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Establishment of a Rat Model of Type II Diabetic Neuropathic Pain

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Editor's Note

The article by Dang et al. published in this issue of Pain Medicine[1] *(PM) is the first fruit of a new collaboration between this journal and the* Chinese Journal of Pain Medicine (CJPM)*, more specifically between the editorial boards of both journals. This article was first selected for publication by the CJPM editorial board and published in that journal, then translated from Chinese into English and accepted for publication in PM. Similarly, an article, previously published in PM, was jointly selected and translated into Chinese for publication*[2] *in CJPM. By facilitating bilingual publication of peer-reviewed articles, the journals hope to create a broader audience for outstanding articles by eliminating the language barrier, which has frequently been the only obstacle to publication. This important initiative may become the new norm for sharing the experience and knowledge accumulated by China and the US in the field of Pain Medicine. We welcome your support and comments.*

Michel Y. Dubois, MD, DABPM
Senior Editor
Pain Medicine

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Abstract

Objective. To establish a rat model of type II diabetic neuropathic pain.

Methods. Sixty Sprague Dawley rats were randomly divided into two groups: group A (N = 10) was fed a normal diet, and group B (N = 50) was fed a high-fat and high-sugar diet. After 8 weeks, the body weight of all rats was recorded, and rats in both groups had their fasting plasma glucose, insulin concentration, and insulin sensitivity index measured and calculated. Subsequently, the rats in group B were randomly divided into three subgroups that were each given different doses of streptozotocin (STZ) by a single intraperitoneal injection (subgroup B1 received 30 mg/kg, subgroup B2 received 35 mg/kg, and subgroup B3 40 mg/kg). Two weeks after the STZ injection, the four groups of rats had their insulin sensitivity index, mechanical withdrawal threshold, and thermal withdrawal latency assessed, allowing us to establish a rat model of type II diabetic neuropathic pain and to determine the optimum dose of STZ. Four weeks after STZ injection (2 weeks after the model was established), the pain threshold was measured in the rats in group A and the group treated with the most effective STZ dose. We also measured the expression of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated cyclic AMP response element-binding protein (p-CREB), and phosphorylated *N*-methyl D-aspartate receptor subtype B (p-NR2B) in the dorsal root ganglion (DRG) and spinal cord dorsal horn regions, which are

closely related to neuropathic pain, and also recorded the TTX-R sodium currents in the acutely isolated DRG neurons.

Results. After 8 weeks of a high-fat, high-sugar diet, the body weight of the rats in group B was significantly increased. Although the fasting blood glucose levels did not change significantly, the fasting insulin levels were slightly elevated, and the insulin sensitivity index was significantly reduced. Two weeks after STZ injection, the blood glucose levels of the rats in subgroup B1 were elevated but did not remain so for a prolonged period. In contrast, the rats in subgroup B3 had elevated blood glucose that was accompanied by a high mortality rate, while the blood glucose levels of the rats in subgroup B2 were moderately elevated and relatively stable. In addition, the pain threshold was significantly decreased ($P < 0.05$), and the mortality was low in this group. Because of this, the dose of STZ that was used in group B2 was considered the most effective dose of STZ for induction of diabetes. Four weeks after STZ injection, the pain threshold in the rats of group B2 was still significantly decreased, and the expression of p-ERK, p-CREB, and p-NR2B in the dorsal root ganglion (DRG) and spinal cord dorsal horn was significantly increased. The tetrodotoxin-resistant sodium current density in DRG neurons was also significantly elevated ($P < 0.05$).

Conclusions. A rat model of type II diabetic neuropathic pain can be established by feeding rats a high-fat, high-sugar diet for 8 weeks, in combination with intraperitoneal injection of 35 mg/kg STZ. This model can be stably maintained for at least 2 weeks.

Key Words. Type II Diabetes; Rats; Neuropathic Pain; Streptozotocin

Diabetic neuropathic pain (DNP), which manifests as spontaneous pain, allodynia, and hyperalgesia, is one of the most common complications of diabetes. It has been estimated by the World Health Organization that in 2030, approximately 360 million people worldwide will suffer from diabetes, of whom type II diabetes will account for 90%. Of these patients, approximately 50% will suffer complications with DNP [1]. Given the extremely high incidence of type II diabetes and its DNP complications, it is essential to establish an optimal animal model of type II diabetic neuropathic pain (type II DNP) to allow in-depth study of its pathogenesis, treatment, and prevention. The aim of this study was to establish a rat model of type II DNP using a high-fat, high-sugar diet in combination with a single intraperitoneal injection of a low dose of streptozotocin (STZ), followed by determination of the pain threshold. This model has not been previously described. A large number of previous studies have shown that the expression of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated cyclic AMP response element-binding protein (p-CREB), and phosphorylated *N*-methyl D-aspartate receptor subtype B (p-NR2B) in the dorsal root ganglion (DRG) and spinal cord dorsal horn is increased and tetrodotoxin-resistant (TTX-R) sodium currents in DRG neurons are elevated, both of which are closely related to neuropathic pain. Because of this, we measured the above-mentioned indicators two weeks after the model was established to confirm the presence and duration of neuropathic pain in our model.

