

Changes in proteins related to early nerve repair in a rat model of sciatic nerve injury

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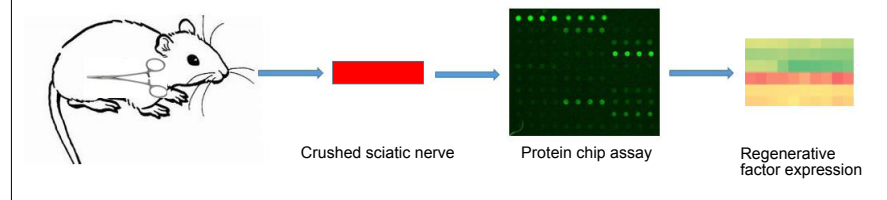
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Yu-Song Yuan^{1,2,#}, Fei Yu^{1,3,#}, Ya-Jun Zhang⁴, Su-Ping Niu⁵, Hai-Lin Xu^{1,6,*}, Yu-Hui Kou^{1,2,*}

Graphical Abstract

Changes in protein factors related to the repair of peripheral nerve injury in the early stage of nerve repair



Abstract

Peripheral nerves have a limited capacity for self-repair and those that are severely damaged or have significant defects are challenging to repair. Investigating the pathophysiology of peripheral nerve repair is important for the clinical treatment of peripheral nerve repair and regeneration. In this study, rat models of right sciatic nerve injury were established by a clamping method. Protein chip assay was performed to quantify the levels of neurotrophic, inflammation-related, chemotaxis-related and cell generation-related factors in the sciatic nerve within 7 days after injury. The results revealed that the expression levels of neurotrophic factors (ciliary neurotrophic factor) and inflammation-related factors (intercellular cell adhesion molecule-1, interferon γ , interleukin-1 α , interleukin-2, interleukin-4, interleukin-6, monocyte chemoattractant protein-1, prolactin R, receptor of advanced glycation end products and tumor necrosis factor- α), chemotaxis-related factors (cytokine-induced neutrophil chemoattractant-1, L-selectin and platelet-derived growth factor-AA) and cell generation-related factors (granulocyte-macrophage colony-stimulating factor) followed different trajectories. These findings will help clarify the pathophysiology of sciatic nerve injury repair and develop clinical treatments of peripheral nerve injury. This study was approved by the Ethics Committee of Peking University People's Hospital of China (approval No. 2015-50) on December 9, 2015.

Key Words: animal model; cell generation; chemotaxis; clamp injury; inflammation; injury; neurotrophic factor; peripheral nerve protein array; repair; sciatic nerve; Wallerian degeneration

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Introduction

Peripheral nerve injury is a common traumatic disease (Korus et al., 2016; Wang et al., 2018) that can cause sensory and motor dysfunction (Ceynowa et al., 2017; Duarte-Moreira et al., 2018). Patients who are not treated promptly may develop permanent disability (Novak et al., 2010; Chui et al., 2018), seriously affecting the quality of life of patients and their families (Evans et al., 1999). Peripheral nerves have a limited capacity for self-repair. Peripheral nerves that are

severely damaged or have significant defects are challenging to repair and a concerning medical issue (Arzillo et al., 2014; Sun et al., 2018). Conservative or surgical methods are the main approaches currently used for treating peripheral nerve injury (Zhang et al., 2013). However, the specific progression, pathogenesis and treatment limitations of peripheral nerve injury often lead to poor prognosis in such patients. Therefore, studies are urgently needed to evaluate the pathogenesis of this disease to inform improvements in its prevention and

¹Department of Trauma and Orthopedics, Peking University People's Hospital, Beijing, China; ²Key Laboratory of Trauma and Neural Regeneration (Peking University), Ministry of Education, Beijing, China; ³National & Local Joint Engineering Research Center of Orthopedic Biomaterials, Department of Bone & Joint Surgery, Peking University Shenzhen Hospital, Shenzhen, Guangdong Province, China; ⁴National Center for Trauma Medicine, Beijing, China; ⁵Office of Academic Research, Peking University People's Hospital, Beijing, China; ⁶Diabetic Foot Treatment Center, Peking University People's Hospital, Beijing, China

*Correspondence to: Hai-Lin Xu, MD, xuhailin@163.com; Yu-Hui Kou, PhD, yuhuikou@bjmu.edu.cn.

<https://orcid.org/0000-0003-1829-3852> (Hai-Lin Xu); <https://orcid.org/0000-0003-1523-825X> (Yu-Hui Kou)

#Both authors contributed equally to this work.

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treatment.

Wallerian degeneration occurs at the distal end of a nerve fiber in the early stage after peripheral nerve injury (Xu et al., 2020). During this process, the distal myelin and axons are structurally altered within a few hours after injury and gradually decompose within 2–3 days. After 5–6 days, phagocytic cells increase in number, and remove the damaged myelin and axons from the lesion (Conforti et al., 2014). At 1 week after injury, repair commences with the proximal axon sprouting and gradually growing into a distal effector at a rate of 1–2 mm/d, eventually restoring function to the nerve (Blanquie and Bradke, 2018). Schwann cells form a new myelin sheath triggered by the distal secretion of nerve growth factors and neurotrophic factors to promote the repair and regeneration of injured nerves. Therefore, changes in related factors within 7 days after peripheral nerve injury can influence injury progression and repair (Capoccia et al., 2014; Lin et al., 2019; Wei et al., 2020). Increased knowledge of the changes would inform the mechanisms involved and aid prevention or reduction in peripheral nerve injury, suggesting avenues of treatment.

