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# Protective effect of *Jiaweibugan* decoction against diabetic peripheral neuropathy<sup>☆</sup>

Yu Wang<sup>1</sup>, Zeqi Chen<sup>1</sup>, Renqun Ye<sup>2</sup>, Yulei He<sup>1</sup>, Yuhong Li<sup>1</sup>, Xinjian Qiu<sup>1</sup>

1 Institute of Integrated Traditional Chinese and Western Medicine, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China

2 Department of Internal Medicine, Chinese Medical Hospital in Bao'an District of Shenzhen City, Shenzhen 518133, Guangdong Province, China

## Abstract

Oxygen free radical damage is regarded as a direct or indirect common pathway associated with diabetic neuropathy and is the main cause of complications in peripheral neuropathies. We speculate that *Jiaweibugan* decoction has a significant effect in treating diabetic peripheral neuropathy through an anti-oxidative stress pathway. In this study, a diabetic rat model was established by intraperitoneal injection of streptozotocin. Rats were treated with *Jiaweibugan* decoction via intragastric administration. The levels of malondialdehyde and glutathione, which are indirect indexes of oxidative stress, in serum were determined using a colorimetric method. The expression levels of nuclear factor kappa B p65 mRNA and p38 mitogen-activated protein kinase, which are oxidative stress associated factors, in the dorsal root ganglion of spinal S<sub>4-6</sub> segments were evaluated by reverse-transcriptase polymerase chain reaction and immunohistochemistry. Results showed that, *Jiaweibugan* decoction significantly ameliorated motor nerve conduction velocity in diabetic rats, effectively decreased malondialdehyde levels in serum and the expression of nuclear factor kappa B p65 mRNA and p38 mitogen-activated protein kinase mRNA in the dorsal root ganglion, and increased glutathione levels in serum. Therefore, our experimental findings indicate that *Jiaweibugan* decoction plays an anti-oxidative stress role in the diabetic peripheral neuropathy process, which has a protective effect on peripheral nerve injury.

Yu Wang<sup>☆</sup>, Studying for doctorate.

Corresponding author: Zeqi Chen, Chief physician, Professor, Doctoral supervisor, Institute of Integrated Traditional Chinese and Western Medicine, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China, czq4471680@yahoo.com.cn.

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## Key Words

neural regeneration; traditional Chinese medicine; peripheral nerve injury; *Jiaweibugan* decoction; diabetic peripheral neuropathy; malondialdehyde; glutathione; nuclear factor kappa B p65; p38 mitogen-activated protein kinase; oxidative stress; taurine; streptozotocin; oxygen free radical damage; grants-supported paper; neuroregeneration

## Research Highlights

- (1) *Jiaweibugan* decoction can effectively improve the clinical symptoms of patients with diabetic peripheral nerve injury; however, the protective mechanisms remain unclear.
- (2) *Jiaweibugan* decoction can markedly improve sciatic motor nerve conduction velocity in diabetic rats, decrease serum levels of malondialdehyde and the expression of nuclear factor kappa B p65 and p38 mitogen-activated protein kinase in the dorsal root ganglion, and increase serum levels of glutathione.
- (3) Oxidative stress contributes to the process of diabetic peripheral neuropathy. In this study, we show that *Jiaweibugan* decoction can reduce oxidative stress levels in a rat model of diabetic peripheral neuropathy.

## INTRODUCTION

Diabetic peripheral neuropathy is one of the most common chronic complications in diabetes mellitus. Nearly 60% of diabetic patients suffer from peripheral neuropathy<sup>[1]</sup>, with the incidence increasing with disease progression<sup>[2]</sup>. The pathogenesis of diabetic peripheral neuropathy is due to many factors, both metabolic and vascular. Among them, the oxidative stress theory is thought to be highly involved, which integrates with four other major pathways of glucose metabolism in the diabetic state. These include: 1) increased polyol pathway activity; 2) advanced glycation end-products accumulation; 3) activation of protein kinase C; 4) increased hexosamine pathway flux<sup>[3]</sup>. Therefore, adjustment of oxidation-reduction processes may provide a new method for diabetic peripheral neuropathy treatment. *Jiaweibugan* decoction has a significant effect on alleviating the clinical symptoms of diabetic peripheral neuropathy, such as limb numbness, pain and muscle weakness<sup>[4-5]</sup>. In a streptozotocin rat model of diabetic peripheral neuropathy, *Jiaweibugan* decoction also reduces the structural and functional impairment to the sciatic nerve<sup>[6-7]</sup>. Malondialdehyde and glutathione, oxidative stress biomarkers, are important in the pathogenesis of diabetic peripheral neuropathy<sup>[8]</sup>. In addition, nuclear factor kappa B and p38 mitogen-activated protein kinase pathways are candidate stress-sensitive signaling systems that can chronically lead to the complications of diabetes<sup>[9]</sup>. To further investigate the mechanisms of *Jiaweibugan* decoction, we chose taurine ( $\beta$ -aminoethanesulfonic acid) as a positive control drug, which functions as an important endogenous antioxidant<sup>[10-11]</sup> and attenuates nerve oxidative stress<sup>[12-13]</sup>. This study aimed to observe the effects of *Jiaweibugan* decoction in a rat model of diabetic peripheral neuropathy by monitoring the expression levels of malondialdehyde and glutathione in serum, and nuclear factor kappa B p65 and p38 mitogen-activated protein kinase in the dorsal root ganglion, in a broader attempt to further understand the protective mechanism of *Jiaweibugan* decoction and provide evidence for the clinical treatment of diabetic peripheral neuropathy.

