The combined effect of mesenchymal stem cells and resveratrol on type 1 diabetic neuropathy

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Abstract. Diabetic neuropathy (DN) is one of the most common diabetic complications that results in an increase in patient discomfort and pain. The present study demonstrated that mesenchymal stem cells (MSCs) or resveratrol (RSV) may improve diabetic hyperglycemia and neuropathy. The aim of the present study was to investigate the combined effect of MSCs and RSV on DN. A total of 100 non-obese diabetic mice were divided into the following six groups: Normal control, MSCs, RSV, MSCs + RSV, insulin and diabetic control groups. Following homologous therapy, the levels of blood glucose and C-peptide, islets, nuclear factor (NF)-κB, nerve growth factor (NGF) and myelin basic protein (MBP), and the sciatic nerve structure in each group were examined and evaluated. Following the administration of therapy, the levels of blood glucose and C-peptide in mice in the MSCs + RSV group were significantly improved when compared with the other diabetic groups, and the dosage of insulin therapy required was the lowest among the six experimental groups ($P<0.05$). The levels of NGF, MBP and NF-κB in the MSCs + RSV group were significantly improved compared with the MSCs and RSV groups ($P<0.05$). Furthermore, the diameter of the axon, number of myelinated nerve fibers and the depth of the myelin sheath in the MSCs + RSV group were greatest among the five examined groups (excluding the control). The combination of RSV and MSCs could relieve hyperglycemia and improve DN. This indicated that the combination of RSV and MSCs may be a novel therapeutic method for the treatment of DN.

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Introduction

The prevalence of diabetes mellitus (DM) is increasing each year. The International Diabetes Federation has estimated that 415 million people have been diagnosed with DM worldwide and anticipates an increase of up to 640 million by 2040 (1). Diabetic neuropathy (DN) is one of the most common diabetic complications and is characterized by complex changes in functional and sensorimotor parameters (2). The pathophysiology of DN involves a complex cascade of specific interrelated mechanisms (3,4). Oxidative stress is an important contributor to the development of DN; this is due to the dramatic increase in the level of free radicals generated in patients with diabetes (5,6).

Resveratrol (RSV), a naturally-occurring polyphenol identified in grapes and red wine, has been demonstrated to exert potent anti-diabetic, anti-oxidative and anti-inflammatory properties (7). RSV was reported to inhibit the apoptosis of pancreatic β-cells and significantly decrease the expression of an inhibitor (nuclear factor-kappa-B-inhibitor alpha) of nuclear factor (NF)-κB and NF-κB p65 in NF-κB signaling (8). In diabetic rats, RSV reduced the levels of malondialdehyde, xanthine oxidase, nitric oxide and DNA fragmentation in the sciatic nerve, and increased glutathione levels in the brain (9). Recent studies have demonstrated that the most beneficial effects of RSV were dependent on sirtuin 1 (SIRT1) activation, through increasing intracellular cyclic adenosine monophosphate (cAMP) and inhibiting cAMP-dependent phosphodiesterase (10,11).

Mesenchymal cells from the umbilical cord possess stem cell-like characteristics, including self-duplication and differentiation (12). Human umbilical mesenchymal stem cells (MSCs) may be induced to differentiate into neuron-like cells *in vitro* (13). Our previous research demonstrated that MSCs could improve hyperglycemia and the number of β-cells in the pancreatic islets of diabetic mice and rats (14-16). Clinical trials have demonstrated that MSCs may improve hyperglycemia in patients with diabetes, reduce the dosage of insulin and oral anti-diabetic drugs, and also decrease the incidence of diabetic complications (17). Furthermore, additional researchers have identified that MSCs may facilitate recovery from spinal cord lesions by releasing brain natriuretic peptide and other vasoactive factors, which reduce edema,

decrease intracranial pressure, and improve cerebral perfusion (18,19). Kim *et al* (20) revealed that the levels of human neurotrophin-3, vascular endothelial growth factor receptor-3 and basic fibroblast growth factor in the stem cell group were higher compared with the PBS group. The mechanism underlying the promotive effect on the regeneration of the injured spinal column following the administration of MSCs likely involved the release of cytokines or growth factors from undifferentiated stem cells, rather than the differentiation of these cells into neuronal or glial cells (20).

Recent findings have demonstrated that RSV may promote the self-renewal and differentiation of MSCs by regulating SIRT1 signaling, which is associated with cell self-renewal, senescence, apoptosis and neural differentiation (21). In addition, RSV may regulate cell growth, osteoblastic differentiation and the expression of osteogenic genes through estrogen receptor/mitogen-activated protein kinase/nitric oxide synthase/cyclic guanosine monophosphate signaling in MSCs cultures (22,23). Previous studies have investigated the therapeutic effects of RSV or MSCs on type 1 (T1)DM and DN, although the combined effect of RSV + MSCs on T1DM and DN, and the mechanism of action, remains unknown at present. The aim of the present study was to investigate the combined therapeutic effect and mechanisms of RSV and MSCs on DN in a mouse model.

Materials and methods

Ethics statement. The present study was approved by the Institutional Animal Ethical Committee (Qingdao, China) and the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China).