The studies range from the level of gene expression to the mechanisms of physiological processes. Many proteins are associated with the injury and repair processes. The traditional methods of detection require large amounts of tissues and cannot detect multiple protein factors simultaneously (Duerr, 2006). Protein chip technology has emerged as a method for tracking related proteins in tissue samples on a supporting medium with captured antibodies. A biotinylated antibody will readily bind to streptavidin. The proteins are quantitatively evaluated by measuring fluorescence. By immobilizing multiple captured antibodies on a supporting medium, a microarray can be prepared to examine multiple protein factors in parallel using a small amount of experimental tissue (Zhu and Snyder, 2003). This technology is useful in studies of molecular biological mechanisms.

Our study aimed to clarify the mechanism underlying the repair of peripheral nerve injury in the early stage. We used protein chip methods to test target protein factors related to Wallerian degeneration following the establishment of a rat model of lower limb sciatic nerve injury.

Materials and Methods

Animals

Thirty female Sprague-Dawley specific pathogen free rats, aged 6 weeks, weighing 140–180 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (license No. SCXK (Jing) 2016-0006). The experimental animals were housed in the specific pathogen-free facility of the Experimental Animal Center of Peking University People's Hospital, maintained on a 12-hour light/dark cycle at $24 \pm 2^\circ\text{C}$ and relative humidity of 50–55%. The experimental procedure and treatment of rats were performed according to the Guide for the Care and Use of Laboratory Animals (Public Health Service, 1996, NIH Publication No. 85-23). The study protocol was approved by the Ethics Committee of Peking University People's Hospital on December 9, 2015 with approval No. 2015-50.

Animal modeling

Thirty rats were randomly divided into normal ($n = 3$) and experimental groups ($n = 27$). Rats in the experimental group were anesthetized using a Matrx Animal Anesthesia Ventilator

System (Midmark Corporation, Dayton, OH, USA). After the rats were placed in the left lateral position and shaved, the right sciatic nerve along the muscle space was exposed. The sciatic nerve was dissociated from the piriformis, and a rubber mat was padded around it. The sciatic nerve was clamped using a needle holder with a bottom width of 4 mm for 30 seconds (Bucan et al., 2019). The muscle was sutured by 4-0 double-needle stitching, followed by injection of 500 μL of normal saline into the muscle space with a 1-mL syringe. The skin was sutured using 4-0 sharp-needle stitching and disinfected with iodophor.

In the experimental group, three rats were randomly selected at 1, 2, 4, 6, 12 hours, 1, 3, 5, and 7 days after modeling, and 1-cm sciatic nerve tissues were collected from the right clamping site, from 3 mm above to 3 mm below the clamping site. In the normal group, a 1-cm sciatic nerve sample was collected from each rat from the equivalent site.

Protein chip assay

Using the GSR-CYT-3 protein chip (Raybiotech, Peachtree Corners, GA, USA), we evaluated four types of proteins: a neurotrophic-related protein (ciliary neurotrophic factor (CNTF)), inflammation-associated proteins (intercellular cell adhesion molecule-1 (ICAM-1), interferon γ (IFN γ), interleukin (IL)-1 α , IL-2, IL-4, IL-6, monocyte chemoattractant protein-1 (MCP-1), prolactin R, receptor for advanced glycation end products (RAGE), tumor necrosis factor- α (TNF- α)), chemotaxis-associated proteins (cytokine-induced neutrophil chemoattractant-1 (CINC-1), L-selectin, platelet-derived growth factor-AA (PDGF-AA)), and the cell generation-associated protein (granulocyte-macrophage colony-stimulating factor (GM-CSF)).

Rat nerve tissue was collected using the Raybiotech kit (Huaying Biomedical Technology Co., Ltd.) according to the manufacturer's instructions. Protein extracts were quantified by the Micro BCA Protein Assay (Huaying Biomedical Technology Co., Ltd.). The chip sample wells were sequentially blocked, then the diluted sample was added and placed on a horizontal shaker (Hengao Technology Development Co., Ltd., Tianjin, China) at 4°C , 70 r/min overnight. On the next day, the sample in each well was removed, washed, shaken, and centrifuged at $100 \times g$ for 3 minutes. Cy3-streptavidin (Huaying Biomedical Technology Co., Ltd.) was added to each well. The sample was incubated in the dark for 1 hour, and then rinsed and centrifuged. The chip was scanned with a GenePix 4000B chip scanner (Molecular Devices, Sunnyvale, CA, USA) (Huang et al., 2018).