Materials and Methods

Materials

Sixty 6-week-old healthy male Sprague Dawley rats (clean-grade) were purchased from the Experimental Animal Center of Wenzhou Medical College (SCXK [Zhejiang] 2005–0019). The rats had free access to food and drinking water during the experiments. STZ was purchased from Sigma (St. Louis, MO, USA; batch number: S0130); citric acid and sodium citrate were purchased from Beijing Boai Gang Trading Co., Ltd. (Beijing, China); and cholesterol and sodium cholate were purchased from Shanghai Blue Season Science and Technology Development Co., Ltd. (Shanghai, China). The blood glucose meter and supporting blood glucose test strips were purchased from Johnson & Johnson (Shanghai, China). The rat insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Haixi Tang Biotechnology Co., Ltd. (Shanghai, China), and the EXL800 microplate was purchased from BioTek Corporation (Winooski, VT, USA). The electronic von Frey tactile pain measurement instrument (2390 series) and plantar/tail flick tester (336) were purchased from IITC (Woodland Hills, CA, USA). The rabbit anti-p-ERK, rabbit anti-p-CREB, and rabbit anti-p-NR2B antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyvinylidene fluoride (PVDF) membranes were purchased from American Solarbio (Beijing, China). The electrophoretic transfer film instrument was purchased from Bio-Rad (Hercules, CA, USA). The MicroChemi exposure machine was purchased from Alpha Innotech (San Leandro, CA, USA). The glass microelectrodes and microelectrode puller (Model P-97) were purchased from Sutter (Novato, CA, USA). Finally, the Axon Multiclamp 700B patch-clamp amplifier was purchased from Molecular Devices (Sunnyvale, CA, USA).

Type II DNP Model

The 60 rats were randomly divided into two groups after three days of feeding adaptation: Group A, the normal-diet group (N = 10); and Group B, the high-fat and high-sugar group (N = 50). The formula of the high-fat and high-sugar diet was as follows: 67% normal diet plus 10% lard, 20% sucrose, 2% cholesterol, and 1% sodium cholate. The body weight, fasting plasma glucose, and fasting insulin of the rats in both groups were measured at 0 and 8 weeks of feeding [2], and the insulin sensitivity index was calculated accordingly. After 8 weeks of the high-fat and high-sugar diet, the rats in group B were randomly divided into three subgroups. After

fasting for 12 hours, with free access to water, the rats were given different doses of STZ (subgroup B1: 30 mg/kg; subgroup B2: 35 mg/kg; and subgroup B3: 40 mg/kg) by a single intraperitoneal injection. Group A received the same dose of vehicle (citrate buffer) as a control. Two weeks after STZ injection, the rats were again measured for fasting blood glucose, insulin levels, insulin sensitivity index, and changes in pain threshold.

Determination of Insulin and Calculation of the Insulin Sensitivity Index

After 12 hours of fasting, but with free access to water, 0.5 mL of tail vein blood was obtained. After standing at room temperature for 30 minutes, the blood samples were centrifuged at 3,000 × *g* for 20 minutes at 4°C. The supernatants were collected as sera, which were used to measure insulin concentrations by sandwich ELISA. The standard curve was calculated based on the OD_{450} values of different concentrations of the standard. The insulin concentrations of the sample sera were calculated using the standard curve. The insulin sensitivity index was then calculated using the following formula: insulin sensitivity index = 1/(fasting glucose × fasting insulin). This value is not normally distributed; therefore, the natural logarithm was used for the calculation [3].

Measurement of Mechanical Withdrawal Threshold

The rats were placed in a transplant organic glass box (22 cm × 22 cm × 22 cm) with a metal mesh (1 cm^2) at the bottom. The glass box was placed 50 cm above the experimental bench. After the rats were given 15 minutes to acclimate to the box, the rear toes of the rats were vertically stimulated with the IITC 2390 series electronic von Frey tactile pain measurement instrument, with a single-stimulus duration of ≤1 second and a stimulus interval of 10 seconds. The intensity of the stimulus was measured when the rats lifted or licked their feet during the test. The test was repeated five times, and MWT was calculated as the average intensity that caused the rats to lift or lick their feet [4].

Determination of Thermal Withdrawal Latency

Thermal withdrawal latency (TWL) was measured using the IITC 336 plantar/tail flick test. The rats were placed on a glass plate 3 mm thick, thermal radiation was applied to the toe, and the time from irradiation to paw withdrawal was recorded. The feet of each rat were tested five times, with time intervals of 5 min. The values of the last three times were averaged as TWL [5].

