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Sulodexide prevents peripheral nerve damage in streptozotocin induced diabetic rats

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ABSTRACT

We investigated whether sulodexide has additional protective effects against peripheral nerve damage caused by microvascular dysfunction in a rat model of diabetes. Female Sprague–Dawley (SD) rats were divided into the following 4 groups (n = 7–9/group): Normal, Normal + Sulodexide (sulodexide 10 mg/kg), diabetic group, and diabetic + Sulodexide (sulodexide 10 mg/kg). We assessed current perception threshold, skin blood flow, superoxide dismutase, and proteinuria in experimental rats after oral administration of sulodexide for 20 weeks. We also performed morphometric analysis of sciatic nerves and intraepidermal nerve fibers of the foot. Superoxide dismutase activity in the blood and sciatic nerve were increased significantly after sulodexide treatment in the diabetic group. Current perception threshold was reduced at 2000 Hz (633.3 ± 24.15 vs 741.2 ± 23.5 μA, $P<0.05$) and skin blood flow was improved (10.90 ± 0.67 vs 8.85 ± 0.49 TPU, $P<0.05$) in the diabetic + Sulodexide group compared with the diabetic group. The mean myelinated axon area was significantly larger (56.6 ± 2.2 vs 49.8 ± 2.7 μm^2, $P<0.05$) and the intraepidermal nerve fiber density was significantly less reduced (6.27 ± 0.24 vs 5.40 ± 0.25/mm, $P<0.05$) in the diabetic + Sulodexide group compared to the diabetic group. Our results demonstrate that sulodexide exhibits protective effects against peripheral nerve damage in a rat experimental model of diabetes. Therefore, these findings suggest that sulodexide is a potential new therapeutic agent for diabetic peripheral neuropathy.

1. Introduction

Peripheral neuropathy is the most common and debilitating complication of diabetes; thus the pathophysiology and therapeutic development of diabetic peripheral neuropathy are active areas of research. Diabetic peripheral neuropathy is thought to occur as a result of hyperglycemia-induced damage to nerve cells and from neuronal ischemia caused by hyperglycemia-induced decreases in neurovascular flow (Edwards et al., 2008; Vinik and Mehrabyan, 2004). Hyperglycemia causes systemic oxidative stress and increases inflammation through cytokine secretion by various cells (Aronson, 2008). Therefore, new agents that improve vascular blood flow by endothelial protection or increasing anti-oxidative stresses have been tested as novel treatments for diabetic peripheral neuropathy.

Sulodexide is a mixture of glycosaminoglycans that includes low molecular weight heparin and dermatan sulfate, both of which have antithrombotic and profibrinolytic properties (Ofosu, 1998). Sulodexide also exhibits endothelium-protective (Kristova et al., 2008) and anti-inflammatory effects in diabetes. Previous studies have shown that glycosaminoglycans and proteoglycans exert anti-inflammatory activity in various cells (Neumann et al., 1999; Wang et al., 2006) and high molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis (Ciszewicz et al., 2009). The clinical efficacy and safety of sulodexide have also been demonstrated in peripheral arterial disease, cardio-cerebrovascular disease, nephropathy, and postphlebitic syndrome (Gambaro et al., 1992; Harenberg, 1998; Williams, 2006).

Potential therapeutic applications of sulodexide for the treatment of diabetic peripheral neuropathy are based on the pathogenesis of diabetic peripheral neuropathy and the diverse effects of sulodexide. The purpose of this study was to demonstrate the protective effects of sulodexide on peripheral nerves in a rat model of experimental diabetes.

2. Materials and methods

2.1. Rats and induction of diabetes

Female Sprague–Dawley (SD) rats (160–180 g, 6–8 weeks old) were purchased from Damool Science (Daejeon, Chungnam, Korea) and allowed to adapt to their new environment for 1 week. Rats were kept in a pathogen-free rat-rearing facility with a 12 h light and dark

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cycle. The temperature (23 ± 1 °C) and humidity (53 ± 2%) of the room were strictly maintained and rats were provided with food and water *ad libitum*. Single intraperitoneal injections of streptozotocin (60 mg/kg body weight) (Sigma Chemical, St. Louis, MO, USA), dissolved in 0.1 mol/l citrate buffer (pH 4.5), was used to induce diabetes. Age-matched rats used as normal glucose controls received an equal volume of vehicle (sodium citrate buffer) administered in the same manner. Forty-eight hours after the injection of streptozotocin, rats with blood glucose levels higher than 350 mg/dl after overnight fasting were considered to be diabetic. The Precision Xtra Plus® (Abbot Laboratories, MediSence Products, Bedford, MA, USA) system was used to measure glucose.

2.2. Intervention schedule

At least 2 weeks after the injection of streptozotocin was needed to induce the typical features of diabetes. Four weeks after the verification of diabetes, normal and diabetic rats were randomly assigned to the following 4 groups (n = 7–9/group): Normal, Normal with sulodexide, diabetic group, and diabetic with sulodexide. Sulodexide (supplied by Asia Pharm. Korea) was dissolved in water and administered orally at a dose of 10 mg/kg. Sulodexide doses were tested from 0.125–0.5 LRU/ml (Ciszewicz et al., 2009) or 2–10 mg/kg for anti-thrombotic effect without bleeding problems (Harenberg, 1998) in previous animal studies and usual doses are 500 LRU/day or 100 mg/day in humans. Therefore, a 10 mg/kg dose was selected in this study to determine the neuroprotective effect. Normal and diabetic rats were treated with compound or placebo once a day for 20 weeks. The protocol for rat care and experimental procedures were approved by the Institutional Rat Care and Use Committee of the Chonbuk National University Medical School.

2.3. Body weight, blood glucose, HbA1c, and urinary protein measurements

Body weight and blood glucose were measured every week after 8 h of fasting. Fasting glucose levels were assessed using blood samples drawn from a tail vein and HbA1c levels were compared using a commercially available kit (NycoCard, Oslo, Norway). On the second day of the 20th week, a glucose tolerance test was performed by administering 50% dextrose (2 g dextrose/kg) into the stomach of the rats after overnight fasting. Before and after administration (0, 0.5, 1, 2, and 3 h), blood glucose levels were also assayed. Plasma was collected after centrifugation for 10 min at 1000 × *g* at 4 °C. The 24 h urine of each rat was collected with a metabolic cage and the 24 h protein excretion and urinary albumin(mg/dl)/creatinine(g/dl) ratio (ACR) levels were determined.

2.4. Current perception threshold

Current perception thresholds were examined to quantify nerve dysfunction during the 20th week, as in our previous study (Jin et al., 2009b). In brief, 2000, 250 and 5 Hz frequency stimuli were used to determine the perception thresholds with respect to pressure, vibration, and temperature, respectively. The sine-waves were delivered by a Neurometer® CPT/C (Neurotron, Inc., Baltimore, MD, USA) and the intensity of the 2000, 250 and 5 Hz stimuli were increased to 0.04, 0.02 and 0.01 mA/s, respectively. The current perception threshold was defined as the minimum intensity value required to elicit a withdrawal reflex of the hind paw or appearance of vocalization or agitation. When the response occurred, the stimulus was immediately stopped and the next stimulus began after an interval of at least 10 min. Every threshold of 2000, 250 and 5 Hz was measured 3 or 4 times and the mean value of the intensities was expressed as the current perception threshold.

