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Effect of Tongxinluo on nerve regeneration in mice with diabetic peripheral neuropathy

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Abstract

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes. This study aims to investigate the effects of Tongxinluo on the nerve regeneration in diabetic peripheral neuropathy mice. Forty Specefic Pathogen Free (SPF) male KK/Upj-Ay mice were divided into diabetes group, diabetes with high dose Tongxinluo (4g/kg) (D+H), diabetes with mid dose Tongxinluo (2g/kg) (D+M), and diabetes with low dose Tongxinluo (1g/kg) (D+L) groups. Fasting blood glucose (FPG), heat pain threshold, motor nerve conduction velocity (MNCV), insulin-like growth factor-1 (IGF1), activator protein 1 (c-fos), nerve growth factor (NGF), and basic fibroblast growth factor (BFGF) were measured. Results indicated that FPG of diabetes group was significantly higher than that of control group. Heat pain threshold and MNCV were significantly lowered in diabetes group. Expression levels of IGF1, NGF and BFGF were significantly lower than that of control, whereas c-fos expression was significantly higher than that of control group. Tongxinluo treatment (D+M and D+H) significantly up-regulated heat pain threshold, MNCV, and IGF1, NGF and BFGF expression, but decreased c-fos expresson when compared to that of diabetes group. In conclusion, Tongxinluo can ameliorate diabetic peripheral neuropathy, improve MNCV, and promote nerve regeneration. The underlying mechanism needs to be further elucidated.

Key words: Diabetic peripheral neuropathy, nerve regeneration, Tongxinluo.

Introduction

The prevalence of diabetes increased rapidly in China. There are more than 110 million patients with diabetes, and only about 1/4 of these patients could receive the drug treatment. China has become one of the countries with biggest diabetes burden (1). Diabetes is often accompanied by complications including ketoacidosis, microvascular disease, retinopathy, kidney disease, nerve disease, a variety of heart, brain, or kidney diseases (2). Diabetic peripheral neuropathy (DPN) is a common complication of diabetes, the prevalence rate of DPN ranges from 60% to 90%. Prolonged course of diabetes increases the risk of DPN (3). Main clinical symptoms of DPN include sensory nerve damage, limb pain and numbness, acute pain, weak reflexes, muscle weakness or atrophy (4). There were some of the growth factors have been proved to be participated in the pathology of the DPN, such as insulin growth factor 1 (IGF1) (5), nerve growth factor (NGF) (6), basic fibroblast growth factor (BFGF) (7) and c-fos (8), therefore, we examined these factors in this study. There is currently no specific treatment of DPN. The commonly used drugs include: aldose reductase inhibitor, ganglioside, nerve growth factor, anticoagulants, vasodilators, etc., but the clinical results are not satisfactory (9). As a risk factor of diabetic foot and amputation, DPN seriously affects the quality of life of patients. Traditional Chinese medicine has exhibited certain advantages in the treatment of DPN. Tongxinluo capsule could inhibit thrombosis, improve function of endothelial cells, and protect the function of microvascular by promoting blood circulation to remove meridian obstruction and smoothing the veins to relieve pain (10). The present study is to investigate the efficacy of Tongxinluo on nerve regeneration in mice with DPN, and to explore the mechanism from the perspective of nerve regeneration to provide scientific references for clinical studies and treatment of DPN.

Materials and methods

Animals

Specefic Pathogen Free (SPF) male KK/Upj-Ay mice (30~40g) and SPF male C57BL/6 mice (25~30g) were puchased from Beijing HuaFuKang Biological Technology Co. (Beijing, China). Animal Certificate of Conformity is SCXK-(BJ) 2009-000. Mice were housed in a normal environment (relative humidity, 40%-70%; temperature, 20 degrees -26 degrees; light, 12h/day).

Reagents

Tongxinluo capsules (Z19980015) were purchased from ShiJiaZhuang YiLing Pharmaceutical Co. (ShiJiaZhuang, China). The primers of Insulin-like growth factors1 (IGF1), activator protein 1 (c-fos), nerve growth factor (NGF), and basic fibroblast growth factor (BFGF) were synthesized by Shanghai Biological Technology Co. (ShangHai, China). Antibodies were purchased from Abcam (Cambeidge, MA, USA).

Laboratory instruments

PowerLab polygraph was purchased from (AD Instrument Company); gel imaging analyzer was purchased from (Biorad Company), 7300 fluorescence quantitative PCR instrument was purchased from (ABI Company).

Table 1. The primers for PCR mRNA expression.