## RESULTS

### Quantitative analysis of experimental animals

Eighty male Wistar rats were equally and randomly divided into four groups: normal group (normal feeding),

model group (diabetic model), *Jiaweibugan* decoction group (diabetic model + *Jiaweibugan* decoction via intragastric administration), and taurine group (diabetic model + taurine via intragastric administration). Both normal and model groups were treated with physiological saline. All 80 rats were included in the final analysis, with no loss.

### *Jiaweibugan* decoction ameliorated the decrease of sciatic motor nerve conduction velocity in streptozotocin-induced diabetic rats

Compared with normal group, the sciatic motor nerve conduction velocity of rats in the model group decreased significantly at 4 and 8 weeks after streptozotocin-induced modeling ( $P < 0.05$ ), and this reduction increased with diabetes progression ( $P < 0.05$ ). Motor nerve conduction velocity in diabetic rats treated with *Jiaweibugan* decoction or taurine increased markedly compared with model rats ( $P < 0.05$ ), and the effect between the two treatment groups showed no significant difference ( $P > 0.05$ ). This evidence indicated that *Jiaweibugan* decoction could distinctly ameliorate the sciatic nerve conduction velocity decrease in streptozotocin-induced diabetic rats (Table 1).

Table 1 Effect of *Jiaweibugan* decoction on the sciatic motor nerve conduction velocity (m/s) of streptozotocin-induced diabetic rats

Group	Time after modeling (week)	
	4	8
Normal	30.9±3.6	30.4±4.4 <sup>b</sup>
Model	20.6±3.2 <sup>a</sup>	17.0±2.5 <sup>ac</sup>
<i>Jiaweibugan</i> decoction	25.8±3.4 <sup>ab</sup>	23.1±4.1 <sup>ab</sup>
Taurine	24.5±2.3 <sup>ab</sup>	22.5±3.6 <sup>ab</sup>

Data are expressed as mean  $\pm$  SD,  $n = 10$ . <sup>a</sup> $P < 0.05$ , vs. normal group; <sup>b</sup> $P < 0.05$ , vs. model group; <sup>c</sup> $P < 0.05$ , vs. the same group at the former time point (one-way analysis of variance and least significant difference method).

### *Jiaweibugan* decoction decreased serum levels of malondialdehyde and increased serum levels of glutathione in streptozotocin-induced diabetic rats

The serum levels of malondialdehyde in the model group at 4 and 8 weeks after modeling were markedly higher than those in the normal group, while the serum levels of glutathione were lower ( $P < 0.05$ ). However, the serum levels of malondialdehyde in the *Jiaweibugan* decoction group or taurine group were significantly decreased compared with the model group ( $P < 0.05$ ). There was no significant difference between the *Jiaweibugan* decoction and taurine groups ( $P > 0.05$ ). This evidence indicates that *Jiaweibugan* decoction could decrease the serum

levels of malondialdehyde and increase the serum levels of glutathione in streptozotocin-induced diabetic rats, and thereby play a role on anti-oxidative stress (Table 2).

