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Tanshinone IIA improves impaired nerve functions in experimental diabetic rats

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ABSTRACT

Diabetic neuropathy is one of the most common complications in diabetes mellitus. Thus far, effective therapeutic agents for restoring the impaired motor and sensory nerve functions in diabetic neuropathy are still lacking. The antioxidant and neuroprotective properties of tanshinone IIA make it a promising candidate for the treatment of diabetic neuropathy. Therefore, the present study investigated the possible beneficial effect of tanshinone IIA on the impaired nerve functions displayed by a rat diabetic model. Insulin-dependent diabetes in rats was developed by a single dose of streptozotocin (STZ) at 50 mg/kg. The diabetic rats were randomly divided into four groups (n = 10 in each group), and were intraperitoneally administrated daily for 4 weeks with tanshinone IIA (20 mg/kg, 50 mg/kg and 100 mg/kg), or normal saline from the fourth day after STZ injection, respectively. At the end of tanshinone IIA administration, thermal and mechanical nociceptive threshold were determined by a hot plate test and Von Frey hairs; motor nerve conducting velocity (MNCV) was determined by an electrophysiological method; nerve blood flow (NBF) was detected using a laser Doppler flow meter; Na^+,K^+ATPase activity, the level of superoxide dismutase (SOD), catalase and malondialdehyde (MDA) in sciatic nerves, and the serum total antioxidant capability were also determined. We found that tanshinone IIA was capable of restoring diabetes-induced deficit in nerve functions (MNCV and NBF), and impairment in thermal and mechanical nociceptive capability. In addition, tanshinone IIA significantly increased the serum total antioxidant capability, improved the activities of Na^+,K^+ATPase, increased the levels of SOD and catalase, and reduced the MDA level in sciatic nerves in diabetic rats. All the findings indicate the beneficial effect of tanshinone IIA on impaired nerve functions and raise the possibility of developing tanshinone IIA as a therapeutic agent for diabetic neuropathy.

1. Introduction

Diabetic neuropathy is one of the most frequently encountered complications in insulin-dependent diabetes, occurring in about 60% of diabetic patients [1]. Many studies have shown that hyperglycemia in diabetes slows motor nerve conducting velocity (MNCV) and alters sensitivity in pain perception. In diabetic neuropathy, 10–20% of the patients experience symptoms such as burning tactile hypersensitivity, spontaneous pain and allodynia [2,3]. For diabetic neuropathy, analgesics have been used for the treatment of nociceptive pain. However, regular analgesics are only partially effective in controlling nociceptive pain, and show little beneficial effect on impaired MNCV in most of the cases. Up to now, besides a tight glycemia control, no viable treatment for diabetic neuropathy is available. Therefore, it is imperative to search for effective drugs, especially the ones of nature origin, for the treatment of diabetic neuropathy.

The role of oxidative damage in the development of diabetes and progression of diabetic complications has been well established [4,5]. Disturbing of oxidant–antioxidant balance system contributes greatly to the development of diabetes and leads to the incidence of complications [5]. Several studies have shown that antioxidants are capable of reducing oxidative damage and lipid peroxidation in diabetic patients and animals [6,7]. Therefore, antioxidants hold great promise in ameliorating diabetic neuropathy. In addition, diabetic neuropathy is a complex process involving degeneration of neurons and nerve fibers in peripheral nervous system [8]. It has been reported that neuroprotective agents, such as neurotrophins and many growth factors, are able to alleviate the extent of nerve degeneration in diabetic animals [9]. All those findings indicate that the agent which exhibits both antioxidant and neuroprotective properties hold great promise in restoring impaired nerve functions under diabetic condition.

Tanshinone IIA, a derivative of phenanthrenequinone, is one of the bioactive components of Danshen, which has been widely used in traditional Chinese medications for cardiovascular and

Abbreviations: STZ, streptozotocin; MNCV, motor nerve conducting velocity; NBF, nerve blood flow; SOD, superoxide dismutase; MDA, malondialdehyde; TBA, thiobarbituric acid; EDTA, ethylene diamine tetraacetic acid.

