Local administration of icariin contributes to peripheral nerve regeneration and functional recovery

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Abstract
Our previous study showed that systemic administration of the traditional Chinese medicine Epimedium extract promotes peripheral nerve regeneration. Here, we sought to explore the therapeutic effects of local administration of icariin, a major component of Epimedium extract, on peripheral nerve regeneration. A poly(lactic-co-glycolic acid) biological conduit sleeve was used to bridge a 5 mm right sciatic nerve defect in rats, and physiological saline, nerve growth factor, icariin suspension, or nerve growth factor-releasing microsphere suspension was injected into the defect. Twelve weeks later, sciatic nerve conduction velocity and the number of myelinated fibers were notably greater in the rats treated with icariin suspension or nerve growth factor-releasing microspheres than those that had received nerve growth factor or physiological saline. The effects of icariin suspension were similar to those of nerve growth factor-releasing microspheres. These data suggest that icariin acts as a nerve growth factor-releasing agent, and indicate that local application of icariin after spinal injury can promote peripheral nerve regeneration.

Key Words: nerve regeneration; peripheral nerve; sciatic nerve; traditional Chinese medicine; icariin; sleeve bridging suture; nerve growth factor; NSFC grants; neural regeneration

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Introduction
The clinical outcomes after peripheral nerve injury remain unsatisfactory. One influential factor is the lack of effective therapies for supporting nerve regeneration (Höke, 2006). Currently, drugs promoting peripheral nerve regeneration are administered by intraoperative spray in the region of damage, or by postoperative oral administration (Aloe et al., 2012). With local administration, the drug concentration is high, but drug metabolism is fast and the duration of action is short, resulting in difficulties in applying such treatments for a long time period. Systemic administration leads to a low local drug concentration, at the same time resulting in adverse reactions and complications (Utley et al., 1996; Wang et al., 2014). However, sustained-release local drug delivery maintains the drug concentration at the injury site while minimizing side effects in other regions, making this an ideal mode of administration of drugs promoting nerve regeneration. Previous studies have attempted administration of such drugs using a micropump at the site of injury (Newman et al., 1996; Utley et al., 1996; Gold, 1997; Hontanilla et al., 2007), but the technique is complex and expensive, hindering its applicability in the clinic. It is important to find an effective method of drug delivery for improving the therapeutic effects of peripheral nerve repair.

To date, studies on local peripheral nerve sustained-release drug delivery systems have mainly focused on promoting neuronal regeneration factors, such as nerve growth factor (Lee et al., 2003; Xu et al., 2003; Aloe et al., 2012; Manni et al., 2013; Bothwell, 2014; Wang et al., 2014), glial cell line-derived neurotrophic factor (Wood et al., 2012a, b, 2013) and brain-derived neurotrophic factor (Quigley et al., 2013). These factors are common polypeptide or protein, and readily denature or decompose in vivo during sustained release, losing their effect of promoting peripheral nerve regeneration (Aloe et al., 2012).

Chinese herbs typically have precise outcomes at multiple targets, and low toxicity with few side effects, and are attracting attention in research as potential treatments for neuronal diseases (Xu et al., 2002; Wei et al., 2008, 2009a, b; Chen et al., 2010; Shindel et al., 2010; Hsiang et al., 2011; Wang et al., 2011; Xue et al., 2012).

Our previous study showed that Epimedium extract and its main component, icariin, effectively contributed to peripheral nerve regeneration (Ma et al., 2011; Kou et al., 2013). Shin-
Materials and Methods

Experimental animals
A total of 32 healthy specific-pathogen-free adult male Sprague-Dawley rats aged 6–8 weeks and weighing 200–220 g were purchased from the Vital River Laboratories, Beijing, China (animal license No. SYXX (Jing) 2011-0010). All rats were housed at 22–26°C under a 12-hour light/dark cycle, and allowed free access to food and water. The use of experimental animals was approved by the Animal Ethics Committee of the People’s Hospital of Peking University, China. The rats were equally and randomly divided into four groups: physiological saline, nerve growth factor solution, icariin, and nerve growth factor sustained-release microspheres.

Drug preparation

Nerve growth factor solution
Nerve growth factor 2.5S (20 μg; Promega Corporation, Madison, WI, USA) was added to 1 mL PBS and stored at 4°C until use.

