

Effects of Hedysari Polysaccharides on Regeneration and Function Recovery Following Peripheral Nerve Injury in Rats

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Abstract: It has been demonstrated that aqueous extract of Radix Hedysari Prescription and modified Radix Hedysari Prescription could improve the regeneration of injured peripheral nerve. Radix Hedysari is a main component in these two formulas. We hypothesized that Hedysari polysaccharides (HPS), a main active ingredient, could also enhance peripheral nerve regeneration after nerve injury in adult animals. In the present study, we examined the effects of HPS on sciatic nerve regeneration for 6 weeks following clamping in rats (administrated orally of 2 ml HPS liquid daily, 0.25 g/ml). The results showed that HPS was able to enhance sciatic function index (SFI) value, tibial function index (TFI) value, peroneal nerve function index (PFI) value, conduction velocity, and the number of regenerated myelinated nerve fibers, suggesting the potential clinical application of HPS for the treatment of peripheral nerve injury in humans.

Keywords: Hedysari Polysaccharides; Nerve Regeneration; Motor Function Recovery; Peripheral Nerve Injury.

Introduction

Traditional Chinese medicine, which has been used in China for hundreds of years, plays an important role in clinical application. Radix Hedysari is the root of Hedysarum polybotrys Hand.-Mazz., which has tonifying and diuretic as well as circulatory effects, is used to blend in various formulas. In spite of unclear effective ingredients, it was found that the Radix Hedysari had a major effect on the circulatory, urological, and immune systems. Radix Hedysari contains a significant amount of polysaccharides, which have been confirmed to own various biological properties.

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Traumatic injury, congenital anomalies and tumor extirpation may result in damage to or the complete sacrifice of critical nerves. Failure to restore injured nerves can lead to loss of muscle function, impaired sensation and/or painful neuropathies (Dahlin and Lundborg, 2001; Frostick *et al.*, 1998). Traditionally, functional nerve defects have been remedied by many methods, including nerve transfer (Oberlin *et al.*, 1994), nerve grafts, artificial nerve conduit bridging (Madhally and Matthew, 1999), and end-to-side neuroorrhaphy (Viterbo *et al.*, 1994). However, these methods only provide a regenerative environment for injured nerves. Recovery of function depends on various local and systemic factors. Regeneration of axons from the proximal stump of an injured nerve to the distal nerve stump is surely one of the most important factors in reinnervation of peripheral tissue. Recent studies have shown that locally applied neurotrophins can enhance survival of damaged neurons and regrowth of lesioned axons in the central and peripheral nervous systems in rats (Chen *et al.*, 2001). However, the beneficial effect of systemically administered neurotrophins on axonal regeneration is largely limited by enzymatic degradation. In addition, systemically delivered neurotrophins can have unexpected side-effects such as the toxicity of the circulating protein (ALS CNTF Treatment Group, 1996). Therefore, it is important to find chemicals that can produce neurotrophin-like effects on axonal regeneration without any enzymatic degradation and toxicity problems.

Based on our previous studies of Radix Hedysari *in vivo*, in the present study we hypothesized that systemic administration of Hedysari polysaccharides in rats could enhance peripheral nerve regeneration. To test this hypothesis, we examined the effect of daily systemic treatment on axonal regeneration and the recovery of motor function following a single clamping lesion of the sciatic nerve.

Materials and Methods

Materials

Radix Hedysari was purchased from Gansu Province, China. All chemicals used were of analytical grade. Physiograph (synergy) was purchased from Oxford Co.

Preparation of HPS

Dried Hedysari (2 kg) was ground into powder and defatted with boiling ethanol for 2 hours. The residue was volatilized at room temperature. Then, the pretreated dry powder was extracted twice with boiling deionized water (water to Hedysari ratio (ml/g) at 10:1) for 2 hours. The extract was filtered and the filtrate was then concentrated to 1/10 volume with a rotary evaporator at 65°C under vacuum. The proteins in the extract was removed by using the Sevag reagent (Navarini *et al.*, 1999). After removal of the Sevag reagent, the supernatant was precipitated by the addition of anhydrate ethanol to a final concentration of 80% (v/v) at 4°C overnight. Finally, the precipitates was collected by centrifugation, washed with acetone, dissolved in deionized water and lyophilized. Dark reddish brown crude water-soluble polysaccharides were obtained. The content of polysaccharides was determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956).