Diagnosis of Type II DNP

Two weeks after STZ injection, the rats in group B with fasting blood glucose ≥16.7 mmol/L and pain threshold ≤ 85% base value were selected for the rat model of type II diabetes neuropathic pain [6,7]. Using statistical analysis of the mortality rate and the model success rate, the optimal dose of STZ was determined, and the rats receiving this dose were defined as Group C.

Measurement of Related Indicators of Type II DNP

Four weeks after STZ injection (2 weeks after the model was established), the pain threshold was determined in rats in Group A and Group C. The levels of p-CREB, p-ERK, and p-NR2B in the DRG and spinal cord dorsal horn were then measured. In addition, the TTX-R sodium currents in the DRG neurons were simultaneously recorded.

Collection of Spinal Cord and Dorsal Root Ganglion Tissues

After the pain thresholds of rats were measured, three rats from each group were sacrificed, thoracotomized, and perfused with 4% paraformaldehyde. The L4–6 spinal cord and the corresponding DRG tissues were then fixed, paraffin-embedded, and sectioned for immunohistochemical staining. In parallel, an additional three rats from each group were sacrificed, and the L4–6 spinal cord and the corresponding fresh DRG tissues were collected and rapidly frozen in liquid nitrogen. The tissues were then stored at –80°C for use in later Western blot analysis. A further three rats per group were sacrificed for rapid separation of the L4–L6 DRG. After digestion with 0.1% collagenase and 0.3% trypsin, the cells of the L4–L6 DRG were placed in a recording groove for 30 minutes until they had adhered. The cells were then used for whole-cell patch-clamp experiments.

Immunohistochemistry

Paraffin sections were dewaxed and stained using the two-step substance P supersensitivity method. The slides were mounted using neutral resin, and the expression of p-ERK, p-CREB, and p-NR2B in the DRG and spinal cord dorsal horn was examined microscopically, where positive staining was observed as brown-yellow granules. Six slides of dorsal horn (I, II lamellar) or DRG were separately chosen for each rat. The numbers of positively stained cells in DRG and spinal cord dorsal horn were then calculated.

Western Blot

Lysis buffer was added, at the ratio of 1:10 wt/vol, to the frozen tissue specimens that had been stored at –80°C. After full grinding and ultrasonication, the lysates were allowed to stand for 30 minutes before being centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was then collected, and the protein concentration was determined using the bicinchoninic acid method. The protein concentration of each sample was adjusted to 35 μg per 15 μL. After denaturing, the protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to a PVDF membrane. The membrane was blocked for 2 hours with Tris-buffered saline–Tween (TBST) solution

Table 1 Changes in weight and fasting plasma glucose in rats fed a high-fat and high-sugar diet

	Weight (g)		Fasting Plasma Glucose (mmol/L)	
Time (Week)	Group A	Group B	Group A	Group B
0	187.90 ± 4.60	183.62 ± 7.19	6.36 ± 0.43	6.21 ± 0.64
8	413.20 ± 16.99	461.08 ± 29.04*	6.34 ± 0.50	6.53 ± 0.36

* $P < 0.05$ vs group A.

containing 5% bovine serum albumin before membranes were incubated with polyclonal antibodies against p-ERK, p-CREB, and p-NR2B. After incubation at 4°C overnight and rewarming at room temperature, the membrane was washed three times with TBST, for 10 minutes each time. The alkaline phosphatase-labeled IgG secondary antibody was then added, and the membrane was incubated at room temperature for 2 hours. After three washes with TBST for 15 minutes each, enhanced chemiluminescent (ECL) reagent was added, and the membranes were exposed and photographed using the DNR MicroChemi ECL imaging system. The optical density of each band was analyzed using AlphaEaseFC software (Alpha Innotech).

Whole-Cell Patch-Clamp

These experiments were conducted at room temperature (20–24°C). The cell-recording groove containing adherent DRG neurons was placed on the stage of an inverted microscope. The neurons that were chosen for this test were those with a small diameter (less than 25 μm), smooth surface, and intact membrane, which mainly express TTX-R sodium channels. When the electrode was placed in contact with the membranes of neurons, the neurons were subjected to continuous negative pressure until a high-resistance seal was formed (>1 GΩ). The cell membranes were then ruptured by the continuous negative pressure. When it was stable, the membrane capacitance was immediately compensated, forming the whole-cell patch-clamp recording mode. In order to record sodium currents, the serial resistance needed to be compensated (prediction > 80%). Under the voltage-clamp mode, the membrane potential was clamped at –80 mV and stimulated with jump voltage (a series of square-wave command voltages from –80 mV to +50 mV, in 10 mV increments, with pulse width of 40 ms). At this time, the recorded inward currents were the total sodium currents. Subsequently, the TTX-R sodium currents were isolated from the total sodium currents by stimulation with a prepulse (square-wave command voltage from –80 mV to –50 mV, pulse width of 500 ms) and recorded [8].