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cerebrovascular diseases. Tanshinone IIA has been shown to exhibit antioxidant property [10,11]. It has been shown that tanshinone IIA is able to reduce the DNA damage by lipid peroxidation in liver cells [10]. In addition, tanshinone IIA has also been shown to protect cardiac myocytes against oxidative stress-triggered damage and apoptosis [11]. In recent years, the neuroprotective property of tanshinone IIA has been increasingly recognized [12,13]. *In vitro* studies have shown that tanshinone IIA is capable of protecting PC12 cells against cytotoxicity induced by serum withdrawal [12]. *In vivo* studies have shown that tanshinone IIA can reduce brain infarct volume and improve neurological functions in a rat model of transient focal cerebral ischemia, which further confirmed the neuroprotective property of tanshinone IIA [13]. Together, the antioxidant and neuroprotective properties of tanshinone IIA make it an attractive candidate for the treatment of diabetic neuropathy. Therefore, the present study was designed to investigate the putative beneficial effect of tanshinone IIA on impaired nerve functions in a rat model of diabetic neuropathy.

2. Materials and methods

2.1. Induction of experimental diabetes

Male adult Sprague–Dawley albino rats ($n = 50$) weighing from 180 to 220 g were obtained from the Laboratory Animal Center of the Fourth Military Medical University (FMMU) and the use of animals was reviewed and approved by the Institutional Ethical Committee of the FMMU. The animals were housed in plastic boxes in groups of 3 with food and water available *ad libitum* in a colony room with controlled temperature (24 ± 2 °C), humidity (50–60%), and a 12:12 h light–dark cycle.

The rats were fasted overnight and insulin-deficient diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO) at 50 mg/kg in sterile saline. Control rats ($n = 10$) were intraperitoneally injected with sterile saline. The animals were allowed to drink glucose solution (5.0%, w/v) overnight to avoid hypoglycemia which might be induced by STZ. The blood glucose levels were detected using a blood glucometer on the third day after STZ injection. Animals with blood glucose above 15 mM were considered as being diabetic and hyperglycemia, and they were randomly divided into four groups ($n = 10$ in each group). Tanshinone IIA (20 mg/kg, 50 mg/kg and 100 mg/kg; Sigma, St. Louis, MO) or sterile saline were injected intraperitoneally on the fourth day after STZ administration and it was given daily for 4 weeks. The blood glucose levels and body weights of the rats were monitored before and at 0, 1, 2, 3 and 4 weeks after STZ injection.

2.2. Thermal and mechanical nociceptive thresholds

At the end of tanshinone IIA administration, the thermal and mechanical nociceptive thresholds were determined in the present study. Thermal nociceptive threshold was determined by a hot plate test. Rats were placed in a transparent observation chamber with floor temperature of 30 °C, and allowed to acclimate for 3 days before testing. A heat source (50 W) was then moved underneath the hind paw of the tested rat. The time between placement of the hot source and appearance of withdrawal or licking of hind paw was recorded as withdrawal latency. The procedure was repeated four times on the same paw, separated by 10-min rest intervals. The median of the four values (withdrawal latencies) was used to represent the thermal nociceptive threshold.

The mechanical nociceptive threshold was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using Von Frey hairs. In brief, the rats were placed in individual transparent observation boxes on a wired grid floor, and allowed to acclimatize for at least 30 min until the rats were calm. Von Frey hairs with a 0.4 mm diameter polypropylene rigid tip were placed perpendicularly against the plantar surface of the hind paw with gradually increasing pressures. The appearance of vocalization, jumping, agitation and avoidance were considered to be a positive withdrawal response. The force which elicited a withdrawal response was recorded as withdrawal latency. The same procedure was repeated on both hind paws with three times for each hind paw. There is a rest interval of 5 min between two successive procedures. The mean of the six values was considered the withdrawal threshold for each rat.