Genes		Primers
IGF1	Forward	5'-CTCTTCAGTTCGTGTGTGGAC-3'
	Reverse	5'-AATGCTGGAGCCATAGCCT-3'
c-fos	Forward	5'-GACAGATACACTCCAAGCGG-3'
	Reverse	5'-GGCAGACCTCCAGTCAAATC-3'
NGF	Forward	5'-CTTCAGCATTCCCTTGACAC-3'
	Reverse	5'-TACAGTGATGTTGCGGGTC-3'
BFGF	Forward	5'-CGTCAAACTACAACTCCAAGCA-3'
	Reverse	5'-CGTCCATCTTCCTTCATAGCA-3'
GAPDH	Forward	5'-TGCTGAGTATGTCGTGGAGTC-3'
	Reverse	5'-TGCTGAGTATGTCGTGGAGTC-3'

Animal administration

Ten SPF male C57BL/6 mice were used as controls. 40 SPF male KK/Upj-Ay mice were divided into diabetes group, diabetes with high dose Tongxinluo (4g/kg) (D+H), diabetes with mid dose Tongxinluo (2g/kg) (D+M), and diabetes with low dose Tongxinluo (1g/kg) (D+L) groups. The tongxinluo was administrated at the age of 9 weeks. The same volume of drinking water was given to the mice of control group and diabetes group. Drugs or water was given to the mice for a period of 12 weeks.

Determination of heat pain threshold

After treatment, the mice were placed on a hot plate (55 ° C). The latency of licking hind foot was used as the heat pain threshold.

Examination of fasting plasma glucose

Fasting blood samples were collected into EDTA tubes. The fasting plasma glucose level was analyzed with a chemical analyzer (Hitachi 7600; Hitachi, Tokyo, Japan) using EDTA-anticoagulated blood.

Measurement of motor nerve conduction velocity

After treatment, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital and connected with PowerLab polygraph to measure MNCV. Briefly, the stimulating electrode was inserted into the right sciatic notch, the recording electrode was inserted the ankle and second toes of left foot. Compound action potential was recorded three times at intervals of 1min to take the average.

Determination of mRNA expression levels of IGF1, c-fos, NGF and BFGF

Total RNA was extract from sciatic nerve tissue and reverse transcripted to cDNA for quantitative PCR. The methods of total RNA extraction, mRNA transcription were perforemed according to the previous report (2). The primers are as listed in Table 1.

Measurement of protein levels of IGF1, c-fos, NGF and BFGF

Western blot was used to measure the levels of IGF1, c-fos, NGF and BFGF protein. Total protein of sciatic nerve tissue was extracted, resolved in SDS-PAGE, trans-blotted onto PVDF membrane, blocked with 5% non-fat milk and incubated with antibodies of IGF1, c-fos, NGF and BFGF. The β-actin was employed as the housekeeping protein control in this study. The bound complexes were detected via enhanced chemiluminesence.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The results were analyzed using the two-sample t-test. In all tests, P values less than 0.05 were considered statistically significant.

Results

Change of FPG

FPG level in diabetes group was significantly higher than that of the control group, the difference was statistically significant ($P < 0.05$); FPG values of Tongxinluo groups decreased slightly compared with that of the diabetes group, but the difference was not statistically significant ($P > 0.05$) (Table 2).

Changes of heat pain threshold and MNCV

Compared with the control group, heat pain threshold and MNCV were significantly lowered in diabetes

Table 2. Changes of FPG.

Group	Dose	Before Administration of Tongxinluo	After Administration of Tongxinluo
Control	-	5.09±0.90*	5.82±1.05*
Diebetes	-	13.48±2.20	17.20±2.10
D+L	1	12.90±2.73	16.06±1.86
D+M	2	14.68±2.24	15.64±2.20
D+H	4	14.69±2.31	14.07±1.91

* P<0.05, vs diabetes group

Table 3. Changes of pain threshold and MNCV.

Group	Dose	Pain threshold	MNCV
Control	-	17.62±2.71*	24.43±0.03*
Diebetes	-	8.36±1.28	5.39±1.07
D+L	1	10.54±0.64	6.19±1.15
D+M	2	12.13±1.50*	8.89±1.25*
D+H	4	12.16±2.95*	9.92±1.59*

* P<0.05, vs diabetes group (same as the untreated Tongxinluo group).

group (P <0.05). However, heat pain threshold and MNCV were significantly increased in D+M and D+H groups (P <0.05) (Table 3).