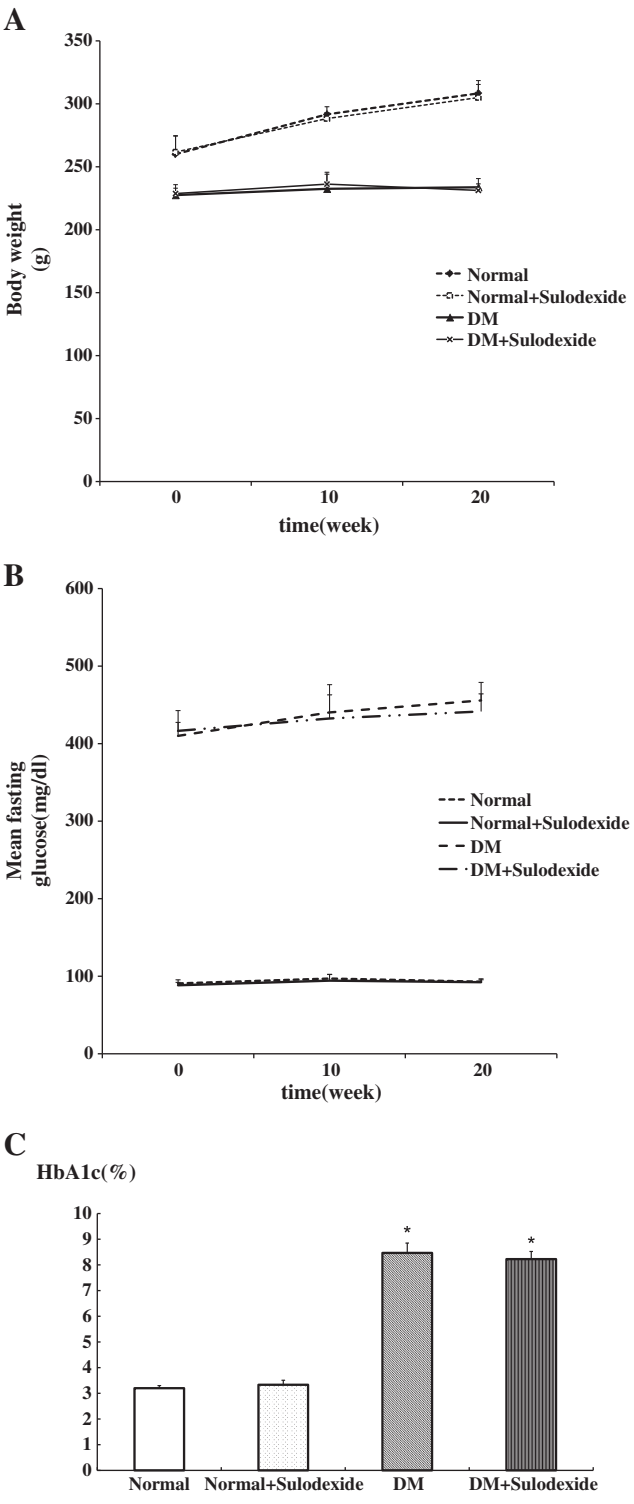

Fig. 1. (A) Body weight changes according to time. Body weight increases were not observed in the diabetes-induced rats, however normal glucose rats gained weight gradually during the experimental period. (B) Fasting blood glucose levels during the experimental period. Sulodexide administration did not result in the lowering of blood glucose in any of the groups. (C) HbA1c levels of the rats in the 20th week. HbA1c levels were also independent of sulodexide treatment in the normal and diabetic group. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

2.5. Skin blood flow measurements

In week 20, dorsum tissue perfusion was evaluated by skin blood flow after treadmill running, as it was difficult to obtain significant

results under resting conditions. The rats were trained for 4 days to run (8–16 m/min) on a motor-driven treadmill. On days 5 to 7, 3 min after treadmill running at an initial speed of 8 m/min, followed by a speed of 16 m/min for 15 min, skin blood flow (tissue perfusion unit: TPU) on the dorsal aspect of the hind leg was measured with a Transonic BLF 21D flow meter (Transonic® Laser Doppler Flow meters, BLF 21 Series, Transonic Systems Inc., Ithaca, NY, USA).

2.6. The measurement of superoxide dismutase activity

In week 20, all rats were anesthetized with intraperitoneal injections of ketamine and xylazine and blood samples were drawn into tubes by cardiac puncture. Serum was collected after centrifugation for 15 min at 2000×*g* at 4 °C. After sacrifice, left sciatic nerve segments were immediately dissected and homogenized in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. The supernatant was obtained after centrifugation at 1.500×*g* at 4 °C for 15 min. Total superoxide dismutase activity in the supernatant and serum were assayed using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of superoxide dismutase activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The protein concentration of the supernatant was quantified by the known Bradford method (Zor and Selinger, 1996).

2.7. Histopathology

Tissue samples (3×3 mm) were taken from the dorsum and toe of the hind leg by a skin biopsy at the 0th and 12th week, and all rats were sacrificed during the 20th week. Segments of the right sciatic nerve were also obtained from each rat after sacrifice. The sciatic nerve tissues were used for morphometric analyses of myelinated fiber and the skin samples were used for immunohistochemical analyses of intraepidermal nerve fiber. Sciatic nerve tissues were post-fixed overnight in 4% paraformaldehyde prior to embedding in JB-4. Then, 1.5 μm transverse sections were stained with toluidine blue. The procedures used for immunohistochemical analysis were the same as those described in a previous report (Jin et al., 2009b). Skin tissue specimens were fixed with periodate-lysine-paraformaldehyde (PLP) (2% paraformaldehyde, 0.075 M lysine, 0.05 M phosphate buffer pH 7.4, 0.01 M sodium m-periodate) solution for 24 h. Tissue specimens were cryoprotected with Tissue-Tec® (OCT compound) (Miles, Elkhart, IN, USA) after thorough rinsing for 48 h in phosphate-buffered saline (PBS) containing 20% glycerol–0.1 M phosphate buffer at 4 °C. Sections of 40 μm in thickness, perpendicular to the dermis, were prepared with a sliding cryostat (Leica CM 1510®, Leica Microsystems AG, Wetzlar, Germany) and were immersed in PBS for 15 min at room temperature. Samples were then transferred into microtubes containing Dako Protein Block Serum-Free® (Dako, Carpinteria, CA, USA) as a blocking buffer supplemented with 3% goat serum. After 30 min of blocking on a shaker table kept at room temperature, sectioned specimens were washed with PBS twice for 10 min and then incubated overnight with the primary antibody, rabbit anti-protein-gene-product 9.5 (PGP 9.5) (Biogenesis, Poole, UK) at a dilution of 1:100 at 4 °C. The antibodies were diluted in antibody diluent (Dako, Carpinteria, CA, USA) supplemented with 1%