Table 2 Effect of *Jiaweibugan* decoction on serum levels of malondialdehyde (nmol/mL) and glutathione (mg/L) in streptozotocin-induced diabetic rats

Group	Malondialdehyde		Glutathione	
	4 weeks	8 weeks	4 weeks	8 weeks
Normal	10.1±1.6	10.6±1.8	589.9±36.0	575.6±51.1
Model	16.3±1.7 <sup>a</sup>	19.8±1.6 <sup>ac</sup>	440.2±14.6 <sup>a</sup>	423.2±23.0 <sup>ac</sup>
<i>Jiaweibugan</i> decoction	13.0±2.8 <sup>ab</sup>	13.9±2.0 <sup>ab</sup>	494.3±53.8 <sup>ab</sup>	520.1±83.0 <sup>ab</sup>
Taurine	13.1±1.7 <sup>ab</sup>	14.8±2.0 <sup>ab</sup>	493.0±33.3 <sup>ab</sup>	500.0±39.5 <sup>ab</sup>

Data are expressed as mean ± SD,  $n = 10$ . <sup>a</sup> $P < 0.05$ , vs. normal group; <sup>b</sup> $P < 0.05$ , vs. model group; <sup>c</sup> $P < 0.05$ , vs. the same group at the former time point (one-way analysis of variance and least significant difference test method).

### *Jiaweibugan* decoction decreased the expression of nuclear factor kappa B p65 mRNA in the dorsal root ganglion (S<sub>4-6</sub>) of streptozotocin-induced diabetic rats

Nuclear factor kappa B p65 mRNA expression in the dorsal root ganglion of rats in the model group was markedly higher than that in the normal group at 4 and 8 weeks ( $P < 0.05$ ), while levels in diabetic rats treated with *Jiaweibugan* decoction or taurine were significantly lower than those in non-treated model rats ( $P < 0.05$ ). There was no significant difference between the two treatment groups ( $P > 0.05$ ). This evidence indicates that, *Jiaweibugan* decoction can down-regulate nuclear factor kappa B p65 mRNA expression in the dorsal root ganglion of diabetic rats with peripheral neuropathy (Table 3, Figures 1, 2).

Table 3 Effect of *Jiaweibugan* decoction on nuclear factor kappa B p65 mRNA expression in the dorsal root ganglion (S<sub>4-6</sub>) of streptozotocin-induced diabetic rats

Group	Time after modeling (week)	
	4	8
Normal	0.9±0.0 <sup>b</sup>	0.9±0.0 <sup>b</sup>
Model	1.3±0.1 <sup>a</sup>	1.3±0.0 <sup>ac</sup>
<i>Jiaweibugan</i> decoction	1.1±0.1 <sup>ab</sup>	1.0±0.0 <sup>ab</sup>
Taurine	1.1±0.1 <sup>ab</sup>	1.0±0.0 <sup>ab</sup>

Expression of nuclear factor kappa B p65 mRNA is expressed as the absorbance ratio of nuclear factor kappa B p65 mRNA/ $\beta$ -actin mRNA.

Data from four independent groups are expressed as mean ± SD,  $n = 10$ . <sup>a</sup> $P < 0.05$ , vs. normal group; <sup>b</sup> $P < 0.05$ , vs. model group; <sup>c</sup> $P < 0.05$ , vs. the same group at the former time point (one-way analysis of variance and least significant difference method).

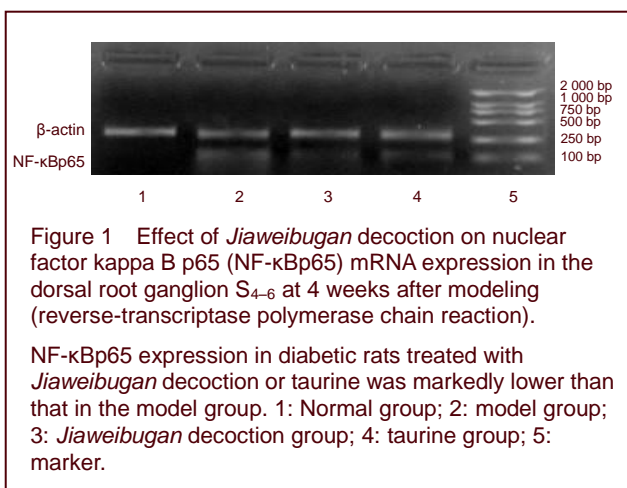


Figure 1 Effect of *Jiaweibugan* decoction on nuclear factor kappa B p65 (NF-κBp65) mRNA expression in the dorsal root ganglion S<sub>4-6</sub> at 4 weeks after modeling (reverse-transcriptase polymerase chain reaction).

NF-κBp65 expression in diabetic rats treated with *Jiaweibugan* decoction or taurine was markedly lower than that in the model group. 1: Normal group; 2: model group; 3: *Jiaweibugan* decoction group; 4: taurine group; 5: marker.