Animals and Treatment

Male Sprague-Dawley rats (Vital River Laboratories, Beijing, China) weighing 200 ± 10 g were maintained under specific pathogen-free laboratory conditions with free access to pellet food and water and kept under a 12 hours light/dark cycle. They were divided into 3 groups at random (30 animals in each group). Beginning from the second day of the operation, rats in the control group and model group were treated with 2 ml 0.9% NaCl by oral gavage once daily for 6 weeks, and rats in the treatment group were treated with 2 ml HPS liquid (0.25 g/ml) in the same style at the same fixed time daily. In the present work, every effort was made to minimize animal suffering and reduce the number of animals used according to the Chinese guidelines for care and use of laboratory animals.

Surgical Procedures

Surgical procedures were carried out under a binocular surgical microscope by using a microsurgical technique. Rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.). After anesthesia, left rat limbs were treated in a sterile manner. The sciatic nerve and its two main branches (the common peroneal nerve and the tibial nerve) were exposed. The sciatic nerves of rats in model group and treatment group were injured by clamping at a level 5 mm distal to the bifurcation for 1 min using pincers with 2 mm width, and the sciatic nerves of rats in control group were not injured. Subsequently, the muscle incision was sutured and the wound was closed by using 4-0 nylon sutures.

Walking Track Analysis

Animals were tested in a confined walkway that had a dark shelter at the end of a corridor (10×50 cm). The rats consistently walked to the dark shelter after several trials during which they often stopped to explore the corridor. The bottom of the track was lined with white paper. Tracks were obtained by dipping 2 rat's hind feet into ink before the animal was placed at the entrance of the corridor. Paw prints from both sides appeared immediately on the paper.

Paired footprint parameters for footprint length, distance from 1st to 5th toe (toe spread) and distance from 2nd to 4th toe (intermediary spread) were recorded for the right normal control foot (NPL, NTS, NIT) and the corresponding left experimental foot (EPL, ETS, EIT) for each rat. Prints for measurement were chosen at the time of walking, based on clarity and completeness at a point when the rat was walking briskly.

Three specific factors were calculated for each of the 3 print measurements (print length, toe spread, intermediary toe spread) by taking the difference between the normal and experiment values and dividing by the normal value as follows:

$$\text{Print length factor (PLF)} = (\text{EPL} - \text{NPL})/\text{NPL};$$

$$\text{Toe spread factor (TSF)} = (\text{ETS} - \text{NTS})/\text{NTS};$$

$$\text{Intermediary toe spread factor (ITF)} = (\text{EIT} - \text{NIT})/\text{NIT}.$$

These factors were then incorporated into the Bain-Mackinnon-Hunter (BMH) sciatic function index (SFI) formula, BMH tibial function index (TFI) formula, and BMH peroneal

nerve function index (PFI) formula:

$$\text{SFI} = -38.3 (\text{PLF}) + 109.5 (\text{TSF}) + 13.3 (\text{ITF}) - 8.8;$$

$$\text{TFI} = -37.2 (\text{PLF}) + 104.4 (\text{TSF}) + 45.6 (\text{ITF}) - 8.8;$$

$$\text{PFI} = 174.9 (\text{PLF}) + 80.3 (\text{TSF}) - 13.4.$$

Electrophysiological Study

Electrophysiological assessment was conducted prior to sacrifice of the animals. First, rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.). The left sciatic nerves were exposed, and stimulating bipolar electrodes were placed at its injured site and distal site in the model group and treatment group, while at a level 5 mm proximal to the bifurcation and its distal site in control group. The recording electrode was placed in the gastrocnemius muscle, while the ground electrode went subcutaneously between the stimulating and recording electrodes. Rectangular pulses (duration 0.1 ms, 0.9 mA, 10 Hz) were used to stimulate the sciatic nerves. Compound muscle action potential was recorded and nerve conduction velocity (NCV, m/s) was obtained semiautomatically by dividing the distance between the 2 stimulating sites by the difference in the onset latency.