Statistical Analysis

SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The measured data were expressed as mean ± standard deviation. The differences between groups were analyzed using the *t*-test, while multiple samples were compared using ANOVA with the least significant difference test. The rates between multiple samples were compared with the χ^2-test. $P < 0.05$ was considered statistical significance.

Results

Body Weight, Fasting Plasma Glucose, Insulin, and Insulin Sensitivity Index in Rats after 8 Weeks of a High-Fat, High-Sugar Diet

Compared with group A, the body weight of the rats in group B was significantly increased after 8 weeks of the high-fat, high-sugar diet ($P < 0.05$). The fasting glucose levels did not change significantly, although the fasting insulin levels were significantly increased ($P < 0.05$; Table 1). In addition, the insulin sensitivity index decreased significantly in animals fed the high-fat, high-sugar diet ($P < 0.05$; Table 2).

Table 2 Changes in fasting insulin and insulin sensitivity index in rats fed a high-fat and high-sugar diet

	Fasting Insulin (μU/L)		Insulin Sensitivity Index	
Time (Week)	Group A	Group B	Group A	Group B
0	5.31 ± 0.60	5.50 ± 0.37	–3.51 ± 0.16	–3.49 ± 0.11
8	3.42 ± 0.32	5.64 ± 0.77*	–3.01 ± 0.12	–3.63 ± 0.14*

* $P < 0.05$ vs group A.

Table 3 Comparison of fasting plasma glucose, fasting insulin, and insulin sensitivity index after intraperitoneal injection with different doses of streptozotocin

		Group B (Streptozotocin)		
Item	Group A (Control)	30 mg/kg	35 mg/kg	40 mg/kg
Fasting plasma glucose	6.16 ± 0.27	22.58 ± 2.17*	22.82 ± 2.74*	23.63 ± 2.60*
Fasting insulin	4.26 ± 0.59	2.28 ± 0.32*	2.20 ± 0.30*	2.04 ± 0.42*
Insulin sensitivity index	–3.26 ± 0.15	–4.00 ± 0.18*	–3.88 ± 0.17*	–3.90 ± 0.27*

* $P < 0.05$ vs group A.

Fasting Plasma Glucose, Insulin, Insulin Sensitivity Index, and Pain Threshold in Rats 2 Weeks after STZ Injection

Two weeks after injection with different doses of STZ, the blood glucose levels in the rats receiving STZ were significantly elevated ($P < 0.05$) compared with group A. In addition, the insulin concentration and insulin sensitivity index were significantly decreased ($P < 0.05$). However, there was no difference between the groups receiving different doses of STZ (Table 3). Finally, the MWT and TWL in the groups receiving STZ were significantly lower than those in group A ($P < 0.05$; Table 4).

Comparison of the Mortality Rate and Model Success Rate Between Different Doses of STZ

Two weeks after STZ injection, the mortality rate in subgroup B1 was 6.7%, and the model success rate was 33.3%. In Subgroup B3, the mortality rate was higher (60%), and the model success rate was 40%. In subgroup B2, the mortality rate was 10%, and the model success rate was further elevated (80%; see Figure 1). Because of these observations, subgroup B2 was regarded as the best model group for diabetes as induced by STZ, and therefore the dose of STZ received by the rats in subgroup B2 was considered the optimal dose.

Neuropathic Pain-Related Indicators in the Rats in Group C 4 Weeks after STZ Injection

Pain Threshold at Different Time Points

After the rats in Group B were fed a high-fat, high-sugar diet for 8 weeks, there were no significant changes in MWT or TWL. However, 2 weeks after STZ injection, MWT and TWL were significantly decreased ($P < 0.05$), and the pain threshold in the rats in subgroup B2 remained low up to 4 weeks after STZ injection ($P < 0.05$, Figure 2).

Immunohistochemical Results

Four weeks after STZ injection, significantly more p-ERK- and p-CREB-positive cells were found in the DRG and spinal cord dorsal horn of subgroup B2 rats than in those in Group A (see Figures 3,4).

Western Blot Analysis

Western blot results demonstrated that the expression of p-ERK and p-CREB in the DRG of subgroup B2 was significantly increased after 4 weeks of STZ injection ($P < 0.05$; Figure 5). In addition, the expression of p-ERK, p-CREB, and p-NR2B in the spinal dorsal horn was also significantly increased ($P < 0.05$; Figure 6).

Whole-Cell Patch-Clamp Results

Four weeks after STZ injection, the amplitude of the TTX-R sodium currents in the DRG neurons acutely isolated from the rats of subgroup B2 were significantly increased (Figure 7A). The peak sodium currents density were also increased significantly ($P < 0.05$, Figure 7B). As seen in the curve of the average current–voltage relationship, the peak sodium currents in both groups were maximal when the voltage was approximately –10 mV, which was significantly decreased when compared with the current–voltage curves in group A or subgroup B2 (Figure 7C).

