Craniocerebral injury promotes the repair of peripheral nerve injury

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Abstract
The increase in neurotrophic factors after craniocerebral injury has been shown to promote fracture healing. Moreover, neurotrophic factors play a key role in the regeneration and repair of peripheral nerve. However, whether craniocerebral injury alters the repair of peripheral nerve injuries remains poorly understood. Rat injury models were established by transecting the left sciatic nerve and using a free-fall device to induce craniocerebral injury. Compared with sciatic nerve injury alone after 6–12 weeks, rats with combined sciatic and craniocerebral injuries showed decreased sciatic functional index, increased recovery of gastrocnemius muscle wet weight, recovery of sciatic nerve ganglia and corresponding spinal cord segment neuron morphologies, and increased numbers of horseradish peroxidase-labeled cells. These results indicate that craniocerebral injury promotes the repair of peripheral nerve injury.

Key Words: nerve regeneration; craniocerebral injury; peripheral nerve; sciatic nerve; sciatic nerve injury; nerve repair; horseradish peroxidase tracer technique; neural regeneration

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Introduction
Traumatic brain injury often occurs in combination with limb fractures and peripheral nerve injury. The promotion of fracture healing by traumatic brain injury has been reported by many studies (Spencer, 1987; Morley et al., 2005). Gibson (1960) first reported that a large number of bone calluses formed in the fracture site of femoral fracture patients that also had brain injuries, which has been verified by many animal and clinical experiments (Perkins and Skirving, 1987; Spencer, 1987). After brain injury, damage to the blood-brain barrier increases its permeability, allowing several osteogenic factors to enter the systemic circulation. In this manner, the expression of such factors in serum was markedly increased, promoting callus formation and accelerating fracture healing (Khare et al., 1995; Liu et al., 2012; Yang et al., 2012b). Cytokines, neuropeptides, neurotrophic factors, humoral factors, and mechanical factors can affect fracture healing after craniocerebral injury (Shen et al., 2012; Yang et al., 2012a; Liu et al., 2013b; Yan et al., 2013; Zhang et al., 2013; Zhao et al., 2014). Numerous studies have demonstrated that serum nerve growth factor, brain-derived neurotrophic factor, and basic fibroblast growth factor expression is higher in patients with fracture and craniocerebral injury than in patients with fracture alone (Wildburger et al., 1994; Yang and Dong, 2012; Zhuang and Li, 2013). Neurotrophic factors play important roles in the repair of peripheral nerve injury (Hong et al., 1999; Liu et al., 2013a; Wang et al., 2013; Yu et al., 2014). The aim of the present study was to determine whether changes in the body, such as neurotrophic factors, caused by craniocerebral injury promote the repair of peripheral nerve.

Materials and Methods
Experimental animals
A total of 80 male specific-pathogen-free Sprague-Dawley rats aged 8 weeks and weighing 200–220 g were purchased from Vital River Laboratories, Beijing, China (license No. SCXK (Jing) 2012-0001). They were housed in a 12-hour light/dark cycle at 23 ± 2°C. The protocols used conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), and the protocol was approved by the Institutional Animal Care Committee of Chengdu Medical University in China. The rats were equally and randomly divided into injury and control groups. The injury group received a craniocerebral injury and sciatic nerve transection, while the control group received only the sciatic nerve transection. Models of craniocerebral injury combined with sciatic nerve transection
Injury group: Using the classical Feeney method (Feeney et
al., 1981), the rats were intraperitoneally anesthetized with 10% chloral hydrate (0.35 g/kg), restrained, and steriley prepared. A sagittal incision was made on the scalp to expose the right parietal bone. A bone window of 5 mm diameter was drilled 1.5 mm posterior to the coronal line and 2.5 mm lateral to the median line using a dental drill. The cerebral dura mater was kept intact. A 20 g falling hammer was dropped freely from a height of 30 cm along the peripheral catheter, striking the bar to make a moderate contusion of right parietal bone. The bone window was sealed with bone wax, and the scalp was sutured. After sterilization, an incision was made in the left buttoc to expose the sciatic nerve. The sciatic nerve was transected 1 cm below the lower hole of the left piriformis of the rats, and then sutured with 9-0 nontraumatic thread using an epicardial suture technique under a microscope (LEL-6A; Zhongtian Optical Instrument Co., Ltd., Zhenjiang, Jiangsu Province, China). Finally, the skin was sutured closed (Cheng and Li, 2006).