Statistical analysis

Data analysis was performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). All measurement data are expressed as the mean \pm standard deviation (SD). GraphPad Prism 5 software (GraphPad, Inc., La Jolla, CA, USA) was used to plot the expression curves of different protein factors at different time points. Proteins up-regulated or down-regulated by more than 15% were considered as differentially expressed, and those up-regulated by over 100% or down-regulated by over 50% were considered as significantly differentially expressed.

Results

Changes of neurotrophic-related proteins in injured sciatic nerve

The expression levels of CNTF protein in healthy nerve tissue

Research Article

and at 1, 2, 4, 6, and 12 hours and 1, 3, 5, and 7 days after nerve injury are shown in **Figure 1** and **Additional Table 1**. The expression of CNTF gradually increased in the early stage of nerve repair after sciatic nerve injury (**Figure 2**), and the expression of the chemotaxis related factor CINC-1 was significantly changed at 6 hours after nerve injury (**Figure 3**).

Changes of inflammation-related protein factors in injured sciatic nerve

The expression levels of ICAM-1, IFN γ , IL-1 α , IL-2, IL-4, and IL-6 in healthy nerve tissue and at 1, 2, 4, 6, and 12 hours and 1, 3, 5, and 7 days after nerve injury are shown in **Figure 4** and **Additional Table 1**. The expression of ICAM-1 first decreased and then increased; the expression of MCP-1, RAGE, and TNF- α first increased and then decreased; and the expression of IFN γ , IL-1 α , IL-2, IL-4, IL-6, and prolactin R showed no significant changes (**Figure 2**). The expression of MCP-1 was significantly higher at 4 hours after nerve injury compared with healthy nerve tissue (**Figure 3**).

Changes of cell chemotaxis-related protein factors in injured sciatic nerve

The expression levels of CINC-1, L-selectin, and PDGF-AA in healthy nerve tissue and at 1, 2, 4, 6, and 12 hours and 1, 3, 5, and 7 days after nerve injury are shown in **Figure 5** and **Additional Table 1**. The expression of CINC-1 and L-selectin increased first and then decreased, showing significantly increased change at 6 hours after nerve injury, whereas the expression of PDGF-AA decreased initially then increased significantly at 5 days after nerve injury (**Figures 2** and **3**).

Changes of cell generation-related protein factors in injured sciatic nerve

The expression levels of GM-CSF in healthy nerve tissue and at 1, 2, 4, 6, and 12 hours and 1, 3, 5, and 7 days after nerve injury are shown in **Figure 6** and **Additional Table 1**. The expression trend of GM-CSF was wavy (**Figure 2**).

Discussion

Peripheral nerve injury is a clinically common traumatic disease with a high incidence that is not easily treated without surgical intervention (Wang et al., 2017; Costales et al., 2019). By evaluating the specific mechanisms underlying the repair process of peripheral nerve injury, corresponding targets can be identified, and related treatments can be performed to promote peripheral nerve regeneration and repair. Few studies have examined the changes in peripheral nerve-related factors during the early stage of repair after peripheral nerve injury, consequently the mechanism is not well-understood. In this study, we prepared a Sprague-Dawley rat model of sciatic nerve clamp injury and used the GSR-CYT-3 high-throughput protein chip produced by Raybiotech to test related proteins at different time points in the early stage of nerve repair after peripheral nerve injury.

Neurotrophic factors (Boyd and Gordon, 2003), inflammatory factors (Micaroni et al., 2013), chemokines (Manjavachi et al., 2014) and cytogenetic factors (Be'eri et al., 1998) are involved in the repair of peripheral nerve injury. In this study, the factors loaded on the GSR-CYT-3 protein chip were classified into these four groups and members listed in the Methods section. The neurotrophic factor, CNTF, is essential in regulating nerve growth (Cen et al., 2017). There are many protein factors related to inflammation, including ICAM-1 (CD54), which is involved in promoting tissue adhesion at the

inflammation site. By binding to its receptor, ICAM-1 promotes interactions between inflammatory cells and endothelial cells and the migration of endothelial cells and leukocytes. Myocardial infarction triggers a significant increase in the expression of ICAM-1 (Ibarra-Lara et al., 2019). IFN γ belongs to the interferon family. Both IFN γ and TNF- α induce nitric oxide synthase and enhance the expression of nitric oxide that promotes autophagy and apoptosis. These two factors have been used as therapeutic drugs for treating inflammatory diseases caused by bone marrow mesenchymal stem cells (Li et al., 2019). IL-1 α , IL-2, IL-4, and IL-6 are members of the interleukin family, which is among the most widely studied inflammatory families involved in the inflammatory or immune processes associated with many diseases. The family members not only stimulate inflammatory injury but are also pivotal in the anti- or pro-inflammatory process (Corpuz et al., 2017; Malik and Kanneganti, 2018; Sharba et al., 2019; Zhang et al., 2019). MCP-1 is an inflammatory factor in the chemokine CC subfamily. Angiotensin II can induce inflammatory responses through MCP-1 and then participate in inflammatory diseases. Prolactin is a neuroendocrine hormone that promotes inflammation and can also be produced in the synovium of patients with arthritis (Tang et al., 2017). Prolactin R is a prolactin receptor that is closely related to inflammation. Secreted RAGE has a pro-inflