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Ameliorative Effect of *Hedysarum polybotrys* Polysaccharide on Neural Tissue Fibrosis in Diabetic Peripheral Neuropathy Mice and its Mechanisms

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Abstract: *Objective*: This study aimed to investigate the role of *Hedysarum polybotrys* polysaccharide (HPS) in ameliorating neural tissue fibrosis in diabetic peripheral neuropathy (DPN) mice. *Methods*: Fifty DPN mice were selected and randomly divided into five groups (n = 10), which were the model group, positive control group (receiving only 4 mg/(kg-d) of α-lipoic acid), high-dose HPS group (200 mg/(kg-d) of HPS was given per day), medium-dose HPS group (100 mg/(kg-d) of HPS was given per day), and low-dose HPS group (50 mg/(kg-d) of HPS given daily). In addition, non-diabetic C57BL/6 wild-type mice were selected as the normal group (n = 10). The expression levels of Keap1 and Nrf2 proteins and their mRNAs in the sciatic nerve tissues of mice in each group were analyzed by Western blot technique and real-time fluorescence quantitative PCR. *Results*: Compared with the normal group, the expression of Keap1 protein and mRNA was increased, while the expression of Nrf2 protein and mRNA was decreased in the sciatic nerve of mice in the model group ($P < 0.05$). Compared with the model group, Keap1 protein and mRNA expression decreased, while Nrf2 protein and mRNA expression increased in the control and high and medium dose HPS groups of mice ($P < 0.05$). *Conclusion*: HPS may inhibit fibrosis of neural tissue and ameliorate nerve injury in DPN mice by regulating the Keap1/Nrf2 signaling pathway. This effect was associated with enhanced antioxidant capacity, promotion of Nrf2 activation, and increased antioxidant gene expression by HPS. Therefore, HPS has a potential therapeutic value to ameliorate DPN-associated nerve injury.

Keywords: Hedysarum polybotrys polysaccharide; Diabetic peripheral neuropathy variant; Kelch1; Nrf2

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1. Introduction

Diabetic peripheral neuropathy (DPN) is a common complication that mainly affects the peripheral nerves leading

to sensory, motor, and autonomic dysfunction [1]. Symptoms of DPN include limb numbness, tingling, pain, and muscle weakness, which in severe cases may lead to foot ulcers and other complications. Effective management and treatment are essential to improve the quality of life of patients [2]. *Hedysarum polybotrys* polysaccharide (HPS), a natural active ingredient extracted from the traditional Chinese medicine *Radix Astragali*, is known for its multiple pharmacological activities, including antioxidant, anti-inflammatory, and immunomodulatory functions [3–4]. Recent studies have found that HPS has some efficacy in DPN treatment, and its mechanism may be related to its ability to enhance the body's antioxidant capacity, thereby reducing the damage caused by oxidative stress [5]. This study aimed to investigate the ameliorative effect of HPS on neural tissue fibrosis of DPN in mice and its potential mechanism and to provide new ideas and theoretical basis for the prevention and treatment of DPN.

2. Materials and methods

2.1. Materials

2.1.1. Experimental animals

Six-week-old male SPF-grade NOD mice and C57BL/6 wild-type mice were selected (Suzhou Saiye Biotechnology Company). They were fed and watered freely in the SPF animal house with a light cycle of 12 hours, a room temperature of 22 ± 2°C, and a humidity of 50% ± 10%. The operation followed the regulations of the Animal Ethics Committee to ensure animal welfare.

2.1.2. Drugs and reagents

The drugs and reagents used are shown in **Table 1**.

Table 1. Sources of drugs and reagents

Drug name	Source
Hedysarum polybotrys polysaccharide (HPS)	Xinyang Mufan Biotechnology Co.
α-Lipoic acid capsule (300 mg/capsule)	Thermo Fisher Scientific
Keap1 monoclonal antibody	Thermo Fisher Scientific
Nrf2 monoclonal antibody	Thermo Fisher Scientific

2.1.3. Main instruments

The main instruments used in this study included SpectraMax Mini enzyme labeling analyzer (Shanghai Meigu Molecular Instrument Co., Ltd.), physiological recorder (Shanghai ShapeZoo Digital Technology Co., Ltd.), protein electrophoresis instrument, transmembrane instrument, and gel imaging analysis system, as well as real-time fluorescence quantitative PCR instrument (all purchased from Thermo Fisher Scientific).

2.2. Experimental methods

2.2.1. Model preparation

In this experiment, 6-week-old male NOD mice were used, which were divided into five groups of 10 mice each, including the model group, the positive control group, and the high-, medium- and low-dose HPS groups. Ten non-diabetic C57BL/6 wild-type mice were also selected as the normal group.