MSC preparation. MSCs were prepared from an umbilical cord obtained from a healthy mother (28 years old) at The Affiliated Hospital of Qingdao University on March 10, 2017 and fully informed consent was obtained 2 weeks prior to delivery. The preparation of MSCs was performed in the laminar flow laboratory using a previously described method (19). The MSCs were cultured for 4 passages to prepare final cell products that were sterile and all qualified for examination, including aerobes, mycoplasma, hepatitis B virus, hepatitis C virus, human immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, syphilis and endotoxin testing. Cells were stained with CD-PE and CD-FITC (from the Human MSC Analysis kit; 562245; BD Biosciences, San Jose, CA, USA) and then analyzed using flow cytometry with a FACSCalibur flow cytometer (BD Biosciences). These cells highly expressed cluster of differentiation (CD) 90 (89.37%), CD105 (82.26%), CD73 (90.63%), and CD146 (65%) but not CD34 (0.23%), CD45 (0.02%) and Human Leukocyte Antigen-D Related (0.03%). The differentiation of MSCs was verified by adipocyte and osteogenic differentiation.

Osteogenic and adipogenic differentiation experiments of MSCs were performed as follows: MSCs were seeded in 6-well plates at $1x10^5$ cells/ml per well. When they reached 80% confluence, the cells were induced using osteogenic medium, containing 10 mmol/l β-sodium glycerophosphate (G9422), 50 μg/ml ascorbic acid (BP461) and 10 nmol/l dexamethasone (D4902; each, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in standard medium (containing DMEM/F12, 10% fetal bovine serum (10099-141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 mg/ml streptomycin and 100 U/ml penicillin) and adipogenic medium, including 1 μmol/l hexadecadrol, 10 μmol/l insulin, 200 μmol/l indomethacin, and 0.5 mmol/l isobutyl-methylxanthine (D8893, I2643, I7378 and I5879, Sigma-Aldrich; Merck KGaA) for 3 weeks. Following this, Alizarin Red staining and Oil Red O staining were used to identify the osteogenic and adipogenic potential, respectively. Cells ($1x10^4$) were fixed with 95% ethanol for 10 min at room temperature, washed with double distillated water three times and stained with 0.1% Alizarin Red for 30 min at room temperature. Samples were then washed with double distillated water 3 times and photographed under a light microscope (magnification, x40). For Oil Red O staining, cells were fixed in ice cold 10% for formalin for 10 min, air dried for 40 min at room temperature and then placed in absolute propylene glycol solution (W550132, Sigma-Aldrich; Merck KGaA) for 5 min. Sections were then stained in pre-warmed oil red O solution (O1516, Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and differentiated in 85% propylene glycol solution for 5 min, washed in water for 3 min and mounted with glycerin jelly. All staining results were observed under a light microscope (magnification, x40).

Non-obese diabetic (NOD) mice. A total of 100 female NOD mice aged 6 weeks old, weighing 16-20 g, were purchased from the Nanjing Laboratory Animal Center (Nanjing, China). Mice were fed specific pathogen-free grade mouse chow and water *ad libitum*. Animals were housed at a temperature of 22-23˚C and a humidity of 60%, with a 12 h light/dark cycle. The fasting plasma glucose (FPG) of all mice were examined at day 7 after mice were obtained. Notably, NOD mice can spontaneously develop type 1 diabetes and are useful animal diabetic models (24). Mice with an FPG ≥16.6 mmol/l that occurred twice on different days that was confirmed by pathological examination were considered as diabetic mice. Cells that exhibited a significant decrease in islet β cells or isletitis were considered diabetic for the purposes of the present study.

Experimental grouping and treatment design. Diabetic NOD mice were randomly divided into the following five groups (n=10): A diabetic control group, MSCs group, RSV group, MSCs + RSV group and insulin group. Mice that maintained normal blood glucose levels throughout the experiment were considered as normal controls (n=10). Due to the incidence of diabetes (~80%) and the difference of onset time, 100 mice were utilized to ensure that there were enough diabetic mice and normal controls. Mice in the MSCs group were treated with an intravenous infusion of MSCs (prepared from a healthy mother as aforementioned) by vena caudalis from the third day following a diagnosis of diabetes, and with a cell number of $1x10^6$ MSCs per mouse. Mice in the RSV group were treated with RSV (R5010, Sigma-Aldrich; Merck KGaA) by intragastric administration from the third day post-diagnosis with diabetes, and the dosage of RSV was 200 mg/kg per day administered for a total of 56 days. Mice in the MSCs + RSV group were treated with the aforementioned MSC infusion and RSV (200 mg/kg per day for 56 days). Mice

in the insulin group were treated with insulin glargine (Lantus; sanofi-aventis, Paris, France) 1 day following the diagnosis of diabetes to maintain normal blood glucose levels. Mice in the diabetic control group were used as diabetic controls and did not receive any treatment. At 8 weeks following diagnosis with diabetes, all mice were sacrificed to acquire blood and tissues for analysis. Mice with two consecutive blood glucose values >400 mg/dl were considered as overt failure (25).