2.3. Motor nerve conducting velocity (MNCV)

At the end of tanshinone IIA administration, the MNCV was measured by an electrophysiological study. The rats were anesthetized by intraperitoneal injection of 1.0% (w/v) sodium pentobarbital solution (40 mg/kg body weight). The left sciatic nerve was exposed and insulated from the surrounding muscle with a rubber dam. A bipolar stimulating electrode was placed under the sciatic nerve at a location 10 mm proximal to the bifurcation. A recording electrode was placed in the gastrocnemius muscle. Nerve stimulation (0.2 ms, 1 mA rectangular pulse wave) and recordings were performed with a PowerLab 4SP distal data acquisition system (Keypoint 3.02, Denmark). Digitized data were stored in a personal computer and the MNCV values were recorded.

2.4. Nerve blood flow

At the end of tanshinone IIA administration, nerve blood flow (NBF) was determined using a laser Doppler flowmeter (Hpsonos 1500, USA). After induction of anesthesia by intraperitoneal injection of 1.0% (w/v) sodium pentobarbital solution (40 mg/kg body weight), the right sciatic nerve was exposed and laser probe was placed just above the exposed nerve. Animals were stabilized for 20 min before a 10-min continuous nerve blood flow recording as described previously [14]. Average values of the 10-min recording were considered as perfusion unit. During the test, body temperature of the rats was maintained at 37 °C.

2.5. Na^+,K^+ATPase

Immediately after recording of MNCV and NBF, the left and right sciatic nerve were rapidly removed, frozen and stored at −80 °C until use. Crude homogenate was prepared by immersion of sciatic nerves in a prechilled solution of 10 mM Tris–HCl (pH 7.5). Na^+,K^+ATPase activity was determined spectrophotometrically using a coupled enzymatic method described previously [15]. The crude homogenate was immersed and homogenized in 10 mM Tris–HCl (pH 7.5), supplemented with 5 mM $MgSO_4$, 2.5 mM phosphoenolpyruvate, 50 μm NADH, 2.5 mM ATP, 0.2 U of pyruvate and 0.2 U of lactate. The ouabain was used at 3 mM. The incubation was performed at 37 °C for 20 min, and the absorbance at 360 nm was followed for 15 min. The activity of Na^+,K^+ATPase was expressed as micromole of NADH oxidized per hour, which was normalized to the protein content of the membrane-enriched fraction.

2.6. MDA, SOD and catalase amount

The levels of superoxide dismutase (SOD) and catalase in sciatic nerves were determined by enzyme assays for all groups. The activity of SOD was measured using a xanthine–xanthine oxidase Cytochrome *c* method and expressed as U/mg protein [16]. The activity of catalase was measured spectrophotometrically according to the methods described previously and expressed as U/mg

protein [17]. The malondialdehyde (MDA) level has been considered as an indicator of lipid peroxidation and free radical generation. The level of MDA in sciatic nerve was estimated by the double heating method which has been described by Draper and Hadley [18]. This method was based on the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) and MDA. The amount of MDA in sciatic nerve was calculated by the absorbance coefficient of MDA–TBA complex and is expressed as μM/mg of protein.

2.7. Total antioxidant capacity

Blood samples were collected after sciatic nerve harvest from the anesthetized animals by cardiac puncture in vacutainer tubes with EDTA (ethylene diamine tetraacetic acid). The blood samples were centrifuged (3000 rpm, 10 min) at 4 °C. The serum was collected and stored at −80 °C until use. The serum total antioxidant capacity was detected using a commercially available kit (Abcam, USA), which is based on suppression of ABTS (2,2′-azino-di-[3-ethylbenzthiazolinesulphonate]) oxidation to produce blue-green ABTS in the presence of metmyoglobin and H_2O_2. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used as a standard antioxidant, and the total antioxidant capacity was expressed as mmol/l serum.