Control group: After anesthesia and restraint, the left sciatic nerve was transected and sutured as detailed above. The rats in both groups were housed in individual cages.

### Measurement of sciatic functional index

Ten rats were collected from both groups at 4, 6, 8, and 12 weeks after injury. Custom-made walking dark chambers were used, as in a previous study (Schiaveto de Souza et al., 2004), to measure footprints. Three parameters were measured on both the experimental side (E) and contralateral normal side (N). (1) Footprint length (PL): from heel to toe; (2) toe width (TS): from the first toe to the fifth toe; (3) toe spacing (IT): from the second toe to the fourth toe. From these parameters, the sciatic functional index was calculated as $-38.3 \times (EPL – NPL)/NPL + 109.5 \times (ETS – NTS)/NTS + 13.3 \times (EIT – NIT)/NIT - 8.8$. The sciatic functional index was used as absolute value ranged from: 0, representing normal, to 100, representing complete nerve damage and loss of function.

### Measurement of gastrocnemius muscle wet weight

At 4, 6, 8, and 12 weeks after injury, 10 rats were selected from both groups. After anesthesia with intraperitoneal injection of chloral hydrate, the entire gastrocnemius muscle was harvested from the medial and external femoral condyles to the calcaneal tuberosity. The wet weight (g) of the gastrocnemius muscle was assessed using an electronic balance (ESJ200-4; Dragon Electronics Co., Ltd., Shenyang, Liaoning Province, China). The recovery of gastrocnemius muscle was further improved. At 12 weeks, the symptoms in rats of both groups were noticeably improved. At 6 weeks, swelling of the left lower extremity. In the control group, 32 rats showed ulcers and movement disorder. At 6 weeks, swelling of the left lower limb subsided, and the ulcers began to diminish in the injury group. In contrast, the swelling was still present in the control group. At 8 weeks, the symptoms started to improve in rats from the control group, and the symptoms in the injury group were noticeably improved. At 12 weeks, the symptoms in rats of both groups were further improved.

### Histopathological observation of nervous tissues using hematoxylin-eosin staining

Sciatic nerve stumps were selected at 4, 6, 8, and 12 weeks after injury. All samples were fixed in 10% neutral formalin for 24 hours, dehydrated through a graded alcohol series, embedded in paraffin, and longitudinally sliced into 5 μm-thick sections. These sections were dewaxed with xylene, hydrated through a graded alcohol series, stained with hematoxylin, differentiated with ethanol hydrochloride, immersed in running water, dipped in eosin, dehydrated, permeabilized, and mounted. Nerve fiber morphology was observed with a light microscope (BH-2; Olympus, Tokyo, Japan).

### Repair of sciatic nerve pathway observed with a horseradish peroxidase tracer

At 4, 6, 8 and 12 weeks after injury, 10 rats were selected from each group. After anesthesia as above, the nerve was slightly clipped 0.5 cm from the left sciatic nerve stump, and 30% horseradish peroxidase solution (Sigma, St. Louis, MO, USA) was infused. After 72 hours, the chest was opened under deep anesthesia, and the ascending aorta of the left ventricle was cannulated. The blood vessels were rinsed with 200 mL of warm physiological saline. Perfusion and fixation were performed with 400 mL of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer. The ganglia of the sciatic nerve and corresponding segments at the T4-L5 spinal levels were serially sliced into 50 μm-thick sections in the cross-sectional plane with a vibratome. These sections were washed with 0.1 mol/L PBS (pH 7.4), and treated with benzidine dihydrochloride. After exposure to 0.3% H2O2, sections were immersed in stabilizing buffer, washed with distilled water, and counterstained with neutral red (Mesulam and Rose, 1977). The number of cell bodies containing blue-stained particles in the ganglia and motor neurons of the spinal anterior horn was observed with a light microscope (Olympus).

