $$ indicates statistical significance within the treated diabetic groups.
The quantity of the nerve fibers innervating the renal cortex was significantly higher in the renal cortex around the glomerulus. Furthermore, it was maintained at a higher level in Groups III, IV and V as compared with Group II (Fig. 7). But we failed to compare the quantity of such nerves due to a lack of standardized methods of quantitative estimation because these methods vary depending on the shape and course of the nerves.

**DISCUSSION**

DPN and the diabetic foot ulcer are the most common serious complications that occur in diabetic patients. They eventually lead to the highest morbidity and mortality, thus causing a huge economic burden for patient care (2). It has been reported that multiple etiologic factors are involved in the pathogenesis of DPN. With the discovery of the polyol pathway, a number of mechanisms have been proposed to explain its pathogenesis and these include advanced glycation end products (AGEPs), protein kinase C (PKC)-β, the hexosamine pathway, and the unifying mechanism of superoxide production (20). Reactive oxygen species (ROS) not only contribute to induction of these various pathways of DPN but also comprise their end products. This eventually leads to an increased oxidative stress and the attenuation of anti-oxidative defense mechanisms (21). In addition, it has also been reported that decreased NGF levels also play a role in the pathogenesis of DPN (4, 5). Low levels of NGF could be due to either decreased production or transport of NGF in diabetes or both, possibly as a result of glucose-induced oxidative stress. In addition, autoimmunity may play a role in the NGF deficiency in diabetes by mechanisms related to immune neutralization of available NGF (22). In experimental diabetic rats, following the treatment with NGF, decreased substance P levels are recovered in the peripheral nerves and this leads to the restoration of the nociceptive threshold to noxious thermal stimuli, the morphology of myelinated nerve fibers and neurogenic vasodilatation in STZ-induced diabetic rats (23). According to a Phase II clinical trial, exogenous recombinant human NGF actually had a significant beneficial effect on diabetic peripheral neuropathy (24). Unfortunately, its efficacy has not been demonstrated in a Phase III trial (25). Presumably, this might be due to a limited delivery to the nervous system and adverse effects (hyperalgesia) after subcutaneous injection which was unavoidable because NGF is a peptide. Therefore, NGF-mimetic peptide and other small agents would be of great interest because they promote the action or synthesis of NGF. In this context, we conducted the current study to examine the beneficial effects of DA-9801, a plant-derived agent that promotes the activity of NGF, in the treatment of patients with DPN.

Dioscorea is a genus of over 600 species of flowering plants that belong to the family Dioscoreaceae, a perennial herb growing in mountainous areas of the Korean peninsula. Various species of Dioscorea have been used as a traditional medicine for the treatment of diverse diseases including metabolic disorders such as obesity and diabetes, inflammatory diseases, pain and neuropathic diseases including diabetic neuropathy (8–11, 26). In particular, dioscin, a major steroid saponin isolated from D. nipponica, and furostanol saponins from D. japonica have a neurotrophic effect by inducing NGF (12, 13). These saponins are considered as a potent stimulant of

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Fig. 7. PGP 9.5-positive small nerve fibers innervating the renal cortex. The number of PGP 9.5-positive, small nerve fibers innervating the renal cortex was significantly lower in the diabetic rats as compared with their non-diabetic controls. Despite a lack of the comparison of the quantity, however, a significantly greater number of nerve fibers occurred in Groups III, IV and V. Horizontal bar indicates 100 μm. a: The control group, b: Group I, c: Group II, d: Group III, e: Group IV and f: Group V.
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NGF release; however, it remains to be determined how these saponins can induce NGF.