2.2.2. Drug administration method

All mice in the experimental groups were administered by gavage for 8 weeks. The specific dosing regimen

was as follows. Positive control group: α-lipoic acid was given at a dose of 4 mg per kg per time. HPS group: HPS was given to the high, medium, and low dose groups at doses of 200, 100, and 50 mg per kg per time, respectively. Model group and normal control group: an equal amount of saline was given at a dose of 5 mL per kg per time.

2.2.3. Sample collection

After 8 weeks of drug administration, blood samples were obtained from mice in each group by eyeball blood sampling. Subsequently, the mice were executed and dissected, and the right sciatic nerve tissue was removed. The collected sciatic nerve tissue was rinsed with pre-cooled saline to remove impurities. After absorbing the surface moisture, it was placed in liquid nitrogen for rapid freezing and finally transferred to a refrigerator at -70°C for storage for subsequent analysis.

2.2.4. Immunoblotting for Keap1 and Nrf2 protein expression

Protein extraction and quantification: about 50 mg was removed from the right sciatic nerve tissue preserved at minus 70°C, and total protein extraction was carried out according to the kit instructions. After the extraction was completed, the protein concentration was determined by the BCA method or other suitable methods.

Protein denaturation and electrophoresis: According to the measured protein concentration, each group of samples was denatured and the protein samples were separated using SDS polyacrylamide gel electrophoresis.

Membrane transfer and closure: membrane transfer is the transfer of separated Keap1 and Nrf2 proteins onto nitrocellulose membranes by electro-transfer method, which is usually carried out under electro-transfer conditions, such as 100 volts for 1 h. The transfer step is carried out at room temperature, using SDS polyacrylamide gel electrophoresis. The sealing step is performed by sealing the membrane using 5%–10% skimmed milk powder or BSA solution at room temperature, usually for 1 hour to reduce non-specific binding.

Antibody incubation: in the primary antibody incubation step, primary antibodies against Keap1 and Nrf2 are diluted, added to the membrane, and incubated overnight at 4 or for 1–2 hours at room temperature. Wash the membrane with PBS or TBST to remove unbound antibodies, add fluorescent labeling, incubate for 1 hour at room temperature, and wash the membrane again.

Develop and analyze: Develop or image the membrane (against the enzyme-labeled antibody) using an appropriate substrate (such as chemiluminescent substrate), or use a fluorescent imaging system. For data analysis, the relative expression levels of proteins are analyzed by the imaging software, which is usually normalized using an internal reference protein (such as β-actin or GAPDH) to ensure the reliability of the results.

2.2.5. Real-time fluorescence quantitative PCR to detect Keap1 and Nrf2 mRNA expression

Total RNA extraction and reverse transcription: 50 mg of right sciatic nerve tissue was collected and total RNA was extracted using TRIzol. RNA concentration and purity were determined by spectrophotometry, gel electrophoresis was performed to check the integrity, and 2 μg of RNA was transcribed into cDNA using a reverse transcription kit for PCR analysis.

Real-time fluorescent quantitative PCR: To construct the qPCR reaction system, a cDNA template, specific primers for Keap1 and Nrf2, fluorescent dye (e.g., SYBR Green), and a PCR reaction buffer were added. Transfer the reaction system to the qPCR instrument, set appropriate cycling conditions for PCR amplification, and record the fluorescence signal of each cycle to monitor the DNA amplification process.

Data analysis: at the end of amplification, the Ct values of Keap1 and Nrf2 were compared with those of the internal reference genes (such as GAPDH or β-actin) by analyzing the Ct values (the number of threshold cycles) to calculate the relative expression.

2.3. Statistical methods

Statistics were performed using SPSS 27.0, and the measurement data were expressed as mean ± standard deviation (mean ± SD), and *t*-tests were performed between groups. Statistical significance was indicated by $P < 0.05$.

3. Results

3.1. Comparison of Keap1 and Nrf2 protein expression in sciatic nerve tissues of mice in each group

Immunoblotting results showed that Keap1 protein was significantly higher and Nrf2 protein was significantly lower in the model group compared with the normal group ($P < 0.05$). Compared with the model group, Keap1 protein was significantly lower and Nrf2 protein was significantly higher in the control group and high and medium dose groups (P<0.05). See Tab 2.