2.8. Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare mean values using SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). If there was a significant overall difference among groups, then Tukey's post hoc test was used to make pair-wise comparisons. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of tanshinone IIA on plasma glucose level and body weight in diabetic rats

A single injection of STZ (50 mg/kg) resulted in a significant rise in plasma glucose (25.8 ± 2.6 mM vs. 5.9 ± 0.7 mM, $p < 0.001$) and a significant decline in body weight (236 ± 13.2 g vs. 473 ± 15.9 g, $p < 0.001$) compared to those in control normal rats. Treatment with tanshinone IIA showed little influence on blood glucose level (20 mg/kg, 25.5 ± 1.4 mM; 50 mg/kg, 24.8 ± 2.1 mM; 100 mg/kg, 25.1 ± 1.9 mM) and body weight (20 mg/kg, 243 ± 14.5 g; 50 mg/kg, 240 ± 12.6 g; 100 mg/kg, 237 ± 11.9 g) in diabetic rats.

3.2. Effect of tanshinone IIA on thermal and mechanical hyperalgesia in diabetic rats

The thermal and mechanical nociceptive threshold decreased by 66.4% and 53.8% (Fig. 1A and B; $p < 0.01$) in diabetic rats compared to those in control normal rats. Treatment with tanshinone IIA significantly reversed the reduction of thermal nociceptive threshold in diabetic rats (Fig. 1A). Similar to the observations for thermal nociceptive threshold, tanshinone IIA significantly reversed the reduction of mechanical nociceptive threshold in diabetic rats (Fig. 1A).

3.3. Effect of tanshinone IIA on MNCV and NBF in diabetic rats

The sciatic MNCV significantly decreased in diabetic rats compared to that in control normal rats (Fig. 2A, $p < 0.01$) 4 weeks after STZ treatment. Treatment with tanshinone IIA restored the impaired MNCV in a dose-dependent manner (Fig. 2A, $p < 0.05$). Consistent with the results of MNCV, the sciatic NBF was significantly lowered by STZ in diabetic rats compared to that in control normal rats (Fig. 2A, $p < 0.01$). The deficit in NBF was restored by application of tanshinone IIA at different doses, of which the highest dose of tanshinone IIA achieved the maximum recovery in both MNCV and NBF in diabetic rats.

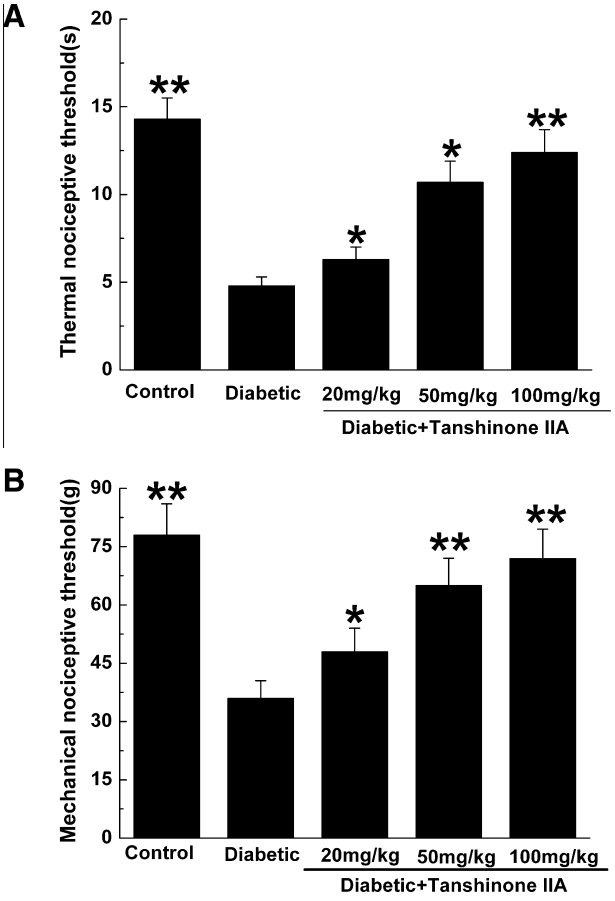

Fig. 1. Effect of tanshinone IIA on thermal (A) and mechanical nociceptive thresholds (B) in diabetic rats. All data are expressed as the mean ± standard error of mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison with diabetic rats treated with saline.