Observation and assessment. Any changes in the mice, including activity, food and drink uptake and body weight, were recorded. Tail-vein blood glucose levels were measured biweekly using OneTouch Horizon glucose measurement strips (Johnson and Johnson Ltd., Milpitas, CA, USA) and a glucometer. C-peptide was measured using ELISA (mouse C-peptide Elisa kit; #90050; NeoScientific, Cambridge, MA, USA), according to the manufacturer's protocol. The initial dosage of insulin was based on the individual body weight and blood glucose level of each mouse. Subsequent insulin dosage was adjusted based on the level of blood glucose as well as body weight. Insulin dosage levels were increased by 0.2 U for every 10-15 g increase in body weight. Diabetic mice in the insulin group were treated with daily injections of insulin glargine. For the MSCs, RSV and MSCs + RSV groups, insulin treatment was administrated as described for the insulin group if blood glucose was beyond glycemic control. All insulin administrations were performed using subcutaneous injection, and the dosage of insulin to maintain glycemic control (FPG ≤8 mmol/l, fed blood glucose ≤10 mmol/l) for diabetic mice was recorded.

Histological examination. All mice were sacrificed at 8 weeks following diagnosis with diabetes, and tissues from the pancreas, heart, liver, kidney and sciatic nerve were acquired. Tissue specimens were fixed in 10% paraformaldehyde at room temperature for 24 h and embedded in optimal cutting temperature compound (OCT, Water-soluble mixture of polyethylene glycol and polyvinyl alcohol). Tissue sections (6 μm-thick) were stained with hematoxylin and eosin (H&E) at room temperature for 2 h and were evaluated with light microscopy (magnification, x400).

Immunohistochemistry. Tissue sections were washed with phosphate buffered saline (PBS) for 5 min, deactivated in H_2O_2 (10 min), washed with distilled water 3 times, fixed with EDTA and blocked with 5% bovine serum albumin (B2064; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. Tissue sections were incubated with primary antibodies anti-insulin (#I2018), anti-glucagon (G2654), anti-NF-κB (17-10060), anti-nerve growth factor (NGF; N3279) and anti-myelin basic protein (MBP; AMAB91062; all Sigma-Aldrich; Merck KGaA; all 1:300) at 25˚C for 60 min. Following washing with PBS, sections were incubated with the secondary antibody (M8770; Sigma-Aldrich Merck KGaA; 1:300) at 25˚C for 45 min, stained with 3,3'-diaminobenzidine (Dako REAL EnVision Detection System; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 sec, counterstained with hematoxylin for 12 min at room temperature, dehydrated with a gradient alcohol series and mounted with neutral gum under light microscopy (magnification, x400).

Masson staining. Six odd number sections of each sciatic nerve were stained with Masson's trichrome. Sections were treated with 10% potassium dichromate and 10% trichloroacetic acid for 30 min, nuclei were stained with hematoxylin for 20 min, differentiated with hydrochloric acid and ethanol for 15 sec, returned to blue coloration with weak ammonia for 15 sec, stained with Masson solution (Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 min, rinsed with 1% acetic acid, dehydrated with an increasing ethanol series for 1 min each, cleared with xylene I and II for 10 min to make the sections transparent and finally sealed in resin. All staining steps were performed at room temperature. Following this, the sciatic fiber was observed under a light microscope at a magnification of x400.

Statistical analysis. All statistical analyses were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± standard deviation. Differences between six groups were tested by one-way analysis of variance (ANOVA) and post hoc analysis with Bonferroni correction for multiple comparisons was applied. For parameters where repeated measurements were taken over time (i.e., FPG and dosage of insulin), a two-way repeated-measures ANOVA was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Blood glucose, dosage of insulin and C-peptide. There were no significant differences in FPG, fed blood glucose and body weight of mice among the six groups (n=10, per group) prior to therapy (data not shown). The diagnosis date of diabetic mice was set as day 1 of the experiment. At day 56, there were a total of 10, 7 (3 mice died of hyperglycemia, >33.3 mmol/l), 8 (2 mice died of hyperglycemia, >33.3 mmol/l), 9 (1 mice died of hyperglycemia, >33.3 mmol/l), 9 (1 mouse died of head injury in fighting) and 10 surviving mice in the normal control, diabetic control, insulin, MSCs, RSV and MSCs + RSV groups, respectively. For the 6 mice that died of hyperglycemia, a glucose level of <22.2 mmol/l was determined. In these circumstances, mice received insulin therapy to attempt to improve hyperglycemia. However, the following day glucose levels continued to rise to >33.3 mmol/l, at which point, mice were sacrificed. All mice in the experiment were female and 5 were placed in a single cage. Although mice were monitored every day, an injury occurred in one mouse as aforementioned. The injured mouse was treated by a veterinarian, but due to subsequent hyperglycemia and wound infection, this mouse succumbed. Following the administration of the respective therapies (after Day 3), the body weight of mice in the RSV, insulin and RSV + MSCs groups were increased compared with the diabetic control group ($P=0.032$, $P=0.019$ and $P=0.013$, respectively), as illustrated in Fig. 1.