Histological Study

The entire sciatic nerve was removed from each rat. Tissues were then harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 12 hours at room temperature. After that, the nerves were rinsed twice in phosphate buffer, and then postfixed in 1% osmium tetroxide for 18 hours. After this step, the tissues were dehydrated through a graded series of ethanol. Specimen were cut into 5 mm long from the injured site to the bifurcation and embedded in paraffin. They were transected into slices with thicknesses of 5 μm and evaluated under a microscope. Myelinated axons were quantified according to the unbiased counting rule. Finally, the total number of myelinated axons was estimated by multiplying the axonal density by the total cross-sectional area of the whole nerve in each animal.

Statistical Analysis

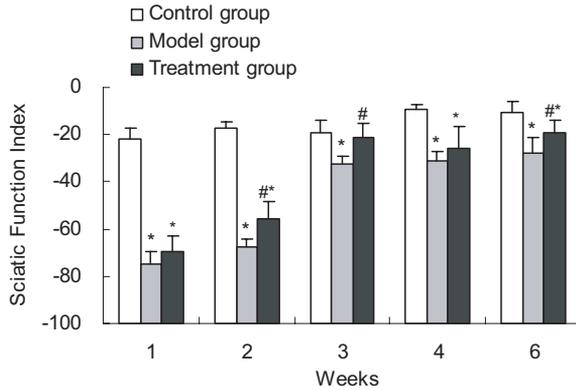
The results were expressed as mean \pm standard deviation. The difference among control group, model group, and treatment group were evaluated using one way ANOVA. p value less than 0.05 was considered statistically significant.

Results

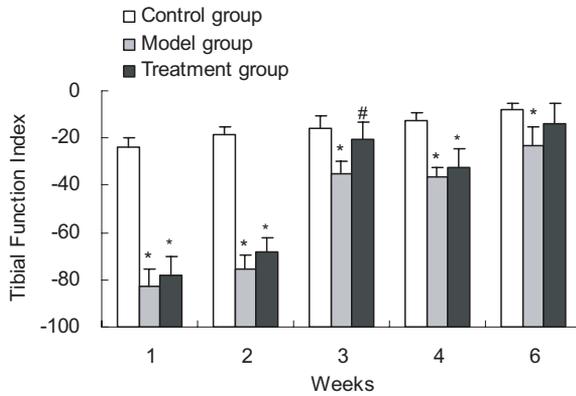
Motor Function Recovery of Injured Sciatic Nerve

The tests for motor function were equivalent for both hind limbs of rats. The hind limb that received a sham operation to the sciatic nerve was used as a blank control, and the hind limb that received an operation and treated with 0.9% NaCl was used as a negative control.

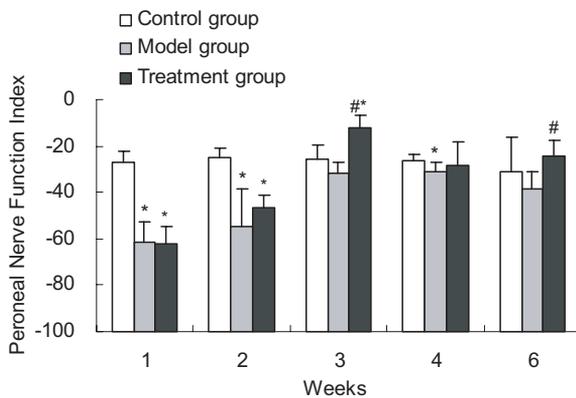
As shown in Figs. 1a and 1b, for SFI and TFI values, the control group values were about -20 at week 1, and rose slowly to about -10 at week 6. Model group values were



(a)



(b)



(c)

Figure 1. Effects of Hedysari polysaccharides on the motor function recovery evaluated by sciatic function index (SFI) (a), tibial function index (TFI) (b), and peroneal nerve function index (PFI) (c). SFI, TFI, and PFI values are significantly better in rats of treatment group than those of model group. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the model group ($n = 6$).

recovered from about -80 up to about -25 , all the values were significantly different from the control group values. Treatment group values were similar to but rose faster than those of the model group; SFI value at week 2 and TFI value at week 3 were significantly different from model group.