### Statistical analysis

The data were analyzed using SPSS 17.0 software, and were expressed as mean ± SD. Intergroup comparisons were made using two-sample t-tests. Values of $P < 0.05$ were considered statistically significant.

### Results

#### Effects of cranioencephal injury on the gross state of rats with peripheral nerve injury

All animals survived the surgeries. Three weeks later, 30 rats in the injury group showed red swelling of their left lower extremity. In the control group, 32 rats showed ulcers and movement disorder. At 6 weeks, swelling of the left lower limb subsided, and the ulcers began to diminish in the injury group. In contrast, the swelling was still present in the control group. At 8 weeks, the symptoms started to improve in rats from the control group, and the symptoms in the injury group were noticeably improved. At 12 weeks, the symptoms in rats of both groups were further improved.

#### Effects of cranioencephal injury on the sciatic functional index of rats with peripheral nerve injury

The dark chamber walking results demonstrated that at 4 weeks after injury, the sciatic functional index was similar between the injury and control groups ($P > 0.05$). After 4 weeks, the sciatic functional index began to decrease in the
Table 1 Effects of craniocerebral injury on sciatic functional index in rats with peripheral nerve injury

| Group   | 4     | 6     | 8     | 12    |
|---------|-------|-------|-------|-------|
| Injury  | 70.16±2.45 | 58.17±2.60 | 37.32±2.34 | 21.56±2.43 |
| Control | 71.27±1.37 | 68.75±2.36 | 43.42±1.88 | 26.27±2.71 |

Data are expressed as the mean ± SD. *n = 10 in each group at each time point. Intergroup differences were compared with two-sample t-tests. *P < 0.01 vs. the control group. Sciatic functional index expressed as absolute value: 0 represents normal, and 100 represents complete nerve damage and loss of function.

Table 2 Effects of craniocerebral injury on the recovery of gastrocnemius muscle wet weight (%) of rats with peripheral nerve injury

| Group   | 4     | 6     | 8     | 12    |
|---------|-------|-------|-------|-------|
| Injury  | 58.96±1.60 | 44.96±1.60 | 55.22±0.46 | 85.76±1.11 |
| Control | 67.35±0.42 | 33.27±0.92 | 43.51±1.41 | 76.90±0.38 |

Data are expressed as the mean ± SD. *n = 10 in each group at each time point. Intergroup differences were compared with two-sample t-tests. *P < 0.01 vs. the control group. Recovery of gastrocnemius muscle wet weight (%) = wet weight on the experimental side (g) / wet weight on the normal side (g) × 100%.

Discussion

After peripheral nerve injury that includes transection of axons, Wallerian degeneration occurs, presenting swollen neuronal bodies, nuclear deviation, and chromatolysis. For example, neuronal bodies died in spinal ganglion and spinal cord of cats following sciatic nerve transection (Risling et al., 1983; Arvidsson et al., 1986). In the present study, a large number of necrotic and degenerated nerve fibers were observed during the repair phase at 4 weeks after sciatic nerve transection in rats in both the injury and control groups. Moreover, neuronal cells were also swollen, degenerated, and apoptotic in the ganglion and corresponding segments of the spinal cord, which confirmed this type of pathological changes. With time after injury, the neurons became regular and function was recovered in the sciatic nerve ganglion and corresponding segments of spinal cord in both groups, which indicates that the key to neurological recovery is the survival and regenerative capacity of damaged neurons (Gillardon et al., 1996; Fan et al., 2009; Dong et al., 2011). After peripheral nerve injury, the death of neuronal bodies was associated with animal age, the axonal injury site, the nature of the damage, and the neuronal type (Yegiyants et al., 2010; Wang et al., 2011). Here, at 6, 8, and 12 weeks, the quantity and quality of neurons in the ganglion and anterior horn of the spinal cord were better in the injury group than in the control group. Whether the craniocerebral injury reduced the death of neurons deserves further investigation.