Table 4 Comparison of mechanical withdrawal threshold and thermal withdrawal latency after intraperitoneal injection with different doses of streptozotocin

		Group B (Streptozotocin)		
Item	Group A	30 mg/kg	35 mg/kg	40 mg/kg
Mechanical withdrawal threshold (g)	39.09 ± 1.54	23.82 ± 2.52*	24.51 ± 2.72*	22.78 ± 3.31*
Thermal withdrawal latency (seconds)	13.14 ± 2.09	8.97 ± 0.74*	8.28 ± 0.96*	8.11 ± 1.03*

* $P < 0.05$ vs group A.

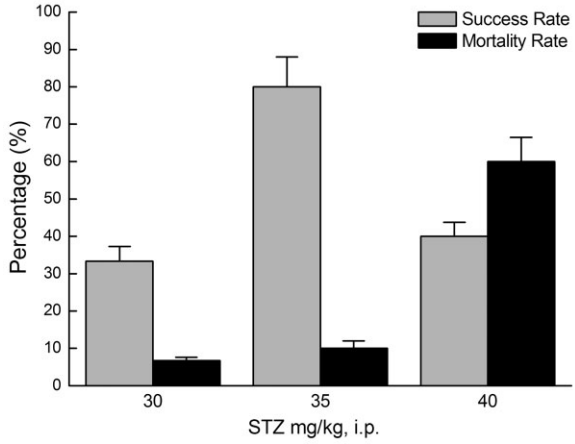

Figure 1 The success rate and mortality rate after intraperitoneal (i.p.) injection with different doses of streptozotocin (STZ).

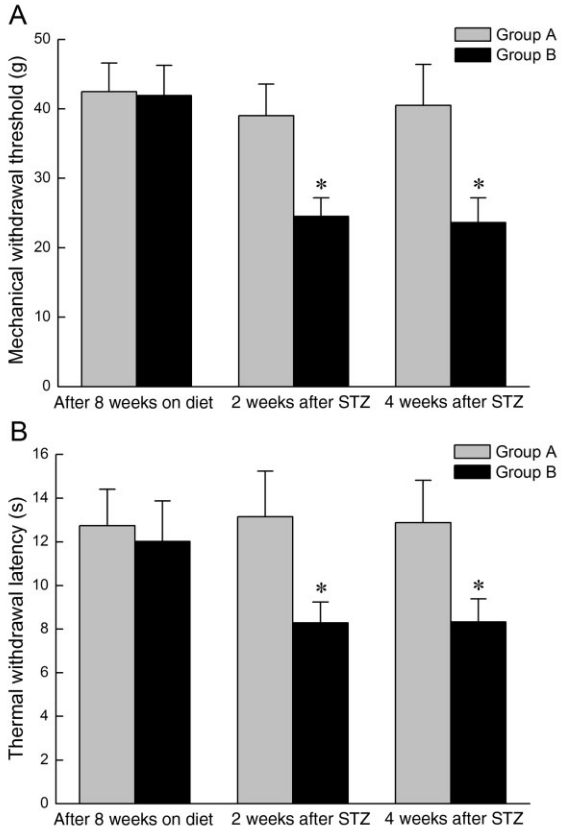

Figure 2 Changes in mechanical withdrawal threshold and thermal withdrawal latency after STZ injection. *$P < 0.05$ vs group A.

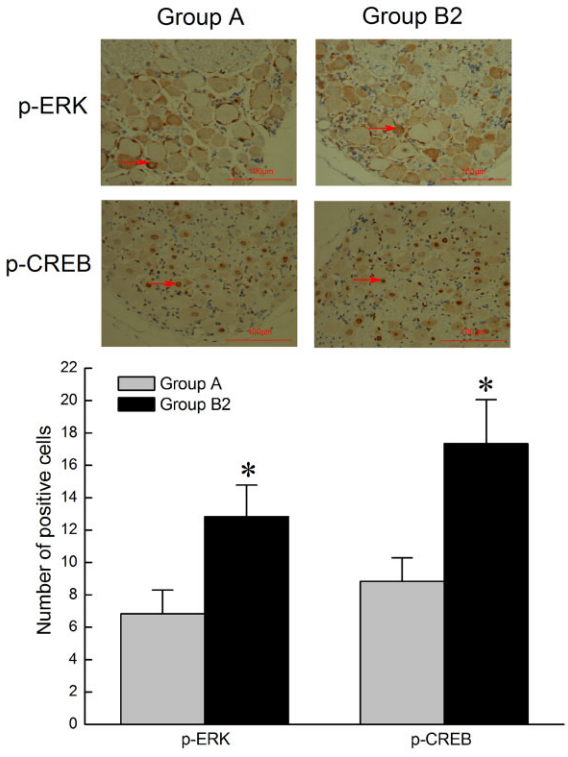

Figure 3 Immunohistochemistry of phosphorylated extracellular signal-regulated kinase (p-ERK) and phosphorylated cyclic AMP response element-binding protein (p-CREB) in dorsal root ganglion. *$P < 0.05$ vs group A.