Icariin suspension
Icariin standard preparation (20 mg; Chinese Drugs and Biological Products Appraisal Office, Beijing, China) was added to 5 mL PBS. The suspension was stored at 4°C until use.

Preparation of nerve growth factor sustained-release microspheres

Nerve growth factor-poly(lactic-co-glycolic acid) sustained-release microspheres were prepared by water-oil-water multiple emulsion solvent evaporation (Wang et al., 2014) and freeze-dried. Twenty milligrams of microspheres were added to 5 mL PBS. The resulting nerve growth factor sustained-release microsphere suspension was stored at 4°C until use.

Establishment of animal models of sciatic nerve injury

Rats were anesthetized intraperitoneally with 2% pentobarbital (30 mg/kg; Sinopharm Chemical Reagent Co., Ltd., Beijing, China). The surgical area was shaved and sterilized, and a transverse incision was made along the lower edge of the femur. Blunt dissection of the muscles was carried out to expose the right sciatic nerve. The nerve was cut 5 mm above its bifurcation into the peroneal and tibial nerves, and 5 mm of the proximal nerve stump was removed to produce a 5 mm defect. Under an operating microscope (Haag-Streit AG, Koeniz, Switzerland), the two nerve stumps were trimmed and a chitin biological conduit (made in-house; patent No. Zl.01134542.X) was placed so that it overlapped 2 mm of each nerve stump. It was fixed to the epineurium using one suture, 1 mm from the stump, at each end (Figure 1). Once the chitin sleeve was in place, 15 μL saline, nerve growth factor solution, icariin, or nerve growth factor sustained-release microspheres were injected into the gap using a microsyringe (Shanghai GaoGe Biological Technology Co., Ltd., Shanghai, China). When we confirmed that there was no significant outflow from the gap, we closed each layer of muscle and skin using a 4-0 suture (Zhang et al., 2013).

Postoperative assessment

The general condition of the animals, as well as wound healing, limb movements, and ulceration were observed every week after surgery. Infection of the wound would exclude the rat from the experiment.

Sciatic functional index

A walking track analysis box (60 cm long, 10 cm wide, 10 cm high; made in-house) was used to calculate the sciatic functional index, a measure of functional recovery after sciatic nerve repair, at 1, 2, 4, 8 and 12 weeks postoperatively. Paper (70 g) was cut to the same dimensions as the floor of the box and placed inside. The hindpaws of each rat were dipped in ink, and the rats were allowed to walk from one end of the box to the other, leaving 5–6 prints. Print length (PL; the maximum length of the print, in mm), toe spread (TS; the distance between the first and fifth toes, in mm) and intermediary toe spread (IT; the distance between the second and fourth toes, in mm) were measured for the left (normal; N) and right (experimental; E) hindpaws. The sciatic functional index was calculated using the Bain-Mackinnon-Hunter formula: sciatic functional index = −38.3 [(EPL − NPL)/NPL] + 109.5 [(ETS − NTS)/NTS] + 13.3 [(EIT − NIT)/NIT] − 8.8. This gave an index value between 100 and 0, where 0 indicated normal sciatic nerve function (de Medinaceli et al., 1982; Hare et al., 1992).

Electrophysiological testing

Twelve weeks after surgery, nerve conduction velocity was measured (Kou et al., 2013). Rats were anesthetized with 2% pentobarbital intraperitoneally. The skin was shaved and sterilized, a transverse incision was made along the lower edge of the femur. Muscle was bluntly dissected and the sciatic nerve was exposed. Stimulating electrodes were placed at the distal and proximal ends of the sciatic nerve trunk. Recording electrodes were placed on the proximal and distal ends of the gastrocnemius muscle. Reference electrodes were placed on the gluteus maximus muscle. Parameters of a Medlec Synergy electrophysiological system (Oxford Instrument Inc., Abingdon, UK) were set at stimulus intensity 0.09 mA and duration 0.1 ms. Compound muscle action potential was recorded under stimulation. The difference (dt) of two recorded latencies was calculated. The distance between
stimulation points of the distal and proximal nerve trunks (dl) was measured. Motor nerve conduction velocity (V) was calculated as dl/dt.

Measurement of recovery of rat triceps muscle
After electrophysiological testing, rats were sacrificed by intraperitoneal injection of an overdose of 2% pentobarbital. Both hind triceps muscles were carefully dissociated and weighed on an electronic balance (FA1604; Shanghai Balance Instrument Factory, Shanghai, China). The recovery of the muscle was calculated as (right muscle wet weight / left muscle wet weight) × 100%.