Table 2. Comparison of Keap1 and Nrf2 protein expression in each group (mean ± SD)

Groups	Nrf2 protein	Keap1 protein
Normal group	0.90 ± 0.03	0.61 ± 0.08
Model group	0.50 ± 0.06[a]	1.13 ± 0.07[a]
Control group	0.87 ± 0.04[A]	0.67 ± 0.06[A]
Low dose group	0.59 ± 0.08	0.97 ± 0.03
Medium dose group	0.75 ± 0.09[A]	0.84 ± 0.11[A]
High dose group	0.84 ± 0.08[A]	0.87 ± 0.12[A]

Note: a indicates $P < 0.05$ compared with the normal group; A indicates $P < 0.05$ compared with the model group

3.2. Comparison of Keap1 and Nrf2 mRNA expression in sciatic nerve tissue of mice in each group

RT-qPCR results in **Table 3** showed that Keap1 mRNA was significantly higher and Nrf2 mRNA was significantly lower in the model group compared with the normal group ($P < 0.05$). Compared with the model group, Keap1 mRNA was significantly lower and Nrf2 mRNA was significantly higher in the control group and high and medium dose groups ($P < 0.05$)

Table 3. Comparison of Keap1 and Nrf2 mRNA expression in each group (mean ± SD)

Groups	Nrf2 protein mRNA	Keap1 protein mRNA
Normal group	1.00 ± 0.00	1.00 ± 0.00
Model group	0.38 ± 0.05[a]	4.13 ± 0.14[a]
Control group	0.84 ± 0.07[A]	1.36 ± 0.09[A]
Low dose group	0.53 ± 0.05	3.87 ± 0.13
Medium dose group	0.72 ± 0.08[A]	2.44 ± 0.10[A]
High dose group	0.83 ± 0.08[A]	1.47 ± 0.15[A]

Note: a indicates P < 0.05 compared with the normal group; A indicates P < 0.05 compared with the model group

4. Discussion

HPS is an active ingredient extracted from the traditional Chinese medicine *Astragalus membranaceus*, which possesses a variety of pharmacological activities, including antioxidant and anti-inflammatory effects. In recent years, the potential role of HPS in the treatment of DPN has attracted widespread attention. Under normal physiological conditions, the binding of Nrf2 protein to Keap1 causes it to stay in the cytoplasm, at which time the activity of Nrf2 is inhibited and cannot perform its antioxidant function. When cells are subjected to oxidative stress or other deleterious stimuli, the conformation of Keap1 changes, and this change causes the binding of Nrf2 to Keap1 to be broken [6–7]. As a result, Nrf2 detaches from Keap1, which in turn translocates to the nucleus. Nrf2 enters the nucleus and binds to the antioxidant response element (ARE), which initiates the transcription of antioxidant genes [8]. Enhanced expression of these genes significantly enhances the antioxidant capacity of cells, enabling them to more effectively protect against oxidative stress and other potential damages. By regulating the activity of antioxidant genes, Nrf2 can enhance cellular defense mechanisms to ensure that cells maintain their normal functional and physiological states in response to environmental stress.

The present study showed that HPS significantly reduced Keap1 expression and elevated Nrf2 expression in mouse sciatic nerve. This suggests that HPS improves the occurrence and development of DPN by activating the Nrf2/Keap1 signaling pathway, enhancing antioxidant capacity, and alleviating oxidative stress damage. The regulation of the Nrf2-Keap1 pathway by HPS can be achieved through the following mechanisms. Promoting the entry of Nrf2 into the nucleus of the cell: HPS may enhance the expression of Nrf2 and Keap1 by decreasing the expression level of Keap1 or promoting its degradation. separation between Nrf2 and Keap1, thereby enhancing the biological activity of Nrf2. This mechanism helps Nrf2 to enter the nucleus more efficiently to fulfill its function of antioxidant action and regulation of cellular stress response and to enhance its transcriptional activity [9]. Enhancement of ARE activity: HPS can directly or indirectly enhance the activity of ARE through a variety of mechanisms, and this enhancement not only contributes to the activation of Nrf2 but also promotes the expression of antioxidant genes related to it, thus effectively enhancing the ability of cells to resist oxidative stress [10]. Thus, HPS can activate the Nrf2-Keap1 signaling pathway by regulating Keap1 and Nrf2 expression in the sciatic nerve, which enhances the antioxidant capacity of neuronal cells and has a significant anti-inflammatory effect, thus contributing to the amelioration of the various symptoms experienced by DPN patients. The findings of this study provide a new perspective and theoretical basis for the treatment of HPS in DPN, suggesting that HPS may be an effective intervention to help alleviate the discomfort associated with this type of neurological disease.

In summary, HPS has an ameliorative effect on mouse neural tissue fibrosis, and its mechanism may be related to the ability to up-regulate the expression of Nrf2, activate the Nrf2/Keap1 signaling pathway, improve the body's antioxidant capacity, and attenuate oxidative stress damage.

Disclosure statement

The authors declare no conflict of interest.

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