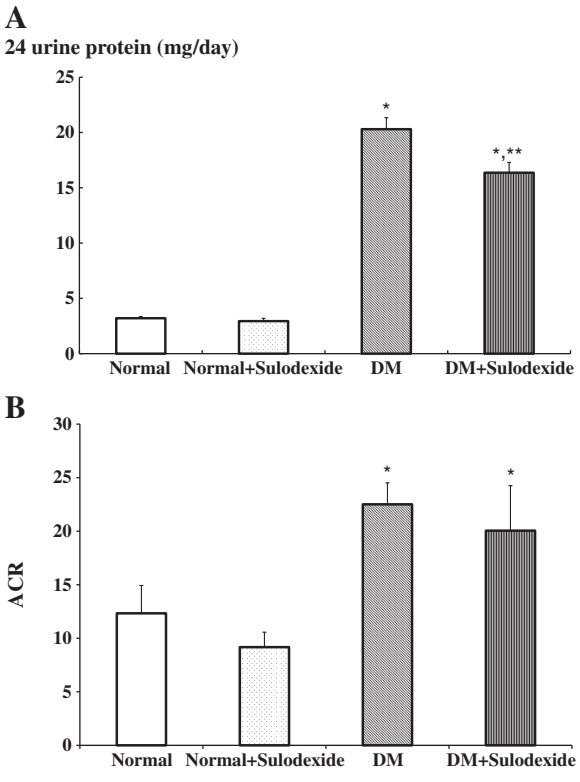

Fig. 2. (A) Twenty-four-hour urinary protein amount and (B) albumin(mg/dl)-to-creatinine (g/dl) ratio (ACR). The 24-hour urinary protein excretion decreased significantly in the diabetic + Sulodexide group compared with diabetic group and ACR was also reduced after sulodexide treatment in each group, although nephropathy was not confirmed in the experimental rats. The value is presented as the mean ± S.D. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

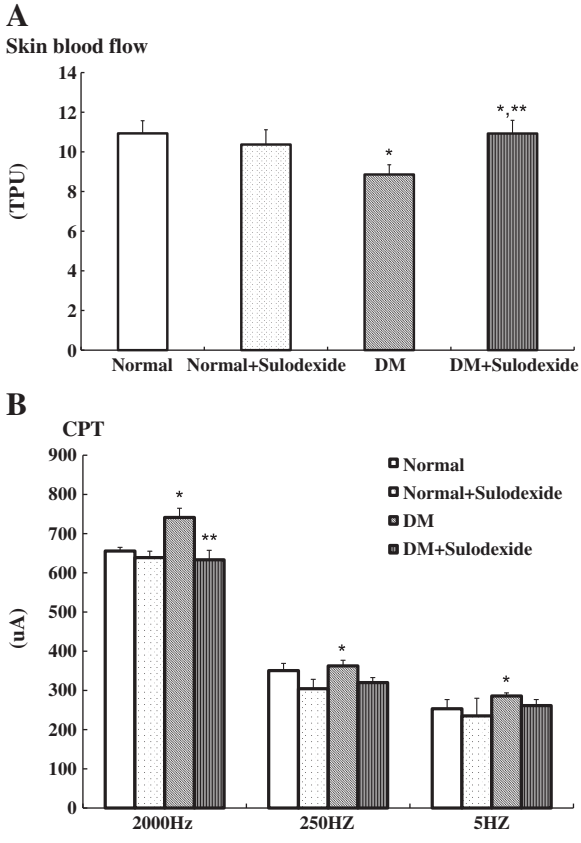

Fig. 3. (A) Skin blood flow values after exercise in each group. The skin blood flow expressed as tissue perfusion unit (TPU) was lower in the DM group than in normal glucose rats, but the value improved significantly, to the range of the normal glucose group, in the diabetic + Sulodexide group. (B) Current perception threshold comparison according to the sulodexide treatment in each group. The thresholds for the different stimuli were higher in the diabetic group than in the normal glucose group and the value was decreased in the diabetic + Sulodexide group at a 2000 Hz stimulus compared with diabetic group. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

goat serum. The relevant secondary antibody, goat anti-rabbit IgG-FITC (1:200, Vector, UK), was loaded for 1 h after complete washing at room temperature in a dark room. After washing with PBS as described above, sections were placed on slides and mounted with a fluorescent mounting media (Dako, Carpinteria, CA, USA).

2.8. Quantitative comparison of myelinated fibers in sciatic nerve and cutaneous intraepidermal nerve fibers

Photomicrographs of the myelinated fiber and intraepidermal nerve fibers were captured using a digital camera (Axiocam HRC®, Carl Zeiss, Goettingen, Germany) with a final magnification of 400 and 100 times, respectively. In the sciatic nerve, the myelinated fiber or axonal area represented by the outer or inner border of the myelin sheath was measured with the aid of analySIS® image software (Soft Imaging Systems GmbH, Munster, Germany) and the axon/fiber area ratio was determined. In addition, the fiber number per mm^2 (fiber density) and the fraction of the endoneurial area occupied by myelinated fibers (fiber occupancy) were also calculated. PGP 9.5-immunoreactive nerve fibers in the epidermis of each section were counted as described previously (Asensio-Pinilla et al., 2009). In cutaneous nerves, each individual nerve fiber with branching points inside the epidermis was counted as one fiber, and the number of intraepidermal nerve fibers per length (fibers/mm) was used to quantify innervation. For epidermal nerve fibers with branching points in the dermis, each individual nerve fiber was counted as a separate fiber. In order to avoid any possible bias during preparation and calculation, two independent investigators were blinded to the experimental groups and the slides were mixed with a set of normal slides before examination.