Hematoxylin-eosin staining
After weighing, a 5-mm section was cut with a scalpel blade from the center of the muscle, fixed in 4% paraformaldehyde for 12 hours at room temperature, rinsed briefly under running tap water, dehydrated through a graded alcohol series, permeabilized, embedded in paraffin, and sliced into transverse sections, 5 μm thick. The sections were dried by baking, dewaxed, permeabilized, stained with hematoxylin and eosin, mounted in neutral resin, and viewed under a light microscope (Leica, Heidelberg, Germany) (Kou et al., 2013).

Osmium tetroxide staining
Following electrophysiological testing, the conduit and sciatic nerve 5 mm distal and proximal to the conduit were dissected out. The sciatic nerve was fixed in 4% paraformaldehyde for 12 hours, rinsed briefly under running tap water, stained with 1% osmium tetroxide (Acros, Waltham, MA, USA) for 12 hours, and rinsed briefly again under running water. The tissue was then dehydrated through a graded alcohol series, permeabilized, embedded in paraffin, and sliced into transverse sections (5 μm thick). The sections were mounted, dried by baking, dewaxed, mounted in neutral resin, and viewed under a light microscope (Leica). Cross-sectional images of the nerve were obtained and the number of myelinated nerve fibers per visual field (400 × magnification)
Figure 5 Effects of icariin and NGF on pathological changes in triceps of rats with sciatic nerve injury (hematoxylin-eosin staining, × 400).
Well-defined structure of left (control) triceps muscle fibers in rats in the physiological saline group (A). Small fibers in the right (experimental) side triceps muscle in the physiological saline group (B), NGF solution group (C), icariin group (D), and NGF sustained-release microsphere group (E), with no observable differences between groups. NGF: Nerve growth factor.

Figure 6 Effects of icariin and NGF treatment on myelinated nerve fibers of rats with sciatic nerve injury.
(A–E) Myelinated nerve fiber morphology (osmium tetroxide staining, × 400) in representative rats from each group. (A) Left (control) sciatic nerve, physiological saline group, showing well-defined structure and uniform myelin sheath thickness. In the right (experimental) sciatic nerve, smaller nerve fibers and thinner myelin sheath were observed in the physiological saline (B), NGF solution (C), icariin (D), and NGF sustained-release microsphere (E) groups. (F) Quantification of myelinated nerve fibers in rats. Data are expressed as the mean ± SD (n = 8 rats per group); *P < 0.05, vs. physiological saline group; #P < 0.05, vs. NGF solution group (one-way analysis of variance and independent samples t-test). NGF: Nerve growth factor; I: physiological saline group; II: NGF solution group; III: icariin group; IV: NGF sustained-release microsphere group.
was calculated using Image tool 3.0 software (Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, TX, USA). Each section was counted three times, and the mean was calculated.

Statistical analysis
Data were processed and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). All data were expressed as the mean ± SD. Intergroup comparisons were performed by one-way analysis of variance, and independent samples t-test. $P < 0.05$ was considered statistically significant.

Results
General condition of rats with sciatic nerve injury
The rats were in good condition following surgery, with no observed wound infection (and therefore no rats excluded). In the physiological saline group, three rats experienced ulceration of the right hindpaw after surgery. Autophagy of the toes was observed at 12 weeks in the same three rats, and their ulcers had not healed. In the nerve growth factor solution group, four rats had ulceration of the right hindpaw. At 12 weeks, autophagy was noted in two of those rats, whereas ulcer healing was visible in the remaining two. In the icariin group, ulceration of the right hindpaw occurred in three rats. At 12 weeks, toe autophagy appeared in two of them, and ulcers had healed in the third rat. In the nerve growth factor sustained-release microsphere group, five rats suffered from ulceration of the right hindpaw. At 12 weeks, toe autophagy was observed in three of those rats, and ulcer healing was detectable in the remaining two.

Sciatic functional index
In all groups, sciatic functional index showed a trend of gradual recovery with time. After 8 weeks, recovery slowed. No significant differences in sciatic functional index were detected between groups at any time point examined ($P > 0.05$; Figure 2).