Discussion

In recent years, the global prevalence of diabetes has rapidly increased. The International Diabetes Federation believes that diabetes, as a noncommunicable disease, is rapidly spreading in modernized nations. In 2011, a multinational joint research study showed that from 1980 to 2008, the number of patients with diabetes worldwide increased from 153 million to 347 million. Accordingly, the prevalence of diabetes increased from 8.3% to 9.8% in adult males and from 7.5% to 9.2% in adult females [9]. Type II diabetes, the most common type, accounts for at least 90% of all people with diabetes. With the rapid growth of today's global economy, the modern lifestyle is helping make it even more prevalent. Diabetes threatens human health and life through a variety of serious complications. In 2000, a study reported that the global number of diabetes-related deaths stood at 2.9 million, among whom the vast majority were patients with type II diabetes [10]. Many of the animal models used to study diabetes and its complications in basic research are based on rats that have been injected with a single dose of STZ. However, the onset of diabetes in this model occurs through the destruction of islet cells, making it essentially

a model of type I diabetes. Given the very high incidence of type II diabetes, this model does not therefore accurately reflect the clinical conditions seen in patients [11,12]. In addition, there are studies reporting that a long-term high-fat, high-sugar diet can be used to induce obesity in rodents and ultimately to establish a model of type II diabetes through the elevation of blood glucose levels. However, this method is very time-consuming, which is not conducive to studies [13,14]. In addition to STZ- and diet-based models, several studies have been published using transgenic or genetic models such as ZDF rats, C57 blk db/db mice, and BBZDR/Wor rats to study type II diabetes and its complications [15]. However, transgenic rodents specific to DNP and other specific complications of type II diabetes are not yet available. It is therefore essential to establish an optimal, stable, and practical animal model for the study of type II diabetes and these complications. This study, to establish an optimal rat model of type II diabetes, was carried out based on the studies of Srinivasan et al. using improved high-sugar and high-fat diet formulations and a single STZ injection with an adjusted dose [2]. This model simulates insulin resistance and islet destruction, which mirrors the typical pathogenesis seen in type II diabetes. Furthermore, this

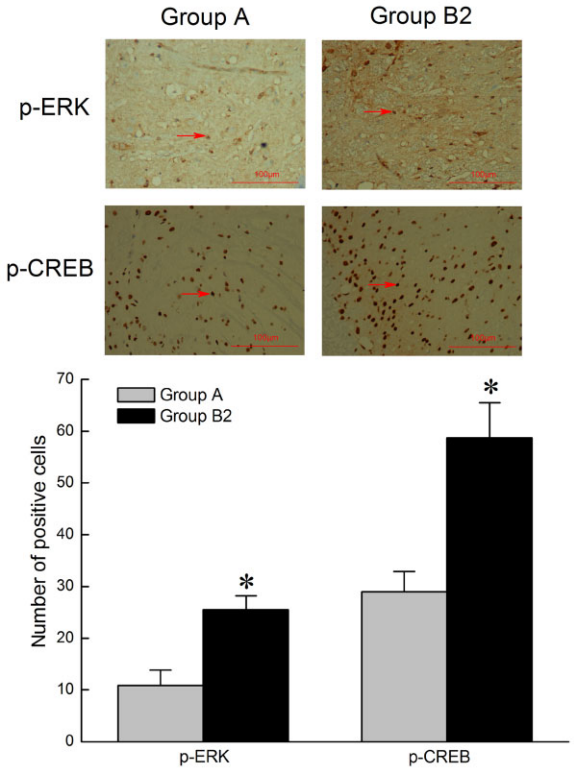

Figure 4 Immunohistochemistry of phosphorylated extracellular signal-regulated kinase (p-ERK) and phosphorylated cyclic AMP response element-binding protein (p-CREB) in spinal dorsal horn. *$P < 0.05$ vs group A.

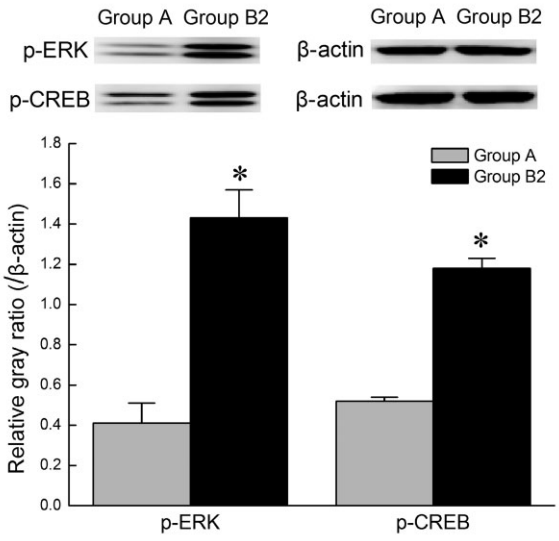

Figure 5 Expression of phosphorylated extracellular signal-regulated kinase (p-ERK) and phosphorylated cyclic AMP response element-binding protein (p-CREB) in dorsal root ganglion. *$P < 0.05$ vs group A.