The conditions for successful repair and regeneration of injured peripheral nerve include the survival and restoration of injured neuronal cell bodies, as well as sprouting and elongation of the proximal axons (English et al., 2011). Mature myelinated nerve fibers in regenerated nerves indicate...
an effective neural regeneration (Xu et al., 2003). In the present study, at 6, 8, and 12 weeks, nerve fibers were observed in the nerve stump, clearly indicating that the injured peripheral nerve here could be repaired and regenerated. In addition, nerve fibers traversed the nerve stump earlier in the injury group (with craniocerebral injury) than in the control group (without craniocerebral injury). Within the same time period, more regularly arranged nerve fibers traversing the nerve stump were found in the injury group compared with the control group, suggesting that craniocerebral injury contributed to the repair of the sciatic nerve injury to some extent. Moreover, the ultimate aim of peripheral nerve repair is to restore the function of a target muscle. The evaluation of the reinnervated rate of a target muscle can also be used to assess nerve repair and regeneration (Liang et al., 2007). In the present study, the recovery of the gastrocnemius muscle was significantly higher in the injury group than in the control group at 6, 8, and 12 weeks, which suggested that the craniocerebral injury promoted the repair of the peripheral nerve injury. Recently, a horseradish peroxidase marking method has been extensively used to trace nerves. Horseradish peroxidase can be absorbed and transported by nerve endings with various functions. It also accumulates in nerve cell bodies, and its transport is mainly associated with microtubules in nerve fibers (Seckel et al., 1984; Mearow et al., 1994). Therefore, nerve fibers that have lost their continuity cannot transport horseradish peroxidase from the distal to proximal end or accumulate it in their cell bodies. Horseradish peroxidase-labeled cells in the spinal ganglion and spinal cord can be used to identify whether regenerated fibers traversed the suture site after end-to-end suture. In this study, at 4 weeks after surgery, labeled neuronal cells were not detected in the rat ganglion or spinal cord in either the injury or control groups, indicating that the regenerated fibers did not traverse the nerve stumps. At 6 weeks, labeled neuronal cells were detected in the rat spinal ganglion in the injury group, but not in the control group, indicating that the regenerated fibers of rats with craniocerebral injury traversed the nerve stumps earlier than those in the control rats. After craniocerebral injury, several factors can promote the repair of injured peripheral nerves. At 8 and 12 weeks after surgery, a large number of labeled cells were visible in the spinal ganglion and spinal cord in both groups, but the number was obviously higher in the injury group than in the control group. These results suggest that changes in the bodies of the

Figure 1 Effects of craniocerebral injury on the pathological changes in sciatic nerve of rats with peripheral nerve injury (hematoxylin-eosin staining, × 100).

After nerve transection, nerve fiber swelling and apoptosis were observed at both stumps. At 4 weeks, partial function of the cells was recovered. At 6 weeks, the nerve fibers traversed the stumps. At 8 and 12 weeks, the number of nerve fibers traversing the stumps increased. The traversing nerves appeared earlier, and more nerve fibers traversed the stumps, in the injury group compared with the control group.
rats after craniocerebral injury protected the neuronal cells, contributed to the better restoration of neuronal cells, and played a role in the repair of the peripheral nerve injury.

In summary, the regeneration of broken sciatic nerves occurred faster in rats with craniocerebral injuries than in control rats without craniocerebral injuries, indicating that the craniocerebral injury promoted the repair of the peripheral nerve injury. However, the precise mechanisms remain poorly understood and deserve further investigation.

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HT and Wang P. Xue JF and Yang ZJ evaluated this study, and provided data and technical support. Wang W wrote the manuscript. Wang P was in charge of manuscript authorization. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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