Compared with the diabetic control group, the blood glucose of mice in the MSCs, RSV (until day 35) and MSCs + RSV groups were decreased following therapy ($P<0.05$). Blood glucose in the MSCs and MSCs + RSV group reached a normal level at day 14, and this was maintained until the end of the experiment; no significant differences were identified in the blood glucose levels between the two groups (MSCs

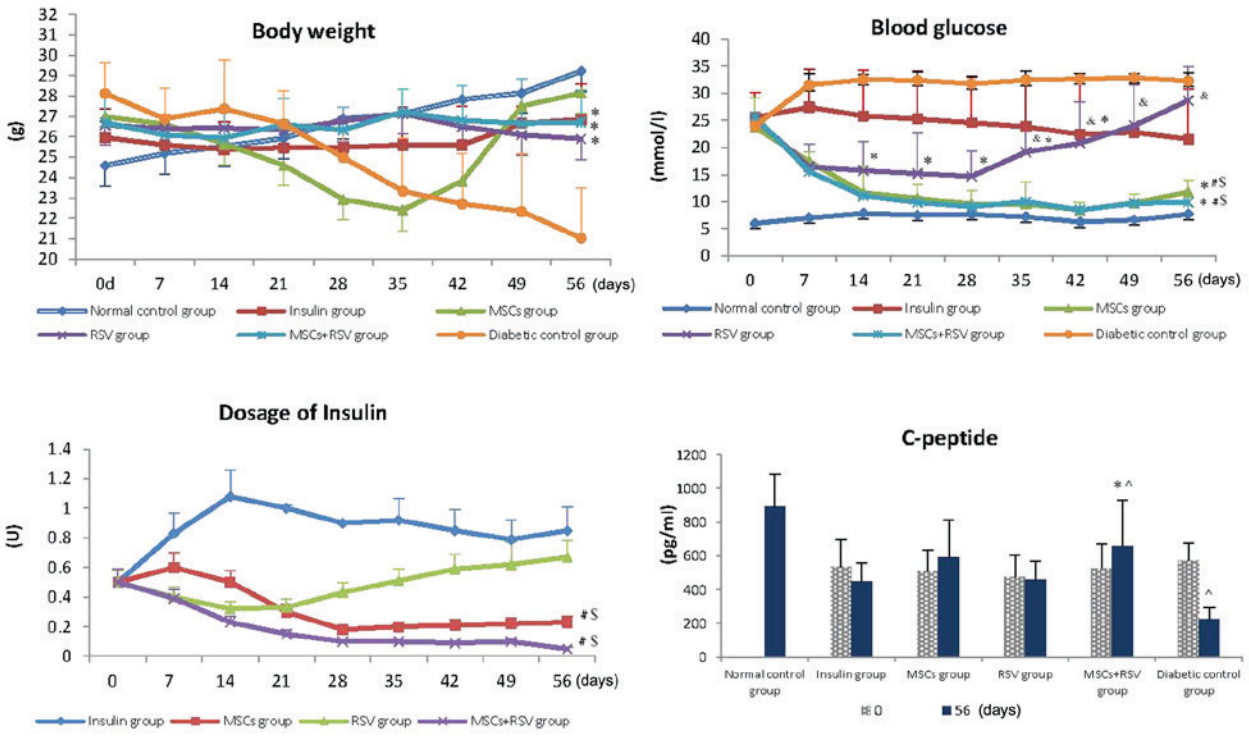

Figure 1. Body weight, blood glucose, dosage of insulin and C-peptide were examined among the six experimental groups. Following the respective administration of therapies, the body weight of mice in the RSV, insulin and RSV+ MSCs groups was increased compared with the diabetic control group. Blood glucose in the MSCs and RSV+ MSCs groups was decreased compared with the diabetic control, insulin and RSV groups following therapy. Furthermore, glucose levels in the MSCs and RSV+ MSCs groups reached a normal level at day 14, which lasted until the end of the experiment. The blood glucose in the RSV group was lower compared with the diabetic control group, yet higher compared with the MSCs group and MSCs + RSV group. The dosage of insulin required in the MSCs group and RSV+ MSCs group was decreased compared with the insulin group and RSV group. Levels of C-peptide in the MSCs + RSV group were improved compared with the diabetic control group, yet remained lower compared with those of the normal control group. *P<0.05 vs. the diabetic control group; $^{\$}$P<0.05 vs. the insulin group; $^{\#}$P<0.05 vs. the RSV group; $^{\&}$P<0.05 vs. the MSCs group and the MSCs + RSV group; $^{\wedge}$P<0.05 vs. the normal control group. MSCs, mesenchymal stem cells; RSV, resveratrol.

group vs. MSCs + RSV group). The blood glucose in the RSV group was lower compared with the diabetic control group, yet higher compared with the MSCs group and MSCs + RSV group (P<0.05), and the lower glucose lasted for a total of 28 days. Subsequently, the blood glucose in the RSV group gradually increased and reached a similar level to the diabetic control group at day 56 (Fig. 1).

Insulin treatment was administered to the mice with blood glucose levels of ≥10 mmol/l. As illustrated in Fig. 1, the dosage of insulin in the MSCs +RSV group decreased from day 7 and the average dosage was 0.15 units during the experiment. The dosage of insulin in the MSCs group decreased from day 14 and the average dosage was 0.27 units. In addition, the dosage of insulin in the RSV group was reduced slightly, followed by an increase until the end of experiment; the average dosage was 0.46 units. The dose of insulin in the insulin group was high, with an average dosage of 0.59 units. The dosages of insulin in the MSCs and MSCs + RSV groups were significantly reduced compared with the insulin and RSV group (P=0.012, P=0.029, P=0.001 and P=0.016, respectively).