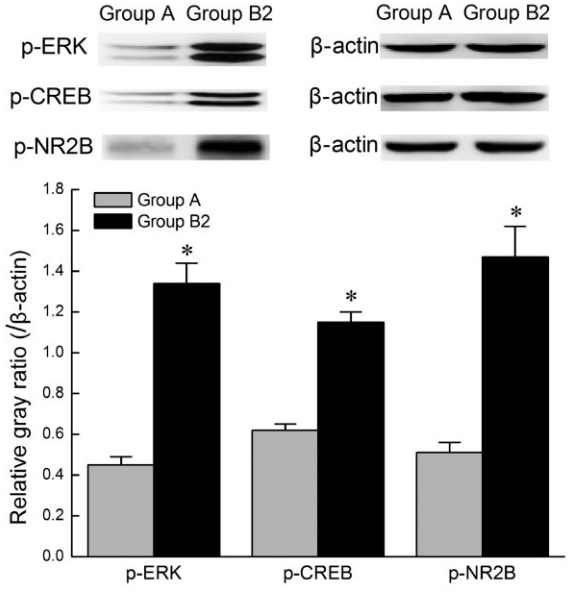

Figure 6 Expression of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated cyclic AMP response element-binding protein (p-CREB), and phosphorylated *N*-methyl D-aspartate receptor subtype B (NR2B) in spinal dorsal horn. *$P < 0.05$ vs group A.

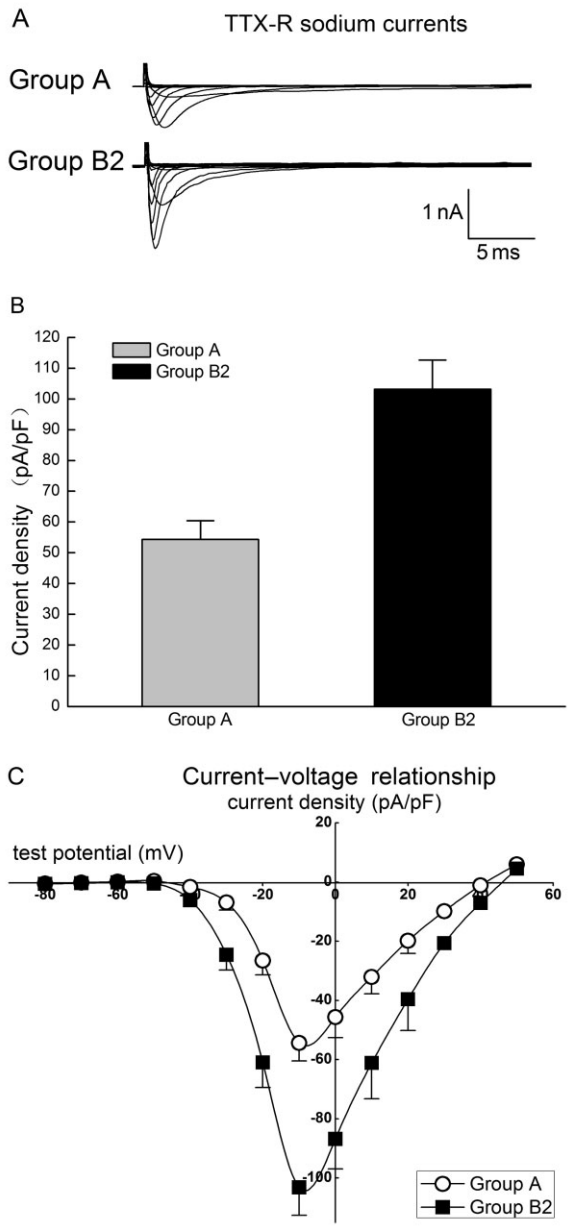

Figure 7 Comparison of tetrodotoxin-resistant (TTX-R) sodium currents recorded from neurons in dorsal root ganglion.

model requires only approximately 2 months to induce insulin resistance, thus greatly reducing modeling time and significantly lowering the cost compared to many transgenic or genetic models. After type II diabetes has been established and has progressed, the pain threshold in these diabetic rats is significantly reduced within 2 weeks, thereby establishing a model of DNP and exhibiting neuropathic pain-related indicators. We have also confirmed that the model can be stably maintained for at least 2 weeks.