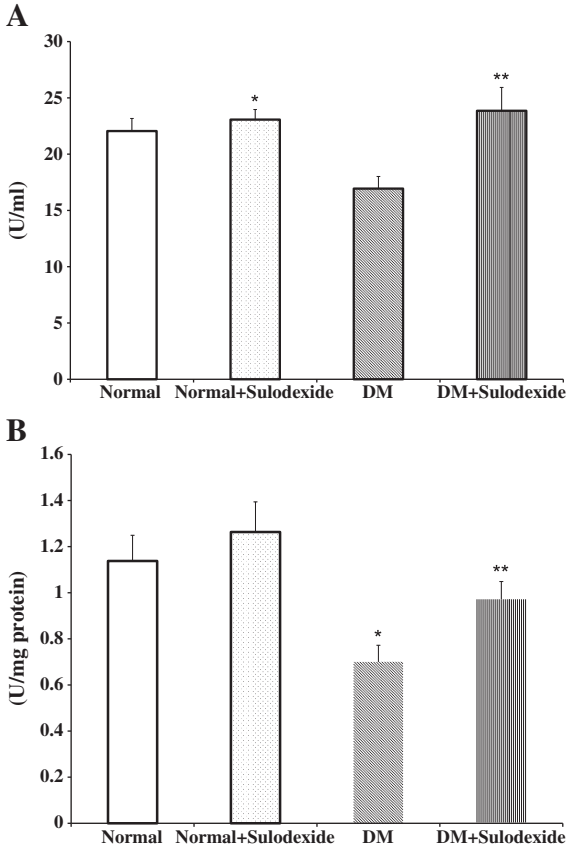

Fig. 4. Superoxide dismutase activity in (A) blood and (B) sciatic nerves of experimental rats. The antioxidative activity-superoxide dismutase activity was significantly recovered in blood and sciatic nerve samples in the diabetic + Sulodexide group compared with the diabetic group. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

2.9. The morphologic comparison of gastric mucosal and renal cortex nerves

The peripheral nerves of organs such as gastric mucosa and renal cortex were also examined to detect any neuro-protective effects of sulodexide. To compare gastric mucosal nerve quantity among

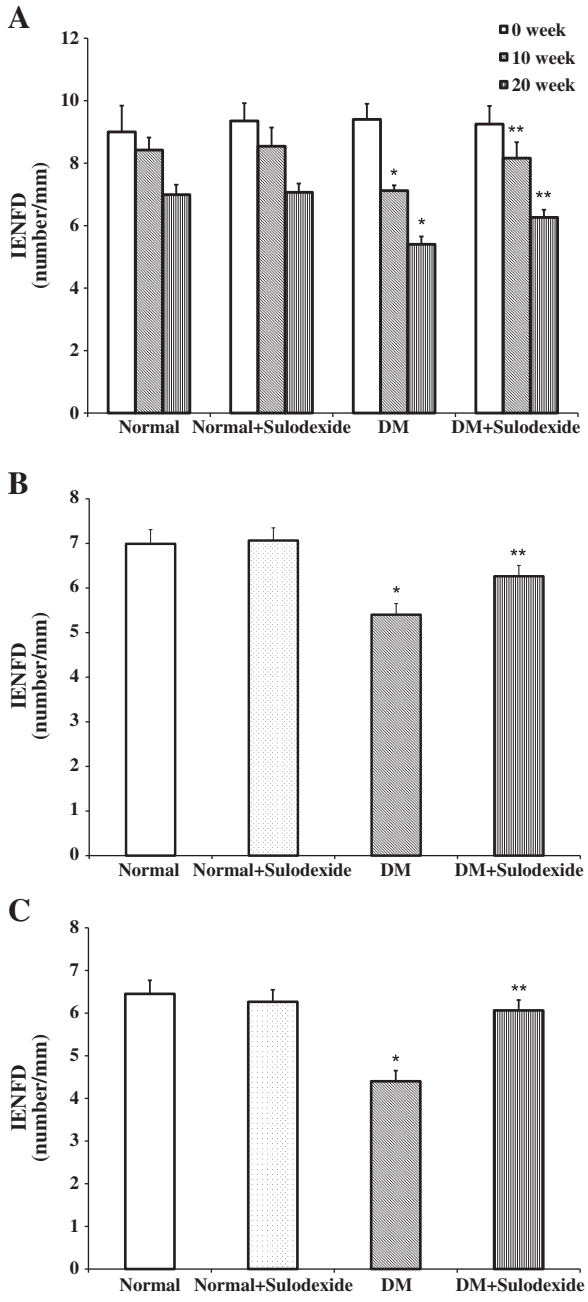

Fig. 5. (A) Intraepidermal nerve fiber density on the dorsum of the foot in experimental rats according to time passage in each group. The decrease in intraepidermal nerve fiber density was blunted significantly in the diabetic + Sulodexide group compared with diabetic group and the degree was similar to that seen in the normal glucose rats. (B) Intraepidermal nerve fiber density for experimental groups at 20th weeks. The density was most reduced in the diabetic group, however it was significantly increased in the diabetic + Sulodexide group compared with diabetic group, although it did not reach the level seen in normal glucose rats. (C) Intraepidermal nerve fiber density of the hind toe of experimental rats. There was a similar trend to that was measured from the dorsum of the foot in each group. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

experimental groups, an arbitrary horizontal line connecting 100 μm or 200 μm distant points from the luminal side was drawn besides morphologic comparison, however, only immunostained nerve fiber patterns were observed in the renal cortex without quantitative comparison among experimental groups.

2.10. Statistical analysis

All data are expressed as the mean ± S.D. One-way ANOVA with Duncan's *post hoc* test was used to compare experimental groups. The power to detect differences was 95% and the data were considered statistically significant if the *P* value was less than 0.05. Statistical analyses were performed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects on body weight and blood glucose level

Body weights increased during the experimental period in the normal glucose group, however weight gain was not observed in the streptozotocin-injected diabetic group with blood glucose levels greater than 350 mg/dl. Body weight was not affected by sulodexide treatment in either group (Fig. 1A). The blood glucose levels in both groups were not significantly changed (Fig. 1B). HbA1c levels were also measured in the 20th week, but significant differences in either normal or diabetic groups were not observed, irrespective of sulodexide treatment (Fig. 1C).

3.2. Effect on urinary protein excretion

The 24 h urinary protein excretion was reduced significantly in the sulodexide-treated diabetic group compared with the non-treated diabetic group (16.4 ± 0.9 vs 20.3 ± 1.0 mg/day, $P<0.05$). However, ACR was not significantly different between groups, although a decreasing trend was observed with sulodexide administration (Fig. 2A, B).

3.3. Effect on skin blood flow and current perception threshold

In week 20, the skin blood flow after treadmill running was increased by 24% in the diabetic + Sulodexide group compared with the diabetic group (10.90 ± 0.67 vs 8.85 ± 0.49 TPU, $P<0.05$). However, there was no difference between the normal and normal + Sulodexide group (Fig. 3A). As shown in Fig. 3B, the current perception thresholds of the diabetic group at all three frequencies were increased compared with those in the normal groups. The thresholds at three frequencies were not markedly changed in the Normal or Normal + Sulodexide group, but the thresholds at 2000 Hz were reduced significantly after sulodexide treatment in the diabetic group ($P<0.05$) (Fig. 3B).

3.4. Effect on superoxide dismutase activity

Superoxide dismutase activities in blood and sciatic nerve samples were significantly higher in the diabetic + Sulodexide group than the diabetic group (Fig. 4A, B) ($P<0.05$). These differences were not observed in the normal glucose group, irrespective of sulodexide treatment. However, superoxide dismutase activity in the diabetic group was significantly lower than the normal group ($P<0.05$) and the diabetic + Sulodexide group showed values of superoxide dismutase activity similar to the levels seen in the normal glucose groups.