The present study also examined the levels of C-peptide in mice on days 0 and 56. Concentrations of the C-peptide were examined when the mouse was diagnosed as diabetic (0 days). Mice that maintained normal blood glucose during the experiment were considered normal controls, so only the concentrations of the C-peptide in the normal control group at day 56 were examined. There were no differences in levels of C-peptide in mice among the insulin, MSCs, RSV, MSCs + RSV and diabetic control groups at the beginning of experiment. However, at day 56, the level of C-peptide in the diabetic control group was significantly decreased compared with the normal control group (P=0.037). The level of C-peptide in the MSCs + RSV group was significantly increased compared with the diabetic control, yet it remained lower compared with normal control group (P=0.041 and P=0.022), as illustrated in Fig. 1.

Histological examination. The sciatic nerves of mice were stained with H&E for light microscopy. As illustrated in Fig. 2, sciatic nerve fibers were degenerated and misarranged with large cracks and a high degree of myelin vacuolization in the diabetic control and insulin groups. In the RSV group, small cracks existed among the sciatic nerve fibers, and visible myelin vacuolization and new capillaries were not observed. In the MSCs group, sciatic nerve fibers were arranged densely and regularly with occasional small cracks, myelin staining was uniform with small air bubbles, and a small number of new capillaries were identified under the microscope. In the MSCs + RSV group, sciatic nerve fibers were densely aligned in a row, the vacuolar degeneration of myelin was decreased and new capillaries were observed under microscope, which were increased compared with those in the MSCs group.

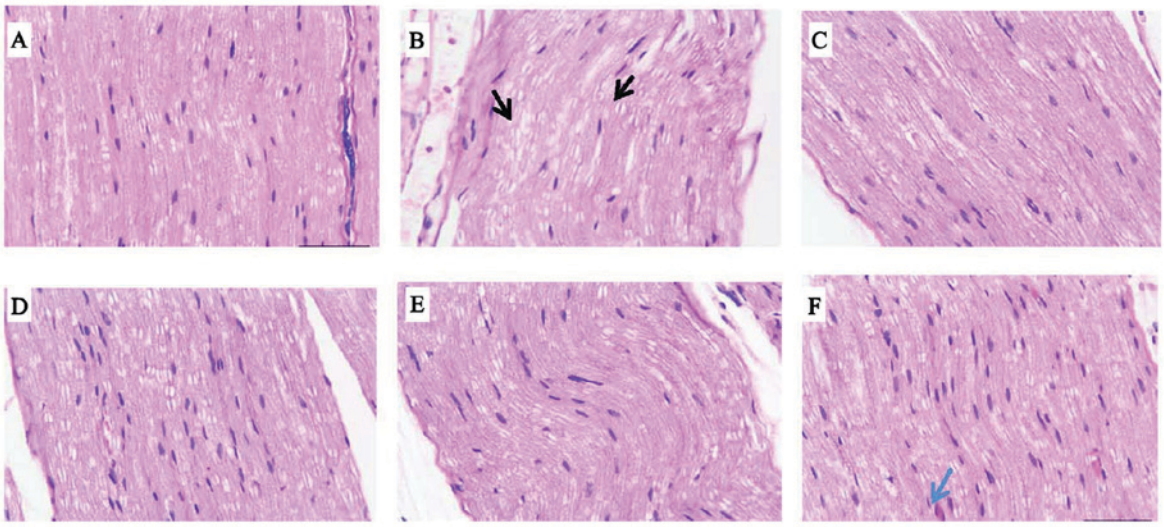

Figure 2. Histological examination of the sciatic nerve with hematoxylin and eosin staining, at a magnification of x40. (A) Normal control group; (B) diabetic control group, with the left arrow indicating that fibers were degenerated and misarranged with large cracks, and the right arrow indicating myelin vacuolization; (C) insulin group; (D) MSCs group; (E) RSV group; and (F) MSCs + RSV group. The blue arrow indicated a new capillary. MSCs, mesenchymal stem cells; RSV, resveratrol.

NF-κB, NGF and MBP. The present study examined the levels of the P56 subunit of NF-κB, NGF and MBP to evaluate the inflammation and recovery of the sciatic nerve on day 56. The level of NF-κB in the diabetic control group was significantly increased, while levels of NGF and MBP were decreased compared with the normal control group ($P<0.05$, Fig. 3A). Notably, levels of NF-κB in the insulin group, RSV, MSCs and MSCs + RSV groups were significantly decreased compared with the diabetic control group ($P=0.041$, $P=0.015$, $P=0.034$ and $P=0.01$, respectively). This indicated that MSCs, insulin and RSV all could improve nerve inflammation in diabetic mice. Following therapy, levels of NGF in the MSCs group and MSCs + RSV group were significantly increased compared with the insulin, RSV and diabetic control groups, yet were significantly decreased compared with the normal control group ($P<0.05$). No statistically significant differences were identified between the diabetic control, insulin and RSV groups. Levels of MBP in the insulin group and diabetic control group were lower than the normal control group, RSV group, MSCs group and MSCs + RSV group, as illustrated in Fig. 3C. No statistical differences were observed between the levels of MBP in the insulin group and the diabetic control group. There were no significant differences in levels of MBP between the normal control group, MSCs group, RSV group and MSCs + RSV group. Taken together, these data suggested that MSCs and RSV relieved inflammation of the sciatic nerve, and that MSCs may secrete NGF to promote recovery in the sciatic nerve.