After the rats were fed on a high-fat, high-sugar diet for 8 weeks in this study, their body weight and insulin concentrations were significantly increased, and the insulin sensitivity index was significantly decreased. This may be due to the inhibition of the molecular signaling pathways of insulin by the high-fat, high-sugar diet [16], but the islets may still be capable of compensation, as they can maintain a pancreatic secretion of insulin that is high enough to overcome the insulin resistance. This may explain why blood glucose levels are not elevated, but the obese diabetic precursor model has successfully been induced [17]. The pain threshold of rats in Group B was not significantly altered, suggesting that the high-fat and high-sugar diet had yet to cause nerve injury. As a chemical inducer, STZ can destroy pancreatic beta cells by oxidation and hydroxylation, so it is widely used to make a model of diabetes. In this experiment, a single intraperitoneal injection of a low dose of STZ was administered after the induction of insulin resistance to destroy the islet beta cells, weakening the compensatory ability of the pancreas and leading to a significant increase in blood glucose levels. The model used in this study therefore accurately mimics the development of human type II diabetes [18]. After STZ injection, the blood glucose levels were elevated 2 weeks after injection with the 30 mg/kg dose. At this dose, only 33.3% rats had blood glucose levels reaching the diabetes diagnostic criterion, whereas at the 40 mg/kg dose, almost all rats had blood glucose levels reaching the criterion. However, the mortality rate in this group was as high as 60%. At the 35 mg/kg dose, the blood glucose levels of 80% rats met the diagnostic criterion, although two rats in this group had excessively high levels of blood glucose and died, probably due to individual differences. In another two rats, the blood glucose levels did not rise, although their insulin concentrations and insulin sensitivity index were significantly decreased. We therefore concluded that 35 mg/kg STZ could be used as the optimal dose for STZ induction of diabetes.

Numerous studies have confirmed that nerve injury can lead to elevated expression of proteins of the mitogen-activated protein kinase (MAPK) family in the DRG and spinal cord dorsal horn. Specifically, significantly increased expression of the MAPK family members p38 MAPK, p-JNK, and p-ERK is observed. Elevated p-ERK expression was also found to be significantly increased in DNP rats [19]. Activated ERK is translocated to the nucleus, where it activates the nuclear transcription factor CREB, causing its phosphorylation, which mediates downstream gene expression. This plays an important role in the long-term alteration of neuronal plasticity that is induced by a variety of noxious agents [20]. Glutamate is a major excitatory neurotransmitter that plays an important role in pain-signaling processes. *N*-methyl D-aspartate receptors, the ionotropic glutamate receptors, also play important functions in the maintenance and development of the central sensitization of chronic pain. The NR2B subunit, which is located in the dorsal horn of the spinal cord, is particularly important in peripheral nerve injury and inflammatory pain. It has been found that NR2B-encoding mRNA was significantly

increased in rat spinal dorsal horn layers III–V [21]. The pain signal transduction pathways and their related protein expression can therefore be good indicators of neuropathic pain. In addition, the altered expression of signaling proteins will ultimately modulate neuronal membrane ion channels via different mechanisms, resulting in altered electrophysiological properties and hyperexcitable neurons. Among these membrane ion channels, voltage-gated sodium channels play an important role in the generation and propagation of action potentials and can lead to ectopic spontaneous discharges. Numerous studies have also confirmed that nerve damage can result in a significantly increased amplitude of the sodium current in DRG neurons, which is less injury-sensitive than the TTX-sensitive sodium current. The TTX-R sodium currents produced by small sized DRG neurons are closely related to DNP and other neuropathic pain. In recent years, certain subtypes of TTX-R sodium channels, such as Nav1.8 and 1.9, have become a focus for research on neuropathic pain [22]. In summary, the increased expression of p-ERK, p-CREB, and p-NR2B in DRG and spinal cord dorsal horn, together with increased TTX-R sodium currents, can be assessed as strong indicators of neuropathic pain. Therefore, in order to clarify the feasibility of our established model of type II diabetes neuropathic pain and to investigate its stabilization time, we performed additional studies and determinations of the pain threshold and related indicators in the rats of subgroup B2 at 4 weeks after STZ injection (2 weeks after the model was established). The rats in this group were treated with STZ at the optimal dose (35 mg/kg). The results showed that, compared with normal rats, the pain threshold in the rats of group C was significantly decreased, and the previously mentioned parameters were also significantly increased. This demonstrates the accuracy and stability of the novel rat model of type II diabetic neuropathic pain developed in this study.

There are still some defects in this study. First, clean-grade Sprague Dawley rats were used, which were more likely to be affected by infectious agents than specific pathogen-free rats. This may therefore have increased the experimental error. In addition, the mortality rate remained as high as 10%, even at the optimal dose of STZ for induction. Finally, although the model can remain stable for at least 2 weeks, observation for a longer period should be carried out.

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