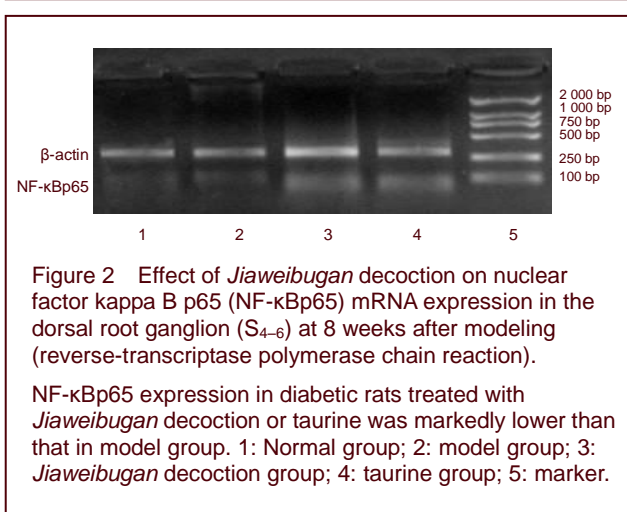


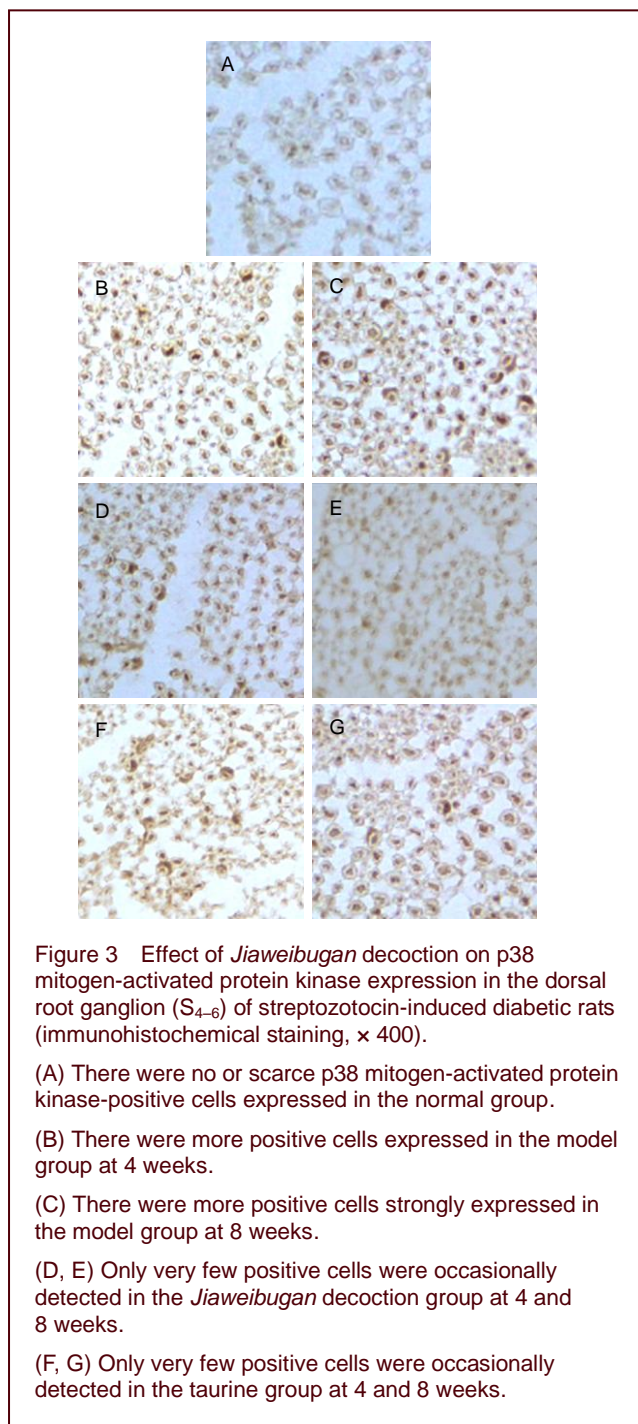
Figure 2 Effect of *Jiaweibugan* decoction on nuclear factor kappa B p65 (NF-κBp65) mRNA expression in the dorsal root ganglion (S<sub>4-6</sub>) at 8 weeks after modeling (reverse-transcriptase polymerase chain reaction).

NF-κBp65 expression in diabetic rats treated with *Jiaweibugan* decoction or taurine was markedly lower than that in model group. 1: Normal group; 2: model group; 3: *Jiaweibugan* decoction group; 4: taurine group; 5: marker.