Masson staining. The sciatic nerve was examined using Masson staining on day 56, as presented in Fig. 4A. Sciatic nerve fibers were sparse and arranged irregularly, with an increased number of visible cracks and uneven axonal myelin staining; the diameters of the axons were markedly different in the diabetic control group compared with the normal control group. Compared with the diabetic control group, sciatic nerve fibers in the insulin, MSCs, RSV and MSCs + RSV groups were improved substantially and arranged regularly, although the myelin staining was marginally inhomogeneous (Fig. 4A). Notably, there were no significant differences in the sciatic nerve between the MSCs + RSV and normal control groups ($P=0.932$).

The diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath (examined under light microscope by two pathologists from the Department of Pathology, the Affiliated Hospital of Qingdao University) in the diabetic control group were decreased compared with the normal control group ($P=0.035$, $P=0.029$ and $P=0.032$ respectively; Fig. 4B). Compared with the diabetic control group, the diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath in the insulin, MSCs, RSV and MSCs + RSV groups were significantly improved ($P<0.05$), most notably in the MSC + RSV group. Furthermore, there were no statistically significant differences in the diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath between the MSCs + RSV group and the normal control group ($P=0.798$, $P=0.836$ and $P=0.147$, respectively; Fig. 4B). Furthermore, myelin sheath depths in the MSCs group and RSV group were significantly improved compared with the insulin group ($P=0.044$ and $P=0.031$). In addition, the axon diameters in the insulin, MSCs and RSV groups were significantly shorter when compared with the normal control group ($P=0.035$, $P=0.041$ and $P=0.026$ respectively; Fig. 4B).

Side effects. There were no symptoms of rejection or toxic effects observed by macroscopic observation, laboratory or histological examination among the six experimental groups.

Discussion

Previous studies revealed that MSCs or RSV may improve hyperglycemia in patients with diabetes; however, there are few reports regarding the combined effect and mechanisms of MSCs + RSV on DN. The present study investigated the