As shown in Fig. 1c, PFI values in the control group were about -25 all the time. Values in the model group and treatment group were about -60 at week 1, and then the values were similar to that of the control group at week 3. However, values in the treatment group were significantly different from those in model group at week 3 and week 6.

Conduction Function Recovery of Injured Sciatic Nerve

Electrophysiological assessment was conducted prior to the collection of the nerves. The motor NCV of the normal sciatic nerve was 42.3 ± 3.3 m/s at week 1, and rose slowly afterwards. No signal was recorded in the distal tibial nerve stump in the model group and treatment group at week 1, but they rose quickly. As shown in Fig. 2, values of the treatment group were significantly different from those of model group at week 2 and week 6.

Axonal Regeneration of Injured Sciatic Nerve

Histochemical staining of cross-sections of rat sciatic nerve with osmium tetroxide is an efficient method for measuring myelinated nerve fiber area as well as for counting myelinated nerve fibers (Fig. 3a). The sizes of the individual myelinated nerve fibers of regenerated nerves in model group and treatment group were significantly smaller than those in the control group. However, there was no significant difference of the sizes of individual myelinated nerve fibers between the model group and the treatment group. The number of myelinated

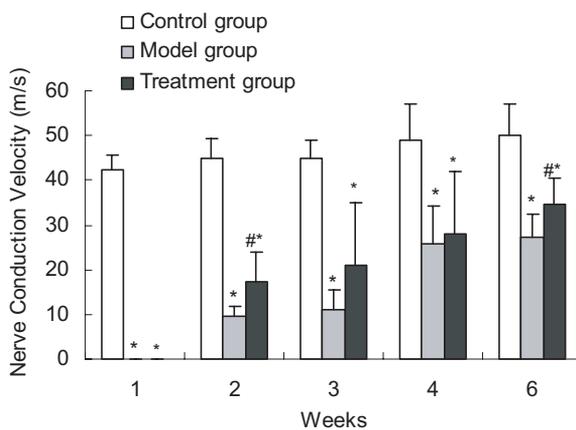


Figure 2. Effects of Hedysari polysaccharides on the conduction function recovery. Conduction values in rats of treatment group are significantly better than those in model group at week 2 and week 6. * $p < 0.05$ vs. the control group, # $p < 0.05$ vs. the model group ($n = 6$).