### *Jiaweibugan* decoction decreased the expression of p38 mitogen-activated protein kinase in the dorsal root ganglion (S<sub>4-6</sub>) of streptozotocin-induced diabetic rats

Immunohistochemical positive cells for p38 mitogen-activated protein kinase were largely located in the cytoplasm and scarcely expressed in the normal group, while more positive cells were expressed in the model, *Jiaweibugan* decoction, and taurine groups. Greater expression and deeper staining were observed in the model group (Figure 3). The gray value of immunopositive cells for p38 mitogen-activated protein kinase in the model group was significantly lower than that in the normal group ( $P < 0.05$ ). This result was evidence that the immunopositive product was highly expressed compared with normal group. In addition, the gray value of immunopositive cells for p38 mitogen-activated protein kinase increased after *Jiaweibugan* decoction or taurine treatment ( $P < 0.05$ ), which indicated low levels of expression compared with the normal group. A high gray value indicated a low quantity of protein expression. On the contrary, a low gray value illustrated a high quantity of protein

expression. These findings infer that *Jiaweibugan* decoction can decrease the expression of p38 mitogen-activated protein kinase in the dorsal root ganglion of diabetic rats with peripheral neuropathy (Table 4).



## DISCUSSION

Among the variety of pathological mechanisms underlying diabetic peripheral neuropathy, oxidative

stress is attracting much attention<sup>[14]</sup>. Oxygen free radical damage is the main contributor to peripheral neuropathy complications and is regarded as the direct or indirect common pathway in which diabetic neuropathy progresses<sup>[15]</sup>. In diabetes mellitus, cell lipid peroxidation increases *via* increases in oxygen free radicals through direct or indirect mechanisms<sup>[16]</sup>. Malondialdehyde is seen as the terminal product of lipid peroxidation, of which levels can reflect the degree of lipid peroxide reactions. In addition, nucleic and lipid cross-linking can induce membrane degeneration and cell mutation, aging or dying<sup>[17]</sup>. These events result in impairments to protein, DNA structure and function, decreases in Na<sup>+</sup>-K<sup>+</sup>-ATPases activity, and destruction of neurotrophic factors, which alters axoplasmic transport, leading to abnormal structure and dysfunction of neural cells<sup>[3, 18-19]</sup>.

Table 4 Effect of *Jiaweibugan* decoction on the gray value of immunopositive products of p38 mitogen-activated protein kinase in the dorsal root ganglion of streptozotocin-induced diabetic rats

Group	Time after modeling (week)	
	4	8
Normal	161.2±10.5 <sup>b</sup>	155.3±7.9 <sup>b</sup>
Model	127.3±9.0 <sup>a</sup>	124.1±9.4 <sup>ac</sup>
<i>Jiaweibugan</i> decoction	140.6±11.3 <sup>ab</sup>	138.7±15.0 <sup>ab</sup>
Taurine	141.3±9.2 <sup>ab</sup>	140.1±9.2 <sup>ab</sup>

The gray value of immunopositive product of p38 mitogen-activated protein kinase from four independent groups are expressed as mean ± SD, *n* = 10. The higher gray value indicates fewer immunopositive products of p38 mitogen-activated protein kinase. <sup>a</sup>*P* < 0.05, vs. normal group; <sup>b</sup>*P* < 0.05, vs. model group; <sup>c</sup>*P* < 0.05, vs. the same group at the former time point (one-way analysis of variance and least significant difference method).

Under conditions of hyperglycemia, the glutathione levels decrease after polyol pathway activation. However, glutathione is a very important free radical scavenging agent *in vivo*, which can effectively remove free radicals produced by biological oxidation and can also restore free radicals directly or promote superoxide dismutase synthesis<sup>[20-22]</sup>. Therefore, polyol pathway activation weakens the antioxidant ability of cells. In diabetes, oxygen free radical formation augments direct or indirect mechanisms leading to an increase in cell lipid peroxidation. Levels of malondialdehyde and glutathione in blood or tissue both can indirectly reflect the degree of oxidative stress. In this study, serum levels of malondialdehyde were significantly increased at 4 and 8 weeks after modeling, while serum levels of glutathione decreased. This result was consistent with the results of Obrosova *et al*<sup>[9]</sup>. Oxygen free radical production increased and the ability of antioxidant defense was

weakened, which confirmed that diabetes could cause the generation of oxidative stress.

Nuclear factor kappa B is a family of five transcription factors that form homodimers and heterodimers known to control the expression of cytokines and other immune-response genes<sup>[23-25]</sup>. At present, nuclear factor kappa B has been shown to play a key role in the morbidity of diabetic peripheral neuropathy by advanced glycosylation end-products and oxidative stress<sup>[26-27]</sup>. This study illustrated that the high expression of nuclear factor kappa B in the dorsal root ganglion is associated with the oxidative stress response generated in experimental diabetic rats with peripheral neuropathy, and that *Jiaweibugan* decoction can decrease this response and play an anti-oxidative role. Taken together, this function may be one of the neuroprotective mechanisms underlying *Jiaweibugan* decoction in treating diabetic peripheral neuropathy.