3.5. Comparison of intraepidermal nerve fiber density

PGP 9.5-positive nerve fibers evident in both the epidermis and the dermis of the foot were analyzed for intraepidermal nerve fiber density comparison. The intraepidermal nerve fiber density of the

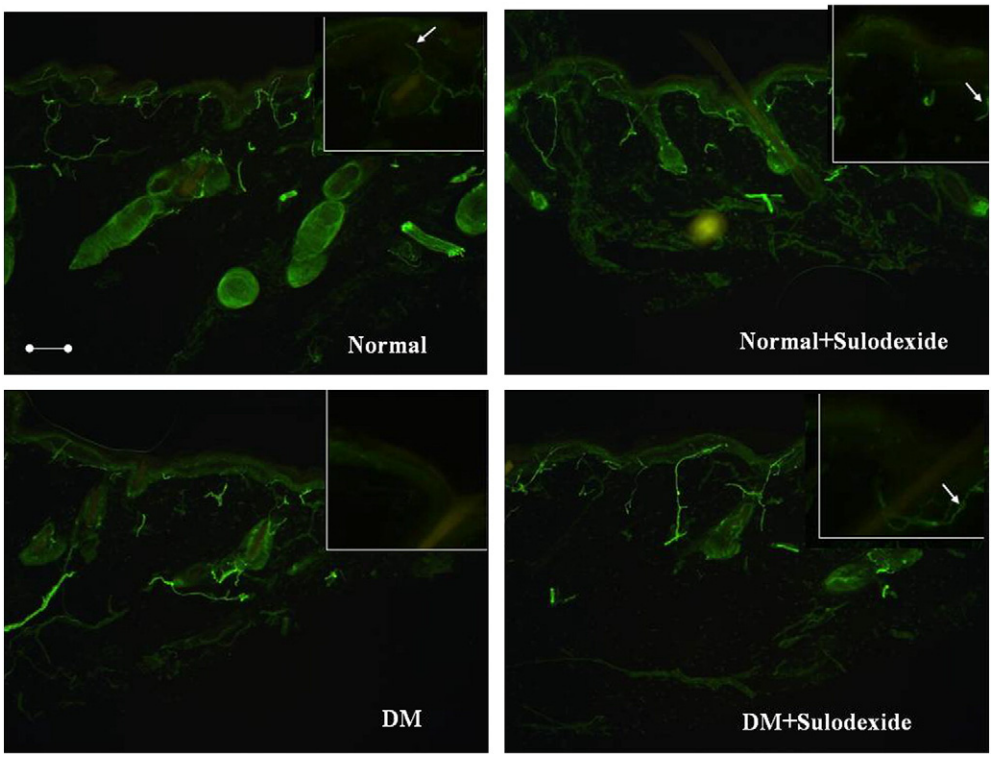

Fig. 6. The morphological finding of intraepidermal nerve fibers of each group at 100× or 200×. The small nerve fibers were more profound and had a less degenerated pattern in the diabetic + Sulodexide group compared with the diabetic. DM: Diabetes. Horizontal bar indicates 100 μm and arrow represents the immunostained small nerve fiber which was used in the intraepidermal nerve fibers count.

dorsum of the foot was similar in all four groups at week 0, and decreased gradually with the passage of time in each group (Fig. 5A). However, at the 20th week, the diabetes group treated with 10 mg/kg sulodexide exhibited significant preservation of nerve fibers in comparison with the non-treated diabetes group, although there were no significant differences between the normal groups (Fig. 5B). This finding was also observed in samples from the hind toes of the rats (Fig. 5C). Fig. 6 shows the morphological pattern of PGP 9.5 positive small nerve fibers extending into the epidermis in each group in which less shortened and less reduced nerve fibers were observed in the diabetic + Sulodexide group compared with the diabetic group.

3.6. Morphological finding of axon and myelin sheath in sciatic nerve

The microscopic analysis of sciatic nerves stained with toluidine blue showed a relative decrease in the number of small- or medium-sized nerve fibers in the diabetic + Sulodexide group compared with diabetic group. The estimated mean fiber area of the diabetic + Sulodexide group was significantly larger than that of the diabetic group (56.6 ± 2.2 vs 49.8 ± 2.7 μm^2, $P<0.05$) and the axon/fiber ratio was also significantly higher in the diabetic + Sulodexide group compared with the diabetic group (40.8 ± 0.9 vs $36.2 \pm 1.0\%$, $P<0.05$) (Fig. 7A, B). However, the estimated total nerve count, including small degenerated nerves, was decreased in the diabetic + Sulodexide group (Fig. 7C). Transverse sections of sciatic nerve samples showed that the endoneurial area appears larger in the diabetic + Sulodexide group compared with diabetic group and nerve fibers with degenerated myelin sheath were decreased pattern in the diabetic + Sulodexide group compared with the diabetic group (Fig. 8).

3.7. Morphologic comparison of gastric mucosal nerves and renal cortex nerves

Immunostained small nerve fibers that passed over an arbitrary line connecting 100 μm or 200 μm points from the luminal side were more abundant in the diabetic + Sulodexide group than the diabetic group (Fig. 9) ($P<0.05$). The immunostained mucosal small nerve fibers exhibited more shortened and more degenerated patterns in the diabetic group than the diabetic + Sulodexide group (Fig. 10). In the renal cortex, immunoreactive nerve fibers with PGP 9.5 were also observed and the renal small nerve fibers immunostained around the glomeruli in the renal cortex were more numerous and exhibited a preserved trend in the diabetic + Sulodexide group compared with the diabetic group; however it was difficult to compare the quantity due to the lack of a standardized method for quantitative estimation of renal small nerve fibers, which are different in shape and passage (Fig. 11).

4. Discussion

Beyond glycemic control, the therapeutic approach to diabetes mellitus must focus on the prevention or reduction of micro- and macrovascular complications through multifactorial interventions including anti-hypertensives, lipid lowering agents, and anti-platelet agents or vasodilators. Among the diverse complications of diabetes, diabetic peripheral neuropathy, which occurs as a result of multiple etiologies and pathogenesis, is a common chronic microvascular complication of diabetes that affects quality of life and causes lower leg problems such as diabetic foot (Gordois et al., 2003). As microcirculation alterations are important pathogenic factors in diabetic peripheral neuropathy (Tesfaye et al., 1994), many pharmacologic agents that improve microcirculation such as pentoxyfylline, gamma-linolenic acid, nitrates, ACE inhibitors, and endothelin antagonists have been tested for treatment of diabetic peripheral neuropathy (Cameron and Cotter, 1993). In this respect, a drug which benefits the microcirculation can be attempted as an additional therapeutic tool for diabetic peripheral neuropathy.