3.4. Effect of tanshinone IIA on Na^+,K^+ATPase activity in diabetic rats

Na^+,K^+ATPase activity significantly decreased in sciatic nerves 4 weeks after STZ treatment in diabetic rats (Fig. 3, $p < 0.01$). Administration of tanshinone IIA significantly reversed the decreased Na^+,K^+ATPase activity of sciatic nerves in diabetic rats dose dependently (Fig. 3, $p < 0.05$). Tanshinone IIA at 100 mg/kg achieved a significantly higher Na^+,K^+ATPase activity than tanshinone IIA at 20 mg/kg and 50 mg/kg (Fig. 3, $p < 0.05$).

3.5. Level of SOD, catalase, MDA and total antioxidant capability

SOD and catalase are two representative antioxidant enzymes. As shown in Fig. 4B and C, the activities of SOD and catalase in sciatic nerves significantly decreased in diabetic rats compared to those in normal control rats ($p < 0.01$). Tanshinone IIA at different doses (20 mg/kg, 50 mg/kg and 100 mg/kg) significantly increased the activities of SOD and catalase in diabetic rats compared to saline treatment. The maximum activity of SOD and catalase was achieved by tanshinone IIA at 100 mg/kg.

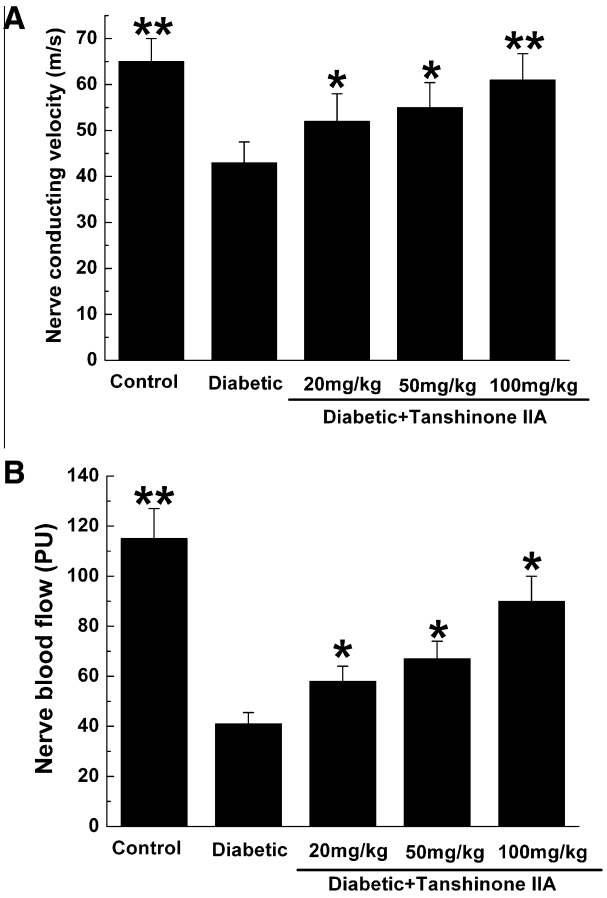

Fig. 2. Effect of tanshinone IIA on motor nerve conducting velocity (A) and nerve blood flow (B) in diabetic rats. All data are expressed as the mean ± standard error of mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison with diabetic rats treated with saline.