The c-Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase are members of the complex superfamily of mitogen-activated protein serine/threonine protein kinases<sup>[28-29]</sup>. They are known as stress-activated kinases. This can be attributed to the fact that the activities of these enzymes are stimulated by a variety of exogenous and endogenous stress-inducing stimuli including hyperglycemia, reactive oxygen species, oxidative stress, osmotic stress, proinflammatory cytokines, heat shock, and ultraviolet irradiation. Chronic activation of the p38 mitogen-activated protein kinase pathway is often associated with disease pathology, including inflammation, ischemia/reperfusion injury, infectious diseases, and neurodegenerative diseases<sup>[30]</sup>. Mitochondrial oxygen radicals, which are powerful activators of the p38 mitogen-activated protein kinase pathway, destroy neurotrophic factors as they increase and effect axoplasmic transport, which induces abnormal expression of gene products and the neuronal apoptosis during peripheral neuropathy. Once mitochondrial oxygen radicals activate the p38 mitogen-activated protein kinase pathway, p38 in the cytoplasm transfers to the nucleus and boosts gene transcription and expression<sup>[1, 3, 31]</sup>. In the glomeruli of streptozotocin-induced diabetic rats, p38 mitogen-activated protein kinase activity is increased compared with controls, followed by an increase in heat shock protein 25 phosphorylation, a downstream substrate of p38 mitogen-activated protein kinase<sup>[32]</sup>. These effects appear to be the result of increased reactive oxygen species production. Excessive levels of reactive oxygen species are known to not only directly

damage cells by oxidizing DNA, protein and lipids, but also indirectly damage cells by activating a variety of stress-sensitive intracellular signaling pathways such as nuclear factor kappa B, p38 mitogen-activated protein kinase and c-Jun N-terminal kinase/Akt<sup>[33-34]</sup>.

The phosphorylation of p38 mitogen-activated protein kinase and p53 has been widely used to monitor oxidative stress<sup>[35]</sup>. Growing evidence indicates that extracellular signal-regulated kinase and p38 mitogen-activated protein kinase signals are implicated in manipulating nuclear factor kappa B and its downstream targets as a response to curcumin in human multiple myeloma cells<sup>[36]</sup>. Moreover, nuclear factor kappa B is involved in apoptosis mediated by p53<sup>[37]</sup>. In our study, p38 mitogen-activated protein kinase did not or was scarcely expressed in the dorsal root ganglion of the normal group, and the gray values of immunopositive products were very high. The level of antigen was low at normal physiological states and the p38 mitogen-activated protein kinase signal pathway was not abnormally activated. In addition, oxygen free radicals and lipid peroxidation were not excessive and the lipid peroxide response was low. However, p38 mitogen-activated protein kinase was highly expressed in the model group, which suggested that oxygen free radicals generated and formed excessively in the dorsal root ganglion and that marked cell lipid peroxidation occurred, with an increase in oxygen free radicals in mitochondria and abnormal activation of the p38 mitogen-activated protein kinase signaling pathway. These data were in accordance with the presumption that the etiology of diabetic neuropathy was induced *via* the direct effect of p38 mitogen-activated protein kinase signaling pathway activation<sup>[38]</sup>. The success of the streptozotocin-induced diabetic peripheral neuropathy model was determined upon a reduction of sciatic motor nerve conduction velocity. This study indicated that elevated malondialdehyde, nuclear factor kappa B p65 and p38 mitogen-activated protein kinase, and reduced glutathione expression occurs during oxidative stress and plays an important role in the progression of diabetic peripheral neuropathy.

Oxidative stress occurs throughout the progression of diabetes mellitus and *Lycium barbarum* polysaccharide has been shown to decrease blood sugar levels, triglyceride and cholesterol levels in rats or mice and reduce the incidence of diabetic complications *via* its antioxidant role<sup>[39-41]</sup>. Total glucosides of peony are active ingredients extracted from the White Peony root, mainly including peoniflorin, hydroxy-peoniflorin, Peonin, and