Sulodexide is a highly purified mixture of glycosaminoglycans, composed of heparin-like (80%) and dermatan fractions (20%), which is used for treating thrombotic disorder. Sulodexide has been reported to have a protective effect against endothelial damage and to improve endothelium-dependent relaxation in small arteries (Kristova et al., 2008; Ofosu, 1998). In addition, Marta Ciszewicz et al. (2009) demonstrated that sulodexide has anti-inflammatory action in endothelial cells and sulodexide is protective against glucose cytotoxicity. Other therapeutic applications of sulodexide include the reduction of albuminuria or proteinuria in the diabetic nephropathy model (Blouza et al., 2010; Gambaro et al., 1992; Williams, 2006). Therefore, in the present study, we addressed whether or not the beneficial effects of sulodexide may be observed regarding other diabetic complications such as peripheral neuropathy in a rat model using streptozotocin-induced diabetic rats. In our studies, the

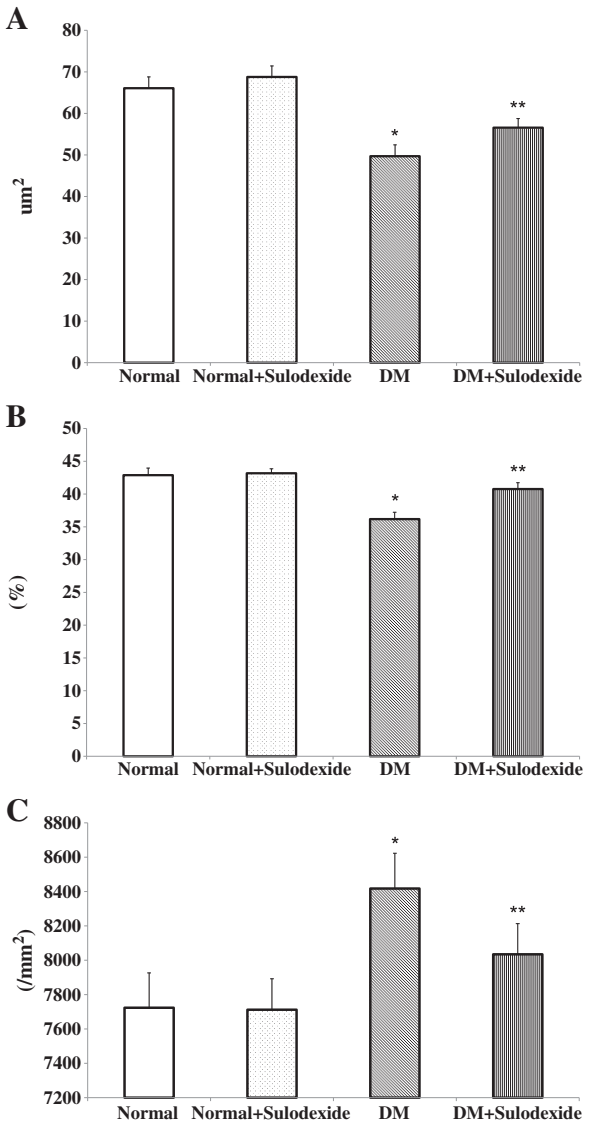

Fig. 7. (A) The average area of small fibers composed of axon and myelin sheaths in the sciatic nerve. The mean area of myelinated small nerve fibers was significantly larger in the diabetic + Sulodexide group compared with the diabetic group. (B) The ratio of axon per total nerve fiber in the sciatic nerve. The occupied area of the axon was increased in the diabetic + Sulodexide group compared with diabetic group. (C) The total estimated number of nerve fibers in the sciatic nerve. The number was highest in the diabetic group due to the smaller size of the nerve fibers. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

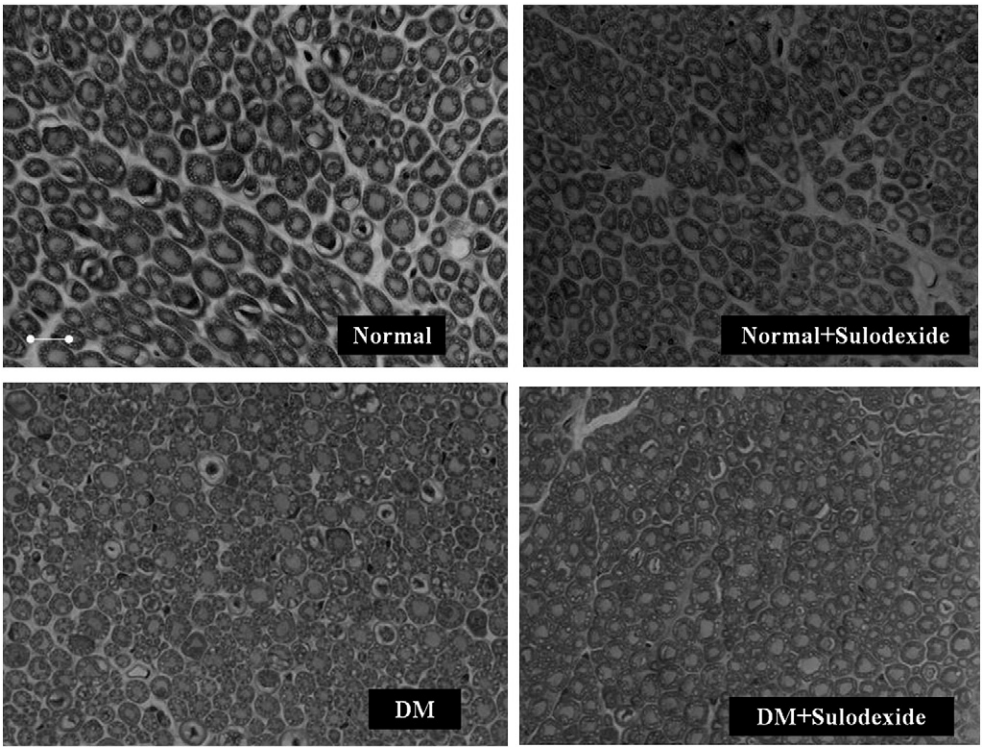

Fig. 8. The transverse section of the sciatic nerve stained with toluidine blue in each group. These photographs showed more degeneration and smaller nerve fibers in the diabetic group, however an improved pattern was observed in the diabetic + Sulodexide group. DM: Diabetes. Horizontal bar indicates 20 μm.

intraepidermal nerve fiber densities gradually decreased in the diabetic condition, and oral sulodexide (10 mg/day/kg) treatment significantly attenuated fiber loss in diabetic rats. Quantifying the density of intraepidermal nerve fibers to assess cutaneous innervation has been considered as a reliable means of both diagnosing and comparing diabetic neuropathy (Beiswenger et al., 2008). An antibody against PGP 9.5 is commonly used for detection of neural elements in immunohistochemical staining of the nerve fiber (Kon et al., 1999).