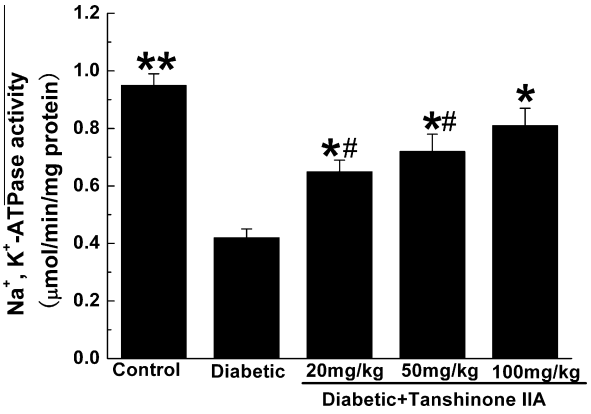

Fig. 3. Effect of tanshinone IIA on Na^+/K^+ATPase activity in sciatic nerve in diabetic rats. All data are expressed as the mean ± standard error of mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison with diabetic rats treated with saline. $^{\#}p < 0.05$ for the comparison with diabetic rats treated with tanshinone IIA at 200 mg/kg.

MAD level was determined and used as an indicator of lipid peroxidation in the present study. As shown in Fig. 4A, the MAD level in sciatic nerve was raised by 61.5% in diabetic rats compared to that in normal control rats ($p < 0.01$). Tanshinone IIA resulted in a significant reduction in the extent of lipid peroxidation in diabetic rats. The MDA levels in tanshinone IIA-treated rats decreased by 14.3% at 20 mg/kg, 28.6% at 50 mg/kg and 33.3% at 100 mg/kg compared to that in saline-treated diabetic rats, respectively.

The total antioxidant capability significantly decreased in diabetic rats compared to that in normal control rats (Fig. 4D, $p < 0.01$). Treatment with tanshinone IIA significantly reversed the reduction of total antioxidant capacity in diabetic rats (Fig. 4D, $p < 0.01$). The total antioxidant capacity in tanshinone IIA-treated rats increased by 6.1% at 20 mg/kg, 10.9% at 50 mg/kg and 11.4% at 100 mg/kg compared to that in saline-treated diabetic rats, respectively.

4. Discussion

The present study investigated the possibility of developing tanshinone IIA as a potential therapeutic agent for the treatment of diabetic peripheral neuropathy. We found that tanshinone IIA was capable of restoring diabetes-induced deficit in MNCV and NBF, and impairment in thermal and mechanical sensation. In addition, tanshinone IIA significantly increased the serum total antioxidant capacity, elevated the activities of SOD and catalase, and reduced the MDA level in sciatic nerves in diabetic rats, suggesting an antioxidant capability of tanshinone IIA against oxidative damage in STZ-induced diabetic rats. All the findings suggest the therapeutic potential of tanshinone IIA for diabetic neuropathy.

In the present study, insulin-dependent diabetes was induced by injection of STZ, which is capable of destructing pancreatic β-cells and causing insulin deficiency in rats. The hyperglycemia induced by STZ results in deficit in nerve functions (decreased MNCV) and impairment in thermal and mechanical sensation [2,3]. In the present study, tanshinone IIA significantly increased MNCV, and reversed thermal and mechanical hyperalgesia in diabetic rats, indicating that tanshinone IIA is capable of restoring the impaired motor and sensory nerve functions in diabetic rats. In addition, decreased NBF was also observed under hyperglycemia conditions, which has been proposed to be responsible for the decreased MNCV in diabetic rats [19]. Tanshinone IIA has been shown to protect myocardium and endothelial cells against ischemia injury [20,21]. The observation that tanshinone IIA partially restored the impaired NBF raises the possibility that tanshinone IIA exerts a beneficial action on vascular endothelial cells in nerve micro-vessels in diabetic rats. Such a speculation needs to be confirmed in future studies.