Albiflorin. These active ingredients have anti-inflammatory and anti-oxidative effects, and modulate the immune response. Total glucosides of peony can prevent renal damage associated with diabetes mellitus by attenuating oxidative stress and play a protective role in diabetic neuropathy<sup>[42-43]</sup>. In addition, angelica sinensis CO<sub>2</sub> supercritical fluid extraction may be a potential antioxidant that plays a major role in eliminating oxygen free radicals, which has a protective effect against oxidative stress injury in human umbilical vein endothelial cells<sup>[44]</sup>. After *Jiaweibugan* decoction treatment for 8 weeks, as compared with the model group, *Jiaweibugan* decoction effectively decreased serum levels of malondialdehyde and the expression of nuclear factor kappa B p65 and p38 mitogen-activated protein kinase in the dorsal root ganglion, and increased serum levels of glutathione. Moreover, *Jiaweibugan* decoction distinctly ameliorated sciatic nerve conduction velocity decreases in streptozotocin-induced diabetic rats. Therefore, we conclude *Jiaweibugan* decoction has antioxidant properties that can reverse the oxidative stress by regulating malondialdehyde, glutathione, nuclear factor kappa B p65 and p38 mitogen-activated protein kinase expression.

## MATERIALS AND METHODS

### Design

A randomized, controlled, animal experiment.

### Time and setting

Experiments were performed at the Institute of Integrated Traditional Chinese and Western Medicine, Xiangya Hospital, Central South University, China, from July 2007 to June 2008.

### Materials

#### Animals

Eighty male Wistar rats, aged 8 weeks, weighing 200–250 g, were purchased from Shanghai Experimental Animal Center of Chinese Academy of Science (certificate No. SCXK (Hu) 2002-0010). All experimental protocols were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animal*, issued by the Ministry of Science and Technology of China<sup>[45]</sup>.

#### Drugs

*Jiaweibugan* decoction was mainly composed of *Lycium barbarum* 15 g, *Fructus Chaenomelis lagenariae* 15 g, *Angelica sinensis* 10 g, *Rhizoma Chuanxiong* 10 g,

*Prepared Rehmannia* root 12 g, *White Peony* root 15 g, *Parasitic Lanthus* 15 g, *Radix Ophiopogon* 15 g and *Radix Trichosanthis* 15 g. The total weight of all drugs was 122 g. All drugs were immersed in 1 200 mL water for 30 minutes, warmed on low heat twice for 30 minutes, and merged. The mass concentration of crude drug was 2 g/mL, and was frozen for future use as an oral liquid. All drugs were purchased from the Department of Pharmacy, Xiangya Hospital of Central South University, China. Taurine (powder) was purchased from Shanghai Boao Biotechnology Co., Ltd., China (batch No. 040508).

### Methods

#### Establishment of diabetic rats by intraperitoneal injection of streptozotocin

Sixty rats were fasted for 12 hours with free access to food before diabetic model establishment. Experimental diabetes mellitus was produced by intraperitoneal injection of streptozotocin in 0.05 M citrate buffer (pH 4.2–4.5; 65 mg/mL; dose 1.32 mL/kg). The rats were accepted as diabetic when their fasting blood glucose exceeded 16.7 mM for 3 days after streptozotocin injection and remained 16.7 mM at the time when they were killed<sup>[46]</sup>. The remaining 20 rats (normal group) received an intraperitoneal injection of 0.05 M citrate buffer after 12-hour fasting.

#### Drug administration

After the diabetes mellitus model was established successfully (fasting blood glucose exceeding 16.7 mM for 3 days after injection of streptozotocin), *Jiaweibugan* decoction was given to rats in the *Jiaweibugan* decoction group at a dosage of 28.6 g/kg body weight per day for 8 weeks using a stomach tube. This oral dosage was converted from an adult (weighing 70 kg) dosage of *Jiaweibugan* decoction according to the Experimental Methodology of Traditional Chinese Medicine Pharmacology<sup>[47]</sup>. Taurine was given to rats in the taurine group at a dosage of 0.3 g/kg body weight per day<sup>[48]</sup> by intragastric administration. Rats in the normal group and model group were treated with physiological saline.

#### Motor nerve conduction velocity measured using the MS92 electromyogram device

Motor nerve conduction velocity was determined using a noninvasive procedure in the sciatic-posterior tibial conducting system in a temperature controlled environment<sup>[49-50]</sup>. All rats were anesthetized with chloral hydrate and placed on a thermostatically controlled heated mat to maintain the body temperature at 37°C. Limb temperature was monitored with an electronic

thermometer (PC-9400 Delta; Sato Keiryoki MFG, Tokyo, Japan) and maintained at 37°C with the aid of a warmed blanket. The left sciatic nerve was electrically stimulated at the Achilles tendon using a general evoked response stimulator (MS92 electromyogram device; Medelec, London, UK) and then at the sciatic notch, and the waves were recorded from the second interosseus muscle of the foot. Motor nerve conduction velocity was calculated by subtracting the distal from the proximal latency measured in milliseconds from the stimulus artifact of the evoked potential, and the difference was divided into the distance between the two stimulating electrodes measured in millimeters using a vernier caliper. The motor nerve conduction velocity was reported in meters per second.