Sciatic nerve conduction velocity
Electrophysiological tests revealed that at 12 weeks after surgery, sciatic nerve conduction velocity was greater in rats that had received icariin or nerve growth factor sustained-release microspheres than in those that received physiological saline or nerve growth factor solution ($P < 0.05$; Figure 3). Moreover, sciatic nerve conduction velocity in the icariin group was similar to that in the nerve growth factor sustained-release microsphere group ($P > 0.05$; Figure 3).

Triceps muscle weight
Twelve weeks after surgery, triceps muscles on the experimental side of rats in all groups were atrophic compared with the control side. The percentage recovery was significantly higher in the icariin and nerve growth factor sustained-release microsphere groups than in the physiological saline and nerve growth factor solution groups ($P < 0.05$), with no significant difference between the icariin and nerve growth factor sustained-release microsphere groups ($P > 0.05$; Figure 4).

Pathological changes in triceps muscle
After hematoxylin-eosin staining at 12 weeks after surgery, left (control) side triceps muscle fibers had clearly-defined boundaries, uniform staining, and regular diameter. On the right (experimental) side, muscle fibers were smaller in diameter than in the left side, but no notable differences in morphology were observed between the groups (Figure 5).

Pathological changes in the sciatic nerve
Osmium tetroxide staining 12 weeks after surgery revealed round or elliptical sciatic nerve fibers, and uniform nerve diameter and myelin sheath thickness in the left (control) side of rats from all four groups. However, in the right (experimental) side, nerve diameter and myelin sheath thickness were varied, and smaller than the control side. There were more myelinated nerve fibers in the icariin and nerve growth factor sustained-release microsphere groups than in the physiological saline group ($P < 0.05$). No significant differences in the number of myelinated nerve fibers were detected between the icariin and nerve growth factor sustained-release microsphere groups ($P > 0.05$; Figure 6).

Discussion
During peripheral nerve repair, neurons and Schwann cells secrete various factors that promote nerve regeneration (Ide, 1996). Nerve growth factor was the first such factor to be discovered and is the most extensively studied. It stimulates synapse growth, promotes differentiation, development and maturation of sympathetic and sensory neurons, and maintains normal neuronal functions (Aloe et al., 2012; Manni et al., 2013; Bothwell, 2014). Nerve growth factor solution and nerve growth factor sustained-release microspheres were therefore used as controls to icariin in the present study. Nerve growth factor is readily absorbed by tissues after direct transient topical application of nerve growth factor solution. However, peripheral nerve regeneration is a long process, with sciatic nerve repair taking at least 6 weeks in rats. Thus, a drug delivery system is advantageous in the promotion of peripheral nerve regeneration (Wang et al., 2014). Wood et al. (2011, 2013) showed that local application of poly (lactic-co-glycolic acid) sustained-release microspheres containing glial cell-derived neurotrophic factor could effectively improve the recovery of motor function in peripheral nerves during delayed repair. Takagi et al. (2012) demonstrated that composite biological sleeves obtained a better outcome when combined with local sustained-release brain-derived neurotrophic factor in bridging of a peripheral nerve defect. In our previous study, we successfully prepared nerve growth factor-poly (lactic-co-glycolic acid) sustained-release microspheres by water-oil-water multiple emulsion solvent evaporation. Our animal studies demonstrated that poly (lactic-co-glycolic acid) sustained-release microspheres containing nerve growth factor effectively elevated the number and maturity of axons and increased the effects of nerve repair in models after small gap bridging suture (Wang et al., 2014). Therefore, nerve growth factor sustained-release microspheres were used as positive controls in the present study.
Here, we found that icariin suspension exhibited identical promoting effects on nerve regeneration to nerve growth factor sustained-release microspheres, indicating that the absorption of icariin suspension in the conduit may also have a sustained-release function.

In summary, local application of icariin suspension contributes to peripheral nerve regeneration, increases the number of regenerating nerve fibers and nerve conduction velocity after sleeve bridging, and ultimately improves functional recovery. The outcomes of icariin suspension are similar to those of nerve growth factor sustained-release microspheres.

Author contributions: BC established animal models and detected sciatic nerve function. SPN performed animal histology staining and analyzed data. ZYW prepared drugs. ZZW made release microspheres. JXD provided literature data. PXZ, XFY and NH were in charge of manuscript authorization. YHK designed this study and wrote the manuscript. BGJ designed this study, analyzed data and was in charge of manuscript authorization. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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