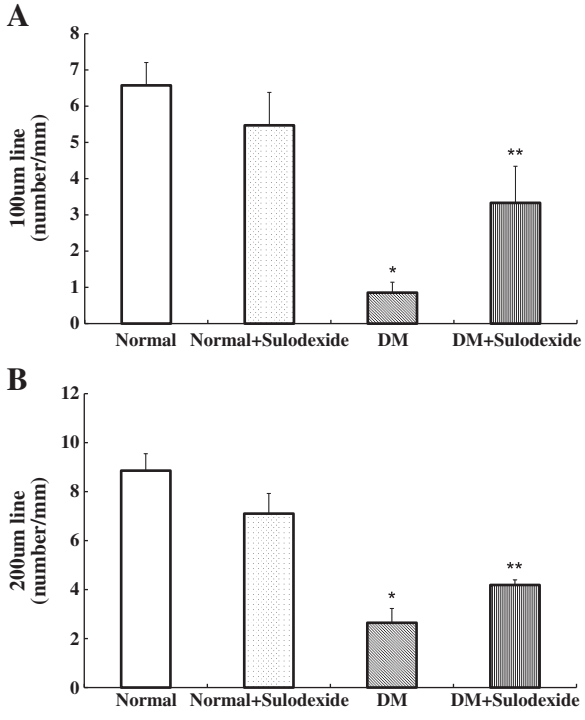

Fig. 9. The average number of gastric mucosal nerve fibers passed over an arbitrary line connecting points located at 100 μm or 200 μm from the luminal side. The nerve count was markedly decreased in the diabetic group, however the number increased in the diabetic + Sulodexide group. DM: Diabetes. N = 7–9 in each group. *: $P<0.05$ DM vs Normal. **: $P<0.05$ DM + Sulodexide vs DM.

In a preliminary study, we detected nerve fiber loss and structural damage with gradual aggravation in the diabetic group from week 8 to 12. The protective effects of sulodexide against decreases in intraepidermal nerve fiber density, as demonstrated in the current study, suggest considerable potential for long-term neuroprotection. It is possible that sulodexide either promotes nerve regeneration or alleviates nerve degeneration. To contextualize this result, peripheral nerves in other tissues were also examined using immunohistochemistry. In sciatic nerves, the average individualized area of small nerve fibers including axon and myelin sheaths was larger in the diabetic + Sulodexide group than in the diabetic group, and the morphological finding, of stained nerve fibers revealed more degenerated patterns in the diabetic group than in the diabetic + Sulodexide group. In the gastric mucosa, small nerve fibers stained with PGP 9.5 were also compared in terms of morphologic findings and quantity. In the diabetic + Sulodexide group, a greater number of small nerve fibers were preserved in the gastric mucosa, compared to the diabetic group, although an arbitrary horizontal line was adapted to assess this parameter. The small nerve fibers in the gastric mucosa were shorter and exhibited more degenerated patterns in the diabetic group than in the diabetic + Sulodexide group. This finding in the diabetic condition is similar to the results of our previous report in which we assessed peripheral nerve damage related to gastric mucosal nerve change (Jin et al., 2009a) and Lin et al. (2008) also reported details regarding this result. In the renal cortex, small nerve fibers were observed using PGP-9.5 and the result showed decreased immunostaining related to the glomerular structure in the diabetic group. This finding was slightly improved in the diabetic + Sulodexide group.

With respect to glucose-lowering, there was no significant difference according to sulodexide treatment in our study in contrast

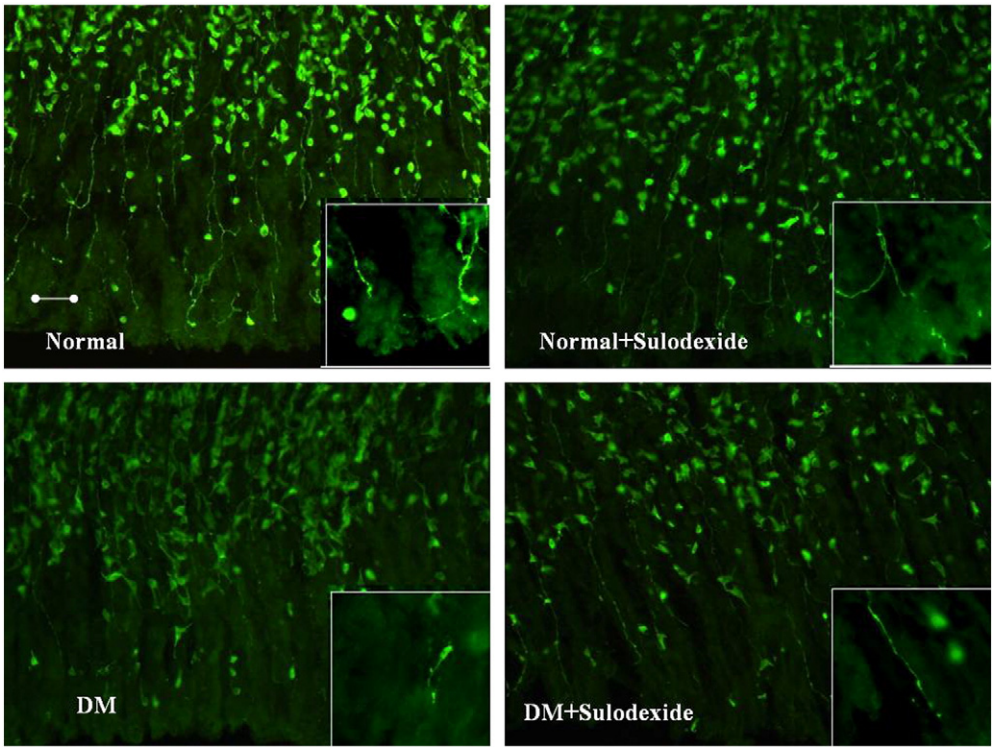

Fig. 10. PGP 9.5 positive small gastric nerve fibers. The nerve fibers were shorter and had a more degenerated pattern in the diabetic group compared to normal group, and were markedly improved in the diabetic + Sulodexide group. DM: Diabetes. Horizontal bar indicates 100 μm.

to a previous report (Kristova et al., 2008). This is due to the complexity of the glucose control mechanism. In the current study, diabetic rats had type 1 diabetes or late-stage type 2 diabetes after administration of 60 mg/kg streptozotocin, which was different from a previous study. In streptozotocin-diabetic rats, glucose lowering is difficult with one medication or one therapeutic modality, and insulin is the only possible therapy for glucose control in this state although the metabolic effect from sulodexide beyond direct glucose control is