Changes of the mRNA expression levels of IGF1, c-fos, NGF and BFGF

The mRNA levels of IGF1, c-fos, NGF and BFGF were detected by using the PCR assay. The mRNA expression levels of IGF1, NGF and BFGF in diabetes group were significantly lower than that of the control group, but c-fos mRNA expression level of diabetes group was higher than that of the control group (Figure 1A, Table 4, P<0.05). Compared with the diatetes group, mRNA expression levels of NGF and BFGF were significantly increased and mRNA expression levels of c-fos was significantly decreased in D+M and D+H groups (P <0.05) (Figure 1A, Table 4).

Changes of the protein levels of IGF1, c-fos, NGF and BFGF

The protein levels of IGF1, c-fos, NGF and BFGF were examined by using the western blot assay. Compared to the control group, the protein levels of IGF1, NGF and BFGF in diabetes group were significantly lower, but c-fos was significantly higher (Figure 1B, Table 5, P <0.05). Compared with the diatetes group, protein levels of NGF and BFGF were significantly increased and protein level of c-fos was significantly decreased in D+M and D+H groups (P <0.05) (Figure 1B, Table 5).

Discussion

According to the International Diabetes Federation, there are about 382 million diabetics 5.1 million death

Table 4. Changes of mRNA levels of IGF1, c-fos, NGF, and BFGF.

Group	Dose	IGF1	c-fos	NGF	BFGF
Control	-	1.02±0.22*	0.90±0.07*	1.11±0.13*	1.13±0.09*
Diebetes	-	0.28±0.01	4.59±0.24	0.33±0.06	0.28±0.03
D+L	1	0.25±0.03	2.63±0.14*	0.47±0.08	0.32±0.08
D+M	2	0.44±0.05*	2.43±0.13*	0.59±0.08*	0.69±0.12*
D+H	4	0.58±0.06*	2.48±0.12*	0.64±0.09*	0.71±0.04*

* P<0.05, vs diabetes group (same as the untreated Tongxinluo group).

Table 5. Changes of protein levels of IGF1, c-fos, NGF, and BFGF.

Group	Dose	IGF1	c-fos	NGF	BFGF
Control	-	0.96±0.11*	0.26±0.04*	0.95±0.16*	0.73±0.05*
Diebetes	-	0.27±0.04	0.86±0.11	0.29±0.05	0.28±0.04
D+L	1	0.36±0.07	0.61±0.10	0.35±0.04	0.62±0.08*
D+M	2	0.50±0.07*	0.56±0.07*	0.53±0.04*	0.61±0.07*
D+H	4	0.62±0.07*	0.41±0.05*	0.57±0.09*	0.61±0.12*

* P<0.05, vs diabetes group (same as the untreated Tongxinluo group).