### Sample processing

At 4 and 8 weeks after modeling, rats were anesthetized by injecting 10% (v/v) chloral hydrate (350 mg/kg) into the abdomen. Rats were bled through cardiac puncture and the separated serum was centrifuged and stored overnight at -20°C. The left lumbar spine dorsal root ganglions (S<sub>4-6</sub>) of rats in each group were taken and soaked in liquid nitrogen and then stored at -80°C for mRNA determination. The right lumbar spine dorsal root ganglions (S<sub>4-6</sub>) of rats in each group were fixed for 24 hours, conventionally dehydrated, embedded in paraffin and serially sectioned for immunohistochemistry experiments.

### Determination of serum levels of malondialdehyde and glutathione using a colorimetric method

The levels of malondialdehyde in serum were detected using the thiobarbituric acid-reactive substance assay. The experiment was carried out with a commercial kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China) according to the manufacturer's specifications. Malondialdehyde values were expressed as nmol/mL. Reduced glutathione levels in serum were determined as previously described<sup>[51]</sup>. This method depends on the reaction of reduced glutathione with 5,5-dithiobis,2-(nitrobenzoic acid) that can be measured colorimetrically. The yellow color developed was measured at 412 nm against a blank reagent.

### Expression of nuclear factor kappa B p65 mRNA in the dorsal root ganglion (S<sub>4-6</sub>) detected by reverse transcription-PCR

Nuclear factor kappa B p65 mRNA primers and  $\beta$ -actin primers were designed and synthesized by Shanghai Boya Biotechnology Co., Ltd., China.

Gene	Sequence (5'-3')	Product size (bp)
Nuclear factor kappa B p65	Upstream: TCC GTT ACA AGT GCG AGG Downstream: TCC CGT GTA GCC ATT GAT	104
$\beta$ -actin	Upstream: TCA CCC ACA CTG TG CCATCT ATG A Downstream: CAT CGG AAC CGC TCA TTG CCG ATA G	296

Total RNA was isolated from the dorsal root ganglion using Trizol reagent. The concentration and purity of the total RNA samples were detected with an ultraviolet spectrophotometer. A volume of 20  $\mu$ L of total RNA was used for reverse transcription-PCR with the superscript preamplification system (Promega, Madison, WI, USA). The PCR conditions were pre-denaturation at 94°C for 7 minutes, denaturation at 94°C for 45 seconds, annealing at 48.1°C for 45 seconds, extension at 72°C for 45 seconds, followed by 36 cycles, and with a final extension at 72°C for 10 minutes. PCR products (5  $\mu$ L) were analyzed by electrophoresis on a 1.5% (w/v) agarose gel. Data were analyzed using Alpha Imager 2200 software (Alpha Innotech, Santa Clara, CA, USA) and the absorbance ratio with the internal reference  $\beta$ -actin was calculated for correction.

### Detection of p38 mitogen-activated protein kinase expression in the dorsal root ganglion (S<sub>4-6</sub>) by immunohistochemical staining

Immunohistochemical staining was used to determine the expression of p38 mitogen-activated protein kinase in the dorsal root ganglion (S<sub>4-6</sub>). Dorsal root ganglion tissue sections were fixed, dehydrated, paraffin embedded, cut into paraffin slices and deparaffinized. After preincubation for 15 minutes with 3% (v/v) H<sub>2</sub>O<sub>2</sub>-methanol and normal serum for 20 minutes, slices were incubated with rabbit anti-p38 mitogen-activated protein kinase monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a moist chamber at 4°C overnight; and with a goat anti-rabbit antibody (Santa Cruz Biotechnology) for additional 15 minutes after washing with PBS. Peroxidase activity was made visible with diaminobenzidine. PBS was chosen as a negative control. The results were analyzed by an image acquisition and analysis system (Image-Pro Plus 6.0, Media Cybernetics Inc., Bethesda, MD, USA) in the form of gray values. A high gray value indicated a low quantity of protein expression. On the contrary, a low gray value illustrated a high quantity of protein expression.

### Statistical analysis

Data are expressed as mean  $\pm$  SD. SPSS 16.0 software

(SPSS, Chicago, IL, USA) was used for statistical analysis. Statistical significance was determined by one-way analysis of variance and least significant difference method. A *P* value less than 0.05 was considered statistically significant.

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**Ethical approval:** This study was approved by the Animal Ethical Committee, Central South University, China.

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