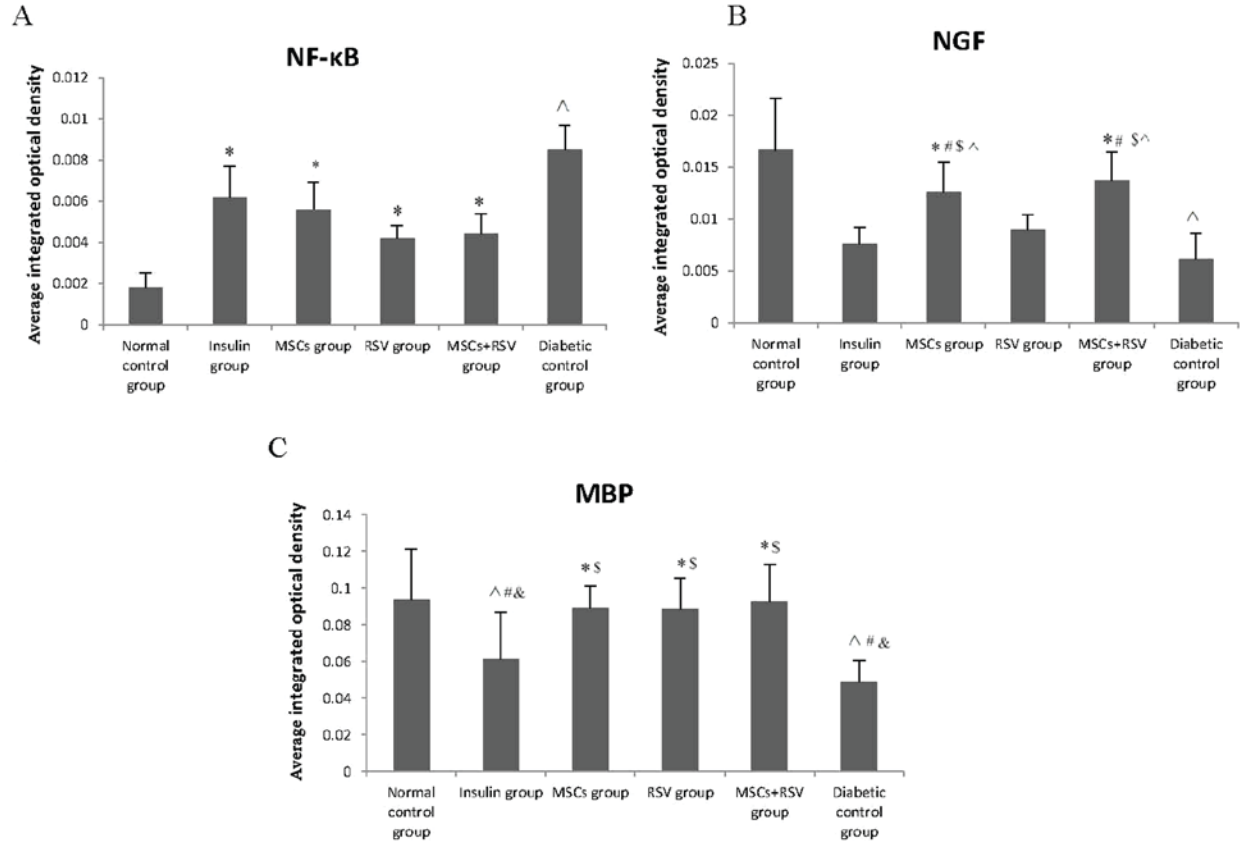

Figure 3. Levels of NF-κB, NGF and MBP in the sciatic nerve, assessed by immunohistochemistry. The level of NF-κB in the diabetic control group was markedly increased, while levels of NGF and MBP were decreased compared with the normal control group). (A) Levels of NF-κB in the insulin, RSV, MSCs and MSCs + RSV group were notably decreased compared with diabetic control group, particularly in the RSV group and MSCs + RSV group. (B) Levels of NGF in the MSC group and MSCs + RSV group were higher compared with the insulin, RSV and diabetic control groups, yet were significantly decreased compared with the normal control group. (C) Levels of MBP in the MSCs group, RSV group and MSCs + RSV group were higher compared with the insulin and diabetic control groups. Levels of MBP in the insulin and diabetic control groups were decreased compared with the other four groups. $^{\wedge}$P<0.05 vs. the normal control group; $^{\$}$P<0.05 vs. the insulin group; $^{\#}$P<0.05 vs. the RSV group; *P<0.05 vs. the diabetic control group; $^{\&}$P<0.05 vs. the MSCs group and the MSCs + RSV group. MSCs, mesenchymal stem cells; RSV, resveratrol; NGF, nerve growth factor; MBP, myelin basic protein; NF-κB, nuclear factor-κB.

therapeutic effect of MSCs + RSV on DN, and demonstrated that levels of blood glucose and C-peptide in mice in the MSCs + RSV group were significantly improved compared with the other experimental groups, and the dosage of insulin required was the lowest among the five diabetic groups.

In our previous research, MSCs were demonstrated to increase the number of islet β-cells due to a paracrine effect; however, this effect did not extend to cell differentiation. Furthermore, MSCs were revealed to regulate regulatory T cells, $CD4^+$ T and $CD8^+$ T cells in diabetic mice to decrease immune attacks on islet cells (15,16). In the present study, RSV was demonstrated to reduce hyperglycemia; however, this reduction was more evident following treatment with MSCs. An increasing amount of evidence has demonstrated that oxidative stress and inflammatory activity serve a role in the pathogenesis of diabetes (26,27). RSV may regulate tissue function by inducing insulin receptor substrate (IRS)-1 and IRS-2 phosphorylation to induce phosphatidylinositol-4,5-bisphosphate 3-kinase and Akt phosphorylation, and increasing endothelial nitric oxide synthase phosphorylation (28,29). RSV may improve SIRT1 gene and protein expression, which is suppressed in DM, thereby regulating Forkhead box O_3 deacetylation *in vitro* and *in vivo* (29,30).

The present study also identified that the levels of NGF in the MSCs and MSCs + RSV groups were significantly increased compared with the RSV, insulin and diabetic control groups. The levels of MBP in the RSV, MSCs and MSCs + RSV groups were increased compared with the insulin and diabetic control groups. In addition, the levels of NF-κB in the RSV and MSCs + RSV groups were significantly decreased compared with the MSCs, insulin and diabetic control groups. These data suggested that RSV may promote the recovery of the sciatic nerve in diabetic mice by regulating inflammation, while MSCs may increase the levels of neurotrophic factors and promote the recovery of the sciatic nerve.

NGF is a small secreted protein that is important for the growth, maintenance and survival of sympathetic and sensory neurons (31). Reduced or absent expression of NGF may lead to cholinergic degeneration and cognitive impairment in rats, which indicates that NGF may serve as a therapeutic agent to protect or restore axonal branching and elongation (32-34). Growing evidence has suggested that numerous growth factors have dual neurotrophic and angiogenic effects, including vascular endothelial growth factor, insulin-like growth factor, NGF, brain derived neurotrophic factor and fibroblast growth factor-2, which are deficient in DN (35,36). It has been demonstrated that human MSCs are able to