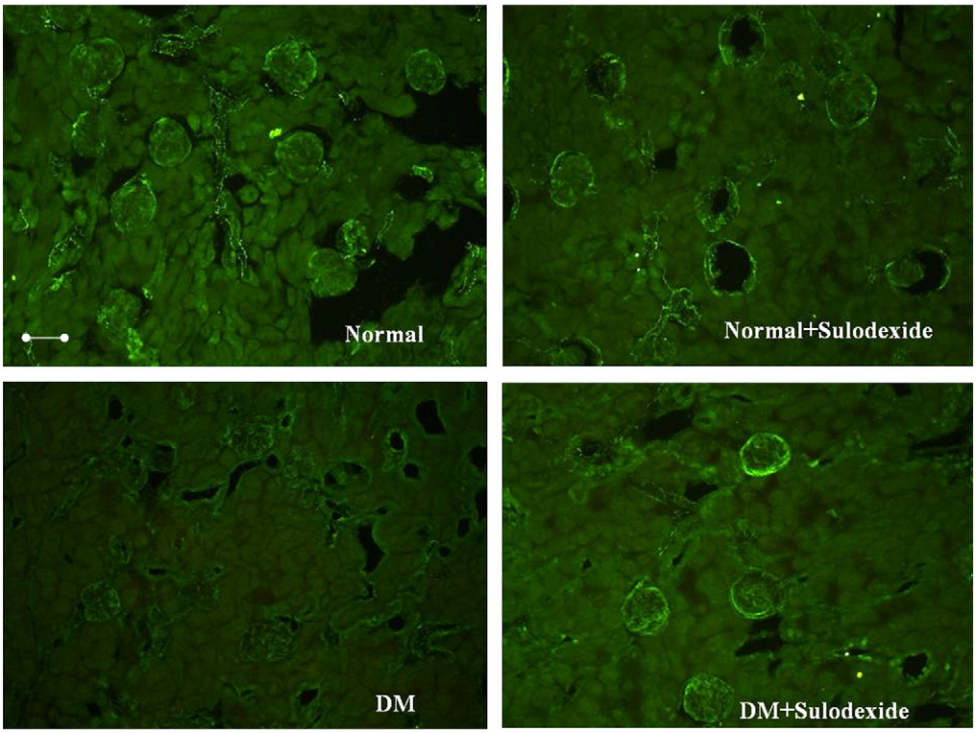

Fig. 11. PGP 9.5 positive small nerve fibers in the renal cortex. The numbers of immunopositive nerve fibers were markedly decreased in the diabetic group compared to normal group, however nerve fibers were detected with improved pattern in the diabetic + Sulodexide group although quantity comparison was not performed. DM: Diabetes. Horizontal bar indicates 100 μm.

expected to occur. Basically, the degree of glucose lowering depends on the insulin secretion capacity, insulin resistance, incretin pathway, and other metabolic or endocrine regulation.

In the current study, although sulodexide did not reduce glucose levels in the blood, the therapeutic potential to prevent or reduce diabetic peripheral nerve damage can be explained by several mechanisms. First, after sulodexide treatment, inflammatory cytokines related to peripheral neuropathy including MCP-1 and IL-6 may be suppressed, and hepatocyte growth factor (HGF) may be increased (Ciszewicz et al., 2009; Gong et al., 2006). Second, the effect on endothelial dysfunction may play a role in the improved microcirculation that was observed in peripheral nerves (Kristova et al., 2008). Third, reversal of glucose toxicity and decrease in oxidative stress are also important aspects of the beneficial effects of sulodexide in experimental diabetes.

Dramatic protective effects of sulodexide in terms of nerve fiber count were not observed in the present study, because diverse multiple factors are usually involved in the progression of peripheral neuropathy. In addition, while any beneficial effects of sulodexide might play a partial role in the prevention of peripheral nerve damage, single corrective interventions cannot completely recover or prevent the progression of complications. Therefore, multifactorial interventions, including glucose control, should be combined to gain maximal preventive outcomes against diabetic peripheral neuropathy.

Harmful oxidative stresses and inflammatory cytokines caused by hyperglycemia in diabetes can be reduced by sulodexide administration, and these effects may be beneficial for the prevention of diabetic complications. Maria et al. (Bilinska et al., 2009) demonstrated the anti-oxidative activity of sulodexide in coronary artery disease. Shu et al. (2009) reported that anti-oxidative stress markers such as superoxide dismutase activity are increased after sulodexide treatment. Similarly, our data also showed similar results in the diabetic + Sulodexide group. In addition, previous studies of sulodexide use in clinical settings have already postulated the beneficial effects of sulodexide against lower leg complications by lowering plasma fibrinogen and viscosity, and decreasing microthrombosis (Corbu et al., 1996; Lunetta and Salanitri, 1992).

In the present study, in addition to assessing morphology, we investigated nerve function by measuring the current perception threshold to assess the degree of neuropathy. A neurometer, which delivers sine-wave stimuli at different frequencies (5250 or 2000 Hz), is widely utilized in clinics to measure perception and pain thresholds. It has been reported that small unmyelinated (C-,0.1–1.5 μm in diameter), small myelinated (Aδ-,2–5 μm in diameter) and large myelinated (Aβ-,5–12 μm in diameter) nerve fibers could be stimulated selectively by 5, 250 and 2000 Hz, respectively (Tay et al., 1997). Different agents, administration methods or doses affected the thresholds at different frequencies (5 or 250 or 2000 Hz) in non-diabetic rats (Kiso et al., 2001). According to our results, additional small C-fibers were not recovered functionally or only protected morphologically. However, we focused the neuroprotective effect of sulodexide in diverse neuronal tissues. Therefore, further research including behavioral tests and sequential current perception threshold measurements showing C-fiber recovery should be performed for application in the treatment of painful diabetic neuropathy. In this experiment, sulodexide improved the threshold of these stimuli, and therefore provided indirect evidence for the beneficial effects of sulodexide on peripheral nerve function.

Our data also demonstrated a reduction in proteinuria, which usually signifies the progression of nephropathy. Proteinuria may be reduced after sulodexide treatment via anion charge reduction in the glomerulus and improvement in micro- and macroangiopathy related to endothelial or mesangial cells (Deckert et al., 1989; Gambaro and van der Woude, 2000). However, it is necessary to further investigate the exact mechanisms and mediators underlying these results. To summarize the limitations, we suggest that neuroprotective effects translating in reduced morphometric and functional damage and potential in the neuroprotection by improvement of the oxidative stress state and an increase in micro and macrovascular blood flow resulted from sulodexide treatment, however, a more exact and detailed mechanism underlying these effects needs to be elucidated in the future. Furthermore, a dose-dependence test is worth performing to detect whether or not there is an increased bleeding tendency. The study to measure the blood flow of a cutaneous peripheral or sciatic nerve directly will be more powerful to support the neuroprotective effect of sulodexide.

In conclusion, we demonstrated the effects of sulodexide on diabetic neuropathy in streptozotocin-induced diabetic rats during a 20-week study with respect to the biochemical, functional, and histological parameters. Sulodexide prevented intraepidermal nerve fiber loss, sciatic nerve damage, and gastric mucosal nerve degeneration and alleviated the increased thresholds in response of sine-wave stimuli at different frequencies. The neuroprotection that we observed may be attributed to alleviated micro- and macrovascular diseases, increased skin blood flow, diminished neuroinflammation and improved oxidative stress state, irrespective of the blood glucose concentration. The results of the present study suggest that sulodexide is a promising novel therapeutic agent for diabetic peripheral neuropathy.

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