DA-9801 is a mixture of extracts from D. japonica, also known as SanYak, and D. nipponica, also known as Buchema. Previous studies have shown that DA-9801 has a neurotrophic activity in an in vitro model and in animal experimental studies. To date, however, few studies have demonstrated the degree and the scientific mechanisms of the neuroprotective effect of Dioscorea species in diabetic animals. In the current study, we assessed blood glucose levels, the functional parameters, the relevant biochemical mediators and the morphometric and quantitative characteristics of DPN in STZ-induced diabetic rats following a 16-wk course of DA-9801 treatment. DA-9801 was administered to the experimental animals at three different doses. Thus, attempts were made to disclose the dose-response relationship. We found that there was a beneficial effect on the blood glucose levels following a 16-wk course of DA-9801 treatment in an animal experimental model of diabetes. But there was no body weight gain. Because DA-9801 had a hypoglycemic effect to a negligible extent and our animal model was late state type 2 diabetic or type 1 diabetic model using STZ, all the experimental rats maintained a sustained hyperglycemia of >20 mmol/L during the experimental period despite the treatment with DA-9801. On functional studies, results need to be delicately interpreted. It is generally known that there are the alterations in the pattern of diabetic nociceptive neuropathy such as hyperalgesia, allodynia and hypalgesia. In addition, with consideration of the affected nerves, it is necessary to support the functional parameters with more objective parameters indicating these symptoms. Our results showed that the IENF density at Week 8 was decreased based on the morphometric findings of the dorsum of the hind foot in diabetic rats. These results suggest that more hypersensitive reactions may arise from the degeneration of small nerve fibers. In Week 16, however, the hypersensitive response disappeared, accompanied by the presence of more blunt responses, including thermal stimuli in particular, in Group II as compared with the control group and Groups III, IV, and V. These results are in agreement with those about the IENF density in peripheral nerves. It is difficult, however, to compare and then to interpret functional parameters at the later stages of diabetes. This is because there is a possibility that there are mixed symptoms and sensitivity. Moreover, there was a difference in the body weight between the non-diabetic groups (the control group and Group I) and the diabetic ones. There is also a possibility that this may cause a difference in the threshold for mechanical allodynia and thermal response. Furthermore, it is also possible that the diabetic groups might be vulnerable to sensory stimuli due to a generalized cachexia rather than the neuronal degeneration or dysfunction.

To show more objective evidence demonstrating the neuroprotective effects of DA-9801, we compared the morphological characteristics of the small cutaneous nerve fibers, sciatic nerves and the peripheral nerves innervating the gastric mucosa and renal cortex among the six groups. The quantity of IENFs that are present in the cutaneous and sciatic nerve fibers was maintained to a higher extent and they were degenerated to a lesser extent in Groups III, IV and V compared to Group II. The degree of the neuroprotective effects of DA-9801 was significantly higher at the highest dose (100 mg/kg/d), which was particularly seen during the late period of the current experiment. In Groups III, IV and V, small nerve fibers innervating the gastric mucosa and renal cortex were protected to a higher extent as compared with Group II. In small nerve fibers innervating the renal cortex, there was a positive immunohistochemistry for PGP 9.5. It was also shown that there was a decreased immunohistochemistry around the glomerulus in Group II. These findings were slightly improved in Groups III, IV and V. To clarify the exact mechanisms of the neuroprotective effect of DA-9801 in more detail, we measured the concentrations of NGF and proinflammatory cytokines such as TNF-α and IL-6 in the sciatic nerve and spinal cord, respectively. The concentrations of NGF were significantly lower in Group II but returned to the normal range in Groups III, IV and V. The concentrations of proinflammatory cytokines were significantly lower in Groups III, IV and V. Based on these results, it can be inferred that DA-9801 has an agonistic activity of NGF, and the anti-inflammatory effects of DA-9801 led to the protection of peripheral small nerve fibers. NGF activity is specifically associated with activation of p75 and trk A, with resultant effects on small sensory and autonomic nerve fibers. Large nerve fibers are dependent on the activity of trk C, whereas medium-sized fibers are affected through the trk B pathway. Therefore, DA-9801 is also expected to effective in diabetic autonomic neuropathy. Although many studies investigated how Dioscoreae inhibits the NGF depletion, the mechanism is unknown. There remains much to be discovered regarding the detailed mechanism of neurotrophic effects. In addition, we divided the experimental animals into three dose groups depending on the dose of DA-9801 (10, 50 and 100 mg/kg/d) and thereby attempted to disclose the dose-response relationship. But there were no consistent results about the effects of DA-9801 on diverse parameters at varying doses. Further studies are therefore warranted to determine the effective dose of DA-9801. Finally, the current study was designed to assess the efficacy of DA-9801 as compared with normal controls. Therefore, further comparative studies are also warranted to demonstrate the efficacy of well-known agents in the treatment of patients with DPN.

In conclusion, anti-inflammation, anti-oxidation or neuronal regeneration are the mechanisms by which the treatment agents act on the therapeutic target in the effective treatment of patients with DPN. In this regard, our results suggest that DA-9801 might play a role in the prevention of nerve degeneration in diabetic rats. Presumably, the efficacy of DA-9801 might arise from the increased neurotrophic activity and anti-inflammatory response, both of which are commonly

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involved in the pathogenesis of DPN.

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