Increased oxidative stress is one of the contributors to the hyperglycemia-induced neural degeneration under diabetic conditions. Oxidative stress causes vascular impairment resulting in endoneurial hypoxia, which leads to impaired motor (decreased conducting velocity) and sensory (thermal and mechanical hyperalgesia) nerve functions [4,5]. Therefore, it is reasonable to assume that inhibition of oxidative stress using antioxidants might be beneficial for recovery of impaired nerve functions in diabetic neuropathy. In the present study, both impaired motor and sensory nerve functions were restored by tanshinone IIA, which has been shown to have antioxidant property in many previous studies [10,11]. The previous findings and the antioxidant properties of tanshinone IIA drove us to investigate whether the beneficial effect of tanshinone IIA on nerve functions could be attributed to its capability in inhibition of oxidative stress in diabetic rats. Therefore, the serum total antioxidant capacity was examined after treatment of tanshinone IIA. We found that tanshinone IIA significantly increased the serum total antioxidant capacity in diabetic rats, suggesting that the beneficial effect of tanshinone IIA on impaired nerve functions in diabetic rats could be, at least in part, attributed to its antioxidant properties.

Tanshinone IIA significantly increased Na^+,K^+ATPase activity in sciatic nerve in diabetic rats. The correction of Na^+,K^+ATPase activity is very important because it plays a key role in cellular homeostasis. Under diabetic conditions, Na^+,K^+ATPase activity is

