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The protective effect of the active components of ERPC on diabetic peripheral neuropathy in rats

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ABSTRACT

Ethnopharmacological relevance: *Euonymus alatus, Radix trichosanthis, Panax notoginseng* and *Coptis chinensis* are popular plants used in traditional Chinese medicine to treat diabetes.

Aim of the study: The aim of the study is to investigate the therapeutic effect of the active components of *Euonymus alatus, Radix trichosanthis, Panax notoginseng* and *Coptis chinensis* (cERPC) on diabetic peripheral neuropathy in the rats and explore the underlying mechanism involved.

Methods: After diabetes was induced in rats for 20 weeks, cERPC or water was administered for 12 weeks. After a hot plate test, motor nerve conduction velocity and sciatic nerve blood flow were determined; the sciatic nerves were isolated for toluidine blue staining; and the fibre area, fibre diameter, axon area, axon diameter and myelin thickness were evaluated. The levels of the myelin basic protein, myelin protein zero, Oct6 and Krox20 were measured by western blot or immunofluorescence.

Results: cERPC was efficient in reducing the response latency, increasing motor nerve conduction velocity, enhancing sciatic nerve blood flow and ameliorating the pathological changes in diabetic rats. cERPC also had a role in increasing the levels of myelin basic protein and myelin protein zero and improving the expression of Oct6 and Krox20 in sciatic nerves of diabetic rats.

Conclusions: cERPC ameliorates diabetic peripheral neuropathy by attenuating electrophysiological, circulatory and morphological alterations, which is mediated by the Oct6-Krox20 pathway.

1. Introduction

Diabetic peripheral neuropathy (DPN) is a prevalent complication of diabetes, affecting more than 50% of diabetics aged >20 years (Pirart, 1977). It is characterised with the segmental demyelination and axonal degeneration of large fibres.

Because DPN can cause infections, foot ulcers, disability, limb amputation and mortality, pharmacologists have made considerable efforts to produce novel drugs, including antioxidants, protein kinase C (PKC)-β inhibitors, nerve growth factors, vasodilators and prostaglandin analogue. Most of these therapies, however, have led to disappointing clinical results, despite numerous promising trials in the cell culture and animal models of DPN (Callaghan et al., 2012). Therefore, new drugs that will improve outcome are urgently needed.

Abbreviations: DPN, diabetic peripheral neuropathy; PKC, protein kinase C; TCM, traditional Chinese medicine; MNCV, motor nerve conduction velocity; AGE, advanced glycation end products; RAGE, receptor of advanced glycation end products; NF-κB, nuclear factor-κB; MDA, malondialdehyde; CGRP, calcitonin gene related peptide (CGRP); cAMP, cyclic adenosine monophosphate; STZ, streptozocin (STZ); WB, western blot; IF, immunofluorescence; MBP, myelin basic protein (MBP); MPZ, myelin protein zero; PPARγ, peroxisome proliferator-agonist receptor γ; NO, nitric oxide; AMPK, adenosine monophosphate-activated protein kinase; DAPI, 4',6-diamidino-2-phenylindole

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Traditional Chinese medicine (TCM) has gained popularity in China because it is cost-effective and safe. In the treatment of chronic diseases, TCM has many advantages because of its ability to target multiple conditions with virtually no side effects. *Euonymus alatus* (Gui-Jian-Yu), *Radix trichosanthis* (Tian-Hua-Fen), *Panax notoginseng* (San-Qi) and *Coptis chinensis* (Huang-Lian) are among the commonly used TCMs to treat diabetes. The TCM theory explains that these four herbal medicines can promote blood circulation, remove blood stasis, nourish yin and remove toxic substances.

Some research studies have reported that the herbs have the potential to ameliorate hyperglycemia, insulin resistance, hypertension, hyperlipidemia and inflammation (Choi et al., 2013; Park et al., 2005; Prabhakar and Doble, 2011; Xie and Du, 2011; Zhai et al., 2016; Zhang and Liang, 2015), all of which are related to diabetes directly or indirectly. In addition, these herbs have been used to treat diabetic nephropathy or retinopathy (Chang et al., 2012; Gao et al., 2013). Further, *Panax notoginseng* has been shown to protect the neuronal cells from apoptosis and *Cortis chinensis* functions as an antioxidant in the neuronal cells (Friedemann et al., 2014; Li et al., 2009).

It remains to be investigated whether the Chinese medicine formula consisting of *Euonymus alatus, Radix trichosanthis, Panax notoginseng* and *Coptis chinensis* (ERPC) can improve diabetic neuropathy. Therefore, we evaluated the effect of ERPC on diabetic peripheral neuropathy in rats. The results indicated that a water extract of ERPC increased motor nerve conduction velocity (MNCV), reduced the response latency in hot plate tests and ameliorated the pathological changes of sciatic nerves in diabetic rats (Han et al., 2012a). Previous studies have shown that ERPC decreases advanced glycation end products (AGE), receptor of advanced glycation end products (RAGE) and nuclear factor-κB (NF-κB) (Han et al., 2012a); reduces the levels of malondialdehyde (MDA) and PKC (Han et al., 2012b); and elevates the expression of calcitonin gene related peptide (CGRP) and cyclic adenosine monophosphate (cAMP) (Han et al., 2012c).