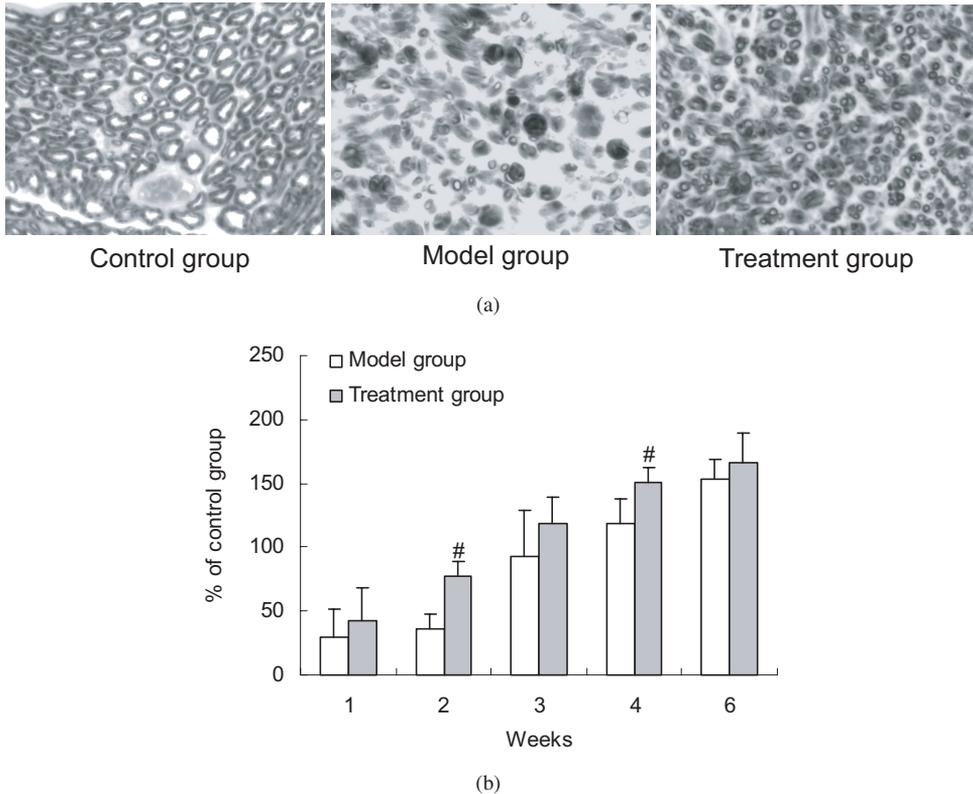


Figure 3. Effects of Hedysari polysaccharides on the axonal regeneration. (a): Histological micrographs of nerve tissues stained with osmium tetroxide (magnification = 400 \times). The nerve tissues were harvested from the same location in control, model, and treatment groups at week 2. The sizes of the individual myelinated nerve fibers of regenerated nerves in both of model and treatment groups were significantly smaller than that in control group. (b): Effects of Hedysari polysaccharides on the total number of regenerated nerve fibers. The total number in treatment group was significantly higher than that in model group at week 2 and week 4. # $p < 0.05$ vs. the model group (n = 6).

fibers of regenerated nerves rose similarly in the model group and the treatment group, but rose faster in the treatment group, especially at week 2 and week 4 (Fig. 3b).

Discussion

Radix Hedysari is a root of *Hedysarum polybotrys* HAND.-MAZZ. or *H. tanguticum* Fedtsch, *H. limprichtii* Ulbr., which belong to the leguminosae. Radix Hedysari is one of the frequently used crude medicines by Chinese physicians for a long time. It was reported that aqueous extract of *H. austrosibiricum* might have anti-aging effect, which is implemented by eliminating oxygen free radicals, raising activities of antioxidases (Hailiqian *et al.*, 2007). Radix Hedysari could prevent osteoporosis and disorder of bone metabolism caused by prednisone acetate (Su *et al.*, 2005), has a significant anti-CB₄V effect at cell