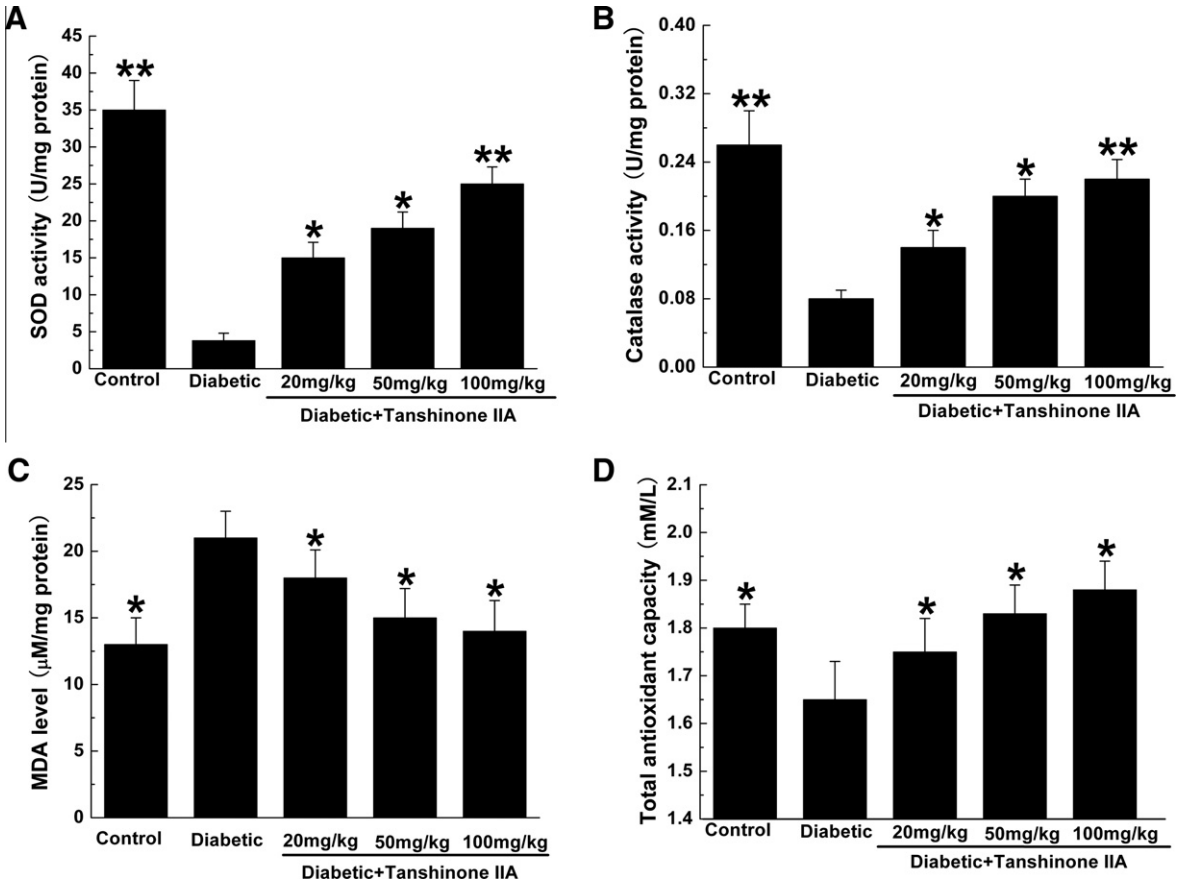

Fig. 4. Effect of tanshinone IIA on SOD activity in sciatic nerve (A), catalase activity in sciatic nerve (B), MDA level in sciatic nerve (C) and serum total antioxidant capability (D) in diabetic rats. All data are expressed as the mean ± standard error of mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison with diabetic rats treated with saline.

significantly inhibited, which results in intraaxonal accumulation of sodium and a decrease in the sodium gradient across the axolemma. The altered membrane sodium gradient could affect axonal sodium conductance, which contributes greatly to nerve dysfunctions in diabetic neuropathy [22]. Despite this knowledge, the mechanisms underlying the decreased Na^+,K^+ATPase activity in diabetic neuropathy has not been well understood. Recently, the role of oxidative damage to Na^+,K^+ATPase activity has been increasingly recognized [23–25]. It has been shown that various oxidative stress are capable of inhibiting Na^+,K^+ATPase activity [23,24]. Oxidative modification, glutathionylation, of the β_1 subunit of Na^+,K^+ATPase can result in a decrease in the α_1/β_1 subunit interaction, which is known to be critical for the maintenance of Na^+,K^+ATPase function [25]. In addition, oxidative stress has been shown to be one of the major contributors to diabetic neuropathy. Therefore, it is possible that the oxidative damage to Na^+,K^+ATPase might be prevented by tanshinone IIA, a potent antioxidant which has been shown to alleviate oxidative stress in diabetic rats in the present study. Such a speculation needs to be confirmed in future studies.

Lipid peroxidation has also been considered as one of the characteristic features in chronic diabetes. Under hyperglycemia conditions, auto-oxidation of unsaturated lipids in membrane lipids produced free radicals, which may react with polyunsaturated free acid in cell membranes resulting in lipid peroxidation [26]. The increased lipid peroxidation in sciatic nerve was observed in the present study, which was evidenced by elevated MDA levels and decreased antioxidant enzyme activities (SOD and catalase) after STZ injection. After treatment with tanshinone IIA, the extent of lipid peroxidation (MDA level) was significantly reduced and antioxidant enzyme activities were significantly increased in sciatic nerve in diabetic rats. All those findings indicate that inhibition of lipid peroxidation might be partially responsible for the improved nerve functions by tanshinone IIA.

In conclusion, the present study investigated the putative beneficial effect of tanshinone IIA on impaired motor and sensory nerve functions in diabetic neuropathy. We found that tanshinone IIA was capable of restoring diabetes-induced deficit in motor and sensory functions. The beneficial effect of tanshinone IIA on impaired nerve functions could be attributed, at least in part, to its antioxidant capacity. All the findings indicate the therapeutic potential of tanshinone IIA as a therapeutic agent for diabetic neuropathy. However, it should be realized that the beneficial effect of tanshinone IIA on diabetic neuropathy still needs to be confirmed in larger animals or even humans. Although no observed adverse effect or toxic effect of tanshinone IIA has been reported in previous literature, further studies are still needed to identify the effect of tanshinone IIA on systematic body functions before its application in the treatment of diabetic neuropathy.

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