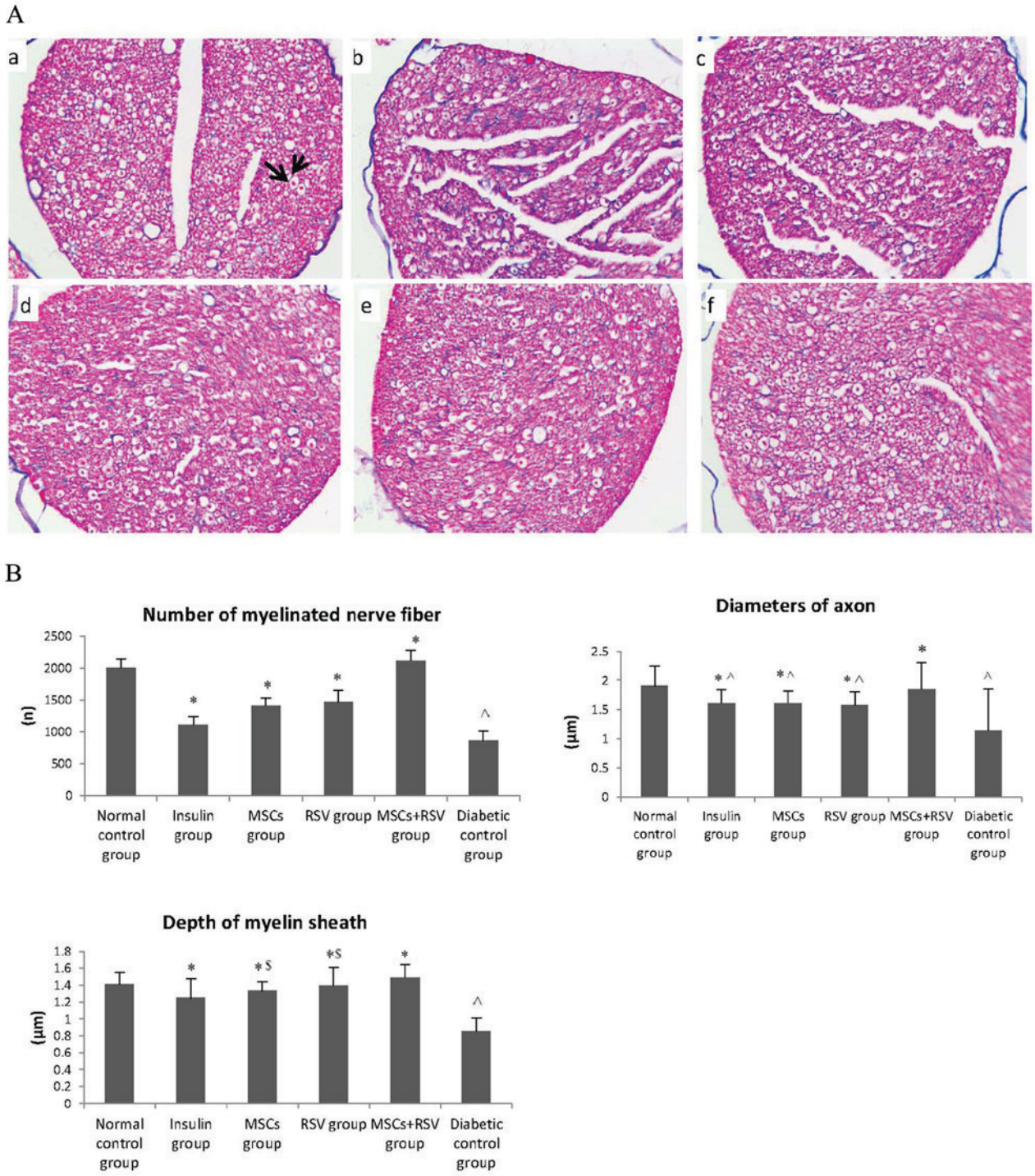

Figure 4. Diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath in the sciatic nerve, assessed by Masson staining. (A) Sciatic nerve, assessed by Masson staining. (a) Normal control group, where the arrows illustrate the axon and myelin sheath; (b) diabetic control group; (c) insulin group; (d) MSCs group; (e) RSV group; and (f) MSCs + RSV group. (B) The diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath in the diabetic control group were decreased compared with the normal control group. Compared with the diabetic control group, the diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath in the insulin, MSCs, RSV group and MSCs + RSV group were improved, particularly in the MSCs + RSV group. The depth of the myelin sheath in the MSCs and RSV groups was improved compared with the insulin group. The axon diameters in the insulin, MSCs and RSV groups were shorter when compared with the normal control group. $^{\wedge}$P<0.05 vs. the normal control group; *P<0.05 vs. the diabetic control group; $^{\$}$P<0.05 vs. the insulin group. MSCs, mesenchymal stem cells; RSV, resveratrol.

produce >84 trophic factors in conditioned medium and cell lysates (20). Treatment with MSCs partially restored nerve function in the sciatic nerve via the secretion of vascular endothelial growth factor, glial cell-derived neurotrophic factor and NGF and modulation of the immune response, rather than engraftment and differentiation of MSCs to the injured site (20,36).

MBP is an important protein synthesized by Schwann cells, which serves a key role in myelination. Decreased expression of the MBP has been observed in the sciatic nerve

of diabetic rats (37). Lower expression levels of MBP signifies the decreased myelination of nerve fibers (38). IN a previous study, following treatment with RSV/MSCs in diabetic mice, an improvement in the expression of myelin proteins in the sciatic nerve was observed (39). Additionally, in the present study, the diameter of the axon, number of myelinated nerve fibers and depth of the myelin sheath in the insulin, MSCs, RSV and MSCs + RSV groups were improved. Furthermore, the therapeutic effect in the MSCs + RSV group was the greatest, and there were no statistically significant differences between the MSCs + RSV group and the normal control group.

In conclusion, the present study investigated the combined effect of RSV and MSCs on type 1 DN. A combination of RSV and MSCs was revealed to relieve hyperglycemia and improve DN by paracrine mechanisms or immune regulation, and the combined administration of RSV and MSCs led to the most marked improvement in DN. This indicated that combination of RSV and MSCs may be a novel therapeutic method of treating T1DM and DN. However, the specific mechanisms of action of RSV and MSCs in DN require further clarification; further investigations should focus on the possible mechanisms of action and potential side effects, due to the complex nature and pathophysiology of DN. Furthermore, the present study did not trace and check the MSC cells *in vivo*. Subsequently, in further research MSC cells will be investigated *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW wrote the manuscript and directed/performed the experiments. JC performed MSC culture and blood examination. KC, XM and MQ performed the animal experiments and the histological examination of the sciatic nerves. ZW and YW designed the experiments, revised and proved the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments comply with the current laws of the country in which they were performed. The present study was approved by the Institutional Animal Ethical Committee (Qingdao, China) and the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China). Informed consent was obtained for the present study.

Patient consent for publication

The patient who donated her umbillical cord provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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