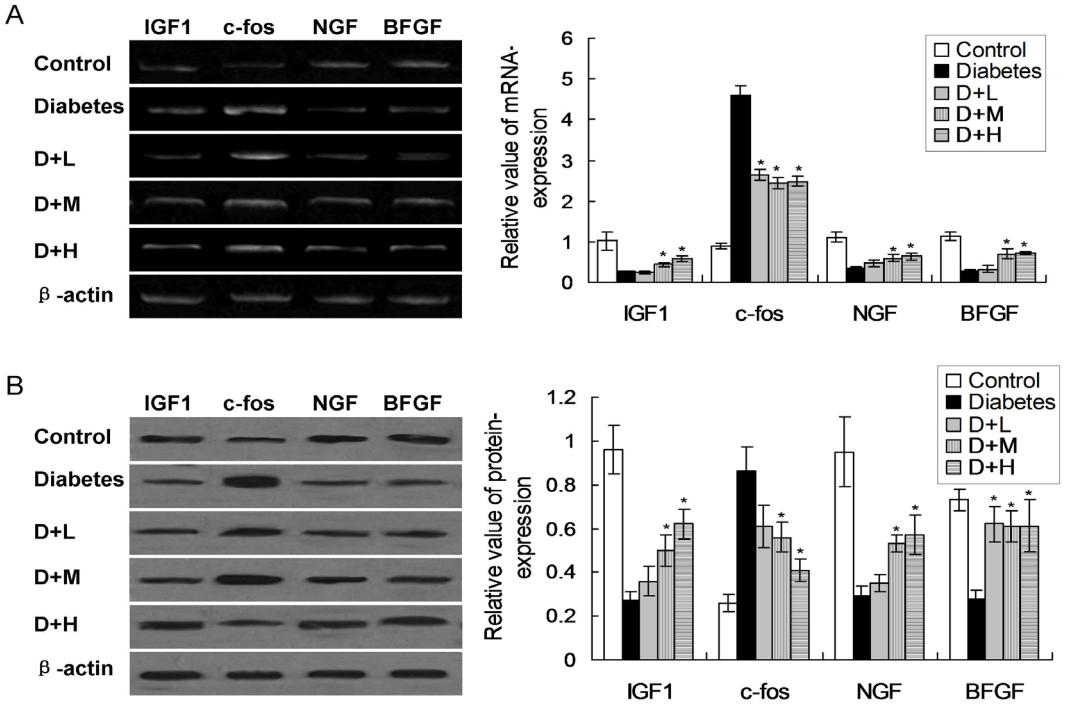

Figure 1. Observtion of the mRNA and protein levels of IGF1, c-fos, NGF and BFGF. A. The mRNA levels of IGF1, c-fos, NGF and BFGF were examined by using PCR assay. B. The protein levels of IGF1, c-fos, NGF and BFGF were examined by using western blot assay. * P<0.05 represents the mRNA or protein levels compared to the Diabetes group.

from diabetes worldwide in 2013. There will be 552 million patients with diabetes by 2030. Diabetes prevention and control has become an urgent problem (11). DPN refers to peripheral nerve dysfunction related symptoms in patients with diabetes. Although high blood sugar is one of the main causes of DPN, the pathogenesis of DPN is not entirely clear. It might be the result of many factors including vascular injury, metabolic disorders, cytokines abnormalities, oxidative stress, immune factors and neurotrophic factor deficiency. The widely accepted theories are theory of vascular and metabolic theory, suggesting that hyperglycemia-induced oxidative stress is an important factor of DPN (12,13). Oxidative stress can cause the polyol pathway activation, non-enzymatic glycation, protein kinase C activation, and hexosamine pathway activation, leading to tissue damage, endothelial function changes, axonal degeneration, and demyelinating disorders, resulting in the occurrence and development of DPN (14,15). There is currently no specific treatment for DPN, the drugs used in the treatment of DPN are based on multiple hypotheses of the pathogenesis of DPN. Common used drugs include an aldose reductase inhibitor, ganglioside, nerve growth factors, anticoagulants, and vasodilator drugs. The efficacy of those drugs differs from different reports.

According to traditional Chinese medicine theory, DPN is a common disorder characterized by numbness, cold, pain, and atrophy caused by consumptive thirst, deficiency of both qi and yin, syndrome of phlegm, and obstruction of meridians and collaterals. Chinese medicine treatment of DPN has some advantages in its multi-target and multi-channel manner. Tongxinluo capsules were made from leech, ginseng, scorpion, red peony, eupolyphaga, cicada, centipede, drop incense, sandalwood, frankincense, semen, and borneol. Tongxinluo can inhibit thrombosis, improve endothelial function, and protection microvascular through promoting blood circulation to remove meridian obstruction and smoothing the veins to relieve pain (16). Studies have shown (17) that Tongxinluo capsule can not only improve the clinical symptoms of DPN patients, but also can improve MNCV. KK/Upj-Ay mouse is a model of type 2 diabetes with DPN pathological features including partial sciatic nerve fiber layer thickness, unmyelinated nerve fibers hollowing, and axonal thinning. Therefore, this study uses KK/Upj-Ay mouse model to explore the efficacy of Tongxinluo capsule on nerve regeneration in mice with DPN to hopefully provide scientific references for clinical research and treatment of DPN.

The results of this study showed that FPG levels were significantly up-regulated in the diabetes group and supplementation of Tongxinluo did not cause significant decrease of FPG level, indicating Tongxinluo does not have significant blood glucose lowering effect. This might be related to the small sample size and needs to be further studied. Significantly reduction of heat pain threshold and MNCV were found in diabetes group. However, supplementation of Tongxinluo at doses of 2 g/Kg and 4g /Kg significantly increased heat pain threshold and MNCV, indicating that Tongxinluo capsule can improve nerve function and protect mice against DPN, which is consistent with other relevant findings (18). Compared to control group, diabetes group has significantly lowered mRNA expression levels of IGF1, NGF and BFGF and significantly elevated mRNA expression level of c-fos. Supplementation of Tongxinluo at doses of 2 g / Kg and 4 g / Kg significantly increased the expression levels of IGF1, NGF and BFGF and decreased the expression level of c-fos, indicating that decreased levels of neurotrophic factors in diabetic mice may lead to diabetic neuropathy or nerve atrophy and Tongxinluo capsule can inhibit this kind of response by promoting neuroregeneration, which is consistent with previous findings (19).

In summary, Tongxinluo capsule can improve nerve function, increase nerve conduction velocity, and promote nerve regeneration in mice with DPN. The underlying mechanism needs to be further elucidated.

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