level (Zhang *et al.*, 2005), improve respiratory distress syndrome in rats (Bai *et al.*, 2003), improve items of hemorrheology and activating blood (Kou *et al.*, 2003), and act as a growth factor of B cells by increasing the proportion of the total B cells and activated B cells (Song *et al.*, 2000). Polysaccharides are a main substance in Radix Hedysari, and exhibited various pharmacological properties. For instance, Hedysari polysaccharides protect the endomembrane barrier and prevent the occurrence and development of arteriosclerosis, delay arteriosclerosis (Zhang *et al.*, 1998), enhance the cytotoxicity of LAK cell or PBMC against EJ cell and BTC when simultaneously applied (Cui *et al.*, 1998), play an important role in reducing free radical and increasing activity of SOD and GSH-px, cut down the blood sugar of diabetic rats and perfect the relevant indexes in tissue fluid of kidney (Jin *et al.*, 2004a; Jin *et al.*, 2004b), control weight, decrease the content of FPG, increase ISI, reduce insulin resistance, improve insulin sensitivity, and reduce the contents of IL-6 in blood serum of rats with insulin resistance of Type 2 diabetes mellitus (Jin *et al.*, 2007), show anti-cancer effects by inhibiting the cell growth, arresting the G2/M phase, inducing apoptosis, and down-regulating bcl-2 protein expression (Li *et al.*, 2007). In addition, it has also been reported that the formulas incorporating the Radix Hedysari may affect the regeneration of injured nerves. Aqueous extract of Radix Hedysari Prescription can promote the growth of Schwann cells by receptor-Camp- PKA signal pathway, and promote the differentiation of Schwann cell by activating PTKs signal pathway (Jiang *et al.*, 2002). Moreover, the local use of aqueous extract of Radix Hedysari Prescription can facilitate the repair of peripheral nerve injury (Dang *et al.*, 2002). In addition, aqueous extract of modified Radix Hedysari Prescription can facilitate the regeneration of peripheral nerves, but has no significant difference with aqueous extract of Radix Hedysari Prescription on the curative efficiency, though the outcome is more special and superior to that of Buyang Huanwu Decoction (Wei *et al.*, 2004). The underlying mechanism is promoting the expression or transportation of neurotrophic factors such as bFGF, NGF, and Trk (Yang *et al.*, 2006).

It is presumed that Hedysari polysaccharides may be the active ingredients in Radix Hedysari which have effects on the regeneration and functional recovery following peripheral nerve injury. In this study, we examined the effect of Hedysari polysaccharides on the motor function recovery, conduction function recovery, and axonal regeneration of injured sciatic nerve in rats.

The rat sciatic nerve model is a widely used model for the evaluation of both motor and sensory nerve function. Bain-Mackinnon-Hunter (BMH) SFI formula is usually used as the sciatic function index. An SFI of 0 is normal, and SFI of -100 indicates total impairment (Kanaya *et al.*, 1992). The walking track analysis clearly demonstrated that there was a direct relationship between individual hind limb muscle function and print measurements (Reynolds *et al.*, 1996). A similar function assay of peroneal and posterior tibial nerve lesions was developed later (Bain *et al.*, 1989). Hare *et al.* (1992) suggest that tibial function index (TFI) and peroneal nerve function index (PFI) may provide a more reliable and sensitive method for evaluating nerve regeneration than the sciatic nerve. In addition, traditional methods of assessing nerve recovery following peripheral nerve injury and repair, such as electrophysiology and histomorphometry were included in the present study. It showed that Hedysari polysaccharides, administered at a clinically relevant dosage, produced a significant

positive effect on the motor function recovery and conduction function recovery in adult rats. The total numbers of regenerated myelinated nerve fibers were increased in both model group and treatment group. However, there was a significant difference between 2 groups at week 2 and week 4. These results suggest that Hedysari polysaccharides are able to improve functional recovery following injury by increasing the total number of regenerated myelinated nerve fibers in adult rats.

At present, with regard to the treatment of peripheral nerve injury, although locally applied neurotrophins is confirmed to be efficient, the cost is high for patients, and the beneficial effects of systemically administered neurotrophins is limited by enzymatic degradation in the blood and obvious toxic effects caused by high-dose, frequent administration (ALS CNTF Treatment group, 1996). Radix Hedysari and Hedysari polysaccharides have been safely used clinically for many years and Hedysari polysaccharides, a mixture of many compounds, may improve regeneration of injured nerve by many mechanisms. Although there was a significant difference between the treatment group and the model group at an earlier time, there was no difference at a later time. It is suggested that the effect of HPS might not be intermittent. Although the difference is not significant, there are some typically high values. At the same time, some typically low values make the mean value low. If more rats were used, there might be a significant difference. In addition, some factors from operators and rats might affect the results as well. Despite being less effective than that of neurotrophins, Hedysari polysaccharides may be used to assist in the application of neurotrophins to enhance axonal regeneration in the nervous system and cut down the dosage of neurotrophins to reduce the toxic effects in humans.

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