The limitations of the water extract are that the bioactive components are unclear and are less in content; moreover, the water extract is too crude to control its quality. Consequently, we are inclined to use the representative components instead of water extract. The components of the four drugs that may be relevant to their putative effects include flavonoids of *E. alatus*, polysaccharide of *R. trichosanthis*, saponins of *P. notoginseng* and alkaloids of *C. chinensis*. Flavonoids of *E. alatus* have been reported to inhibit the α-glucosidase activities (Lee et al., 2007), improve the glucose uptake in the adipocytes (Yang et al., 2004), reduce the plasma glucose, ameliorate the glucose tolerance and alleviate the oxidative stress (Fang et al., 2008a, 2008b). Polysaccharide of *R. trichosanthis* lowers the blood glucose (Hikino et al., 1989). Saponins of *P. notoginseng* have been found to reduce the glucose in diabetic mice, lower the levels of triglycerides and total cholesterol and decrease inflammation and oxidation (Uzayisenga et al., 2014). Alkaloids of *C. chinensis* reduce glucose uptake (Yang et al., 2014), inhibit oxidative stress and attenuate neurodegenerative disease (Zhang et al., 2009).

Thus, the purpose of this study is to evaluate the effect of the putative active components of ERPC (cERPC), which consist of flavonoids of *E. alatus*, polysaccharide of *R. trichosanthis*, saponins of *P. notoginseng* and alkaloids of *C. chinensis* on DPN in rats and elucidate the underlying mechanisms. The findings of the study will reveal the bioactive components of ERPC and clarify the pharmacological mechanisms of cERPC. Furthermore, this study will lay a foundation for its clinical application for DPN.

2. Materials and methods

2.1. Ethical statement

All procedures involving animals and their care were carried out according to the governmental guidelines on animal experimentation and the National Institutes of Health's "Principles of Laboratory Animal Care". All the experimental protocols were approved by the Institutional Animal Ethics Committee of Beijing University of Traditional Chinese Medicine, Beijing, China (Permit Number: 12-1214).

2.2. Plant material

E. alatus (Gui-Jian-Yu), *R. trichosanthis* (Tian-Hua-Fen), *P. notoginseng* (San-Qi) and *C. chinensis* (Huang-Lian) were purchased from Tongrentang (Beijing, China). The herbs were authenticated by Dr. Peng Tan (College of Chinese Medicine, Beijing University of Chinese Medicine, Beijing). Voucher specimens (No TCM120305, 120306, 120307 and 120308) were deposited in the YIFU Building of Beijing University of Chinese Medicine.

2.3. Preparation of plant extract

E. alatus was extracted with 60% ethanol for two times, 2 h every time. Then, the sample was loaded on the AB-8 resin, desorbed with 50% ethanol after removing impurity, concentrated and dried.

P. notoginseng was extracted with 70% ethanol for three times, 1.5 h every time. The extract was merged and concentrated. Then, the sample was loaded on the HPD-300 resin, desorbed with 70% ethanol after removing impurity, concentrated and dried.

C. chinensis and *R. trichosanthis* were immersed for 2 h with 80 °C water. They were extracted twice, and the extracts were merged and vacuum concentrated at 60 °C. 95% ethanol was added to the concentrate to make the volume fraction of ethanol reach 60%. The sample was centrifuged and the precipitation was abandoned. The supernatant was concentrated and dried.

Ultraviolet spectrophotometry was used to determine the component content in the sample. Rutin was used as the standard to determine the content of total flavonoids, and its content was 1.021%; ginsenoside-Rb1,Rg1 and notoginsenoside R1 were used as the standard to determine the content of total saponins, and the content was 6.123%; berberine was used as the standard to determine the content of total alkaloids, and its content was 7%; the glucose was used as the standard to determine the content of total polysaccharides, and its content was 12.3%. The extracts of *E. alatus, R. trichosanthis, P. notoginseng* and *C. chinensis* were mixed and dissolved in water. The mixture (per g) contains flavonoids of *E. alatus* 0.14 g, polysaccharide of *R. trichosanthis* 0.33 g, saponins of *P. notoginseng* 0.36 g, and alkaloids of *C. chinensis* 0.17 g.

2.4. HPLC-MS^n analysis

HPLC-MS^n analysis was performed on a LTQ-Orbitrap XL (Thermo, Fisher, USA). The HPLC instrument was equipped with an auto sampler, a quaternary pump and a column compartment. Samples were separated on a Zorbax Extend-C18 column (150 mm×4.6 mm I.D., 5 µm). The mobile phase consisted of acetonitrile (A) and water containing 0.05% (v/v) ammonia water (B). A gradient program was used as follows: 0 min, 5:95 (A:B, v/v); 10 min, 16:84; 45 min, 32:68; 60 min, 49:51. A 10 min post-run time was set to fully equilibrate the column. The flow rate was 0.8 ml/min. The column temperature was 30 °C. The sample injection volume was 10 µl. The HPLC eluent was introduced into the ESI source of the mass spectrometer in a post column splitting ratio of 5:1. For MS detection, high purity nitrogen (N_2) was used as the nebulizing gas, and ultra-high pure helium (He) as the collision gas. Positive ion polarity modes were used for compound ionization. In the negative ESI ion mode, the capillary voltage was 15 V, and the tube lens offset voltage was 37 V. For full scan MS analysis, spectra were recorded in the range of *m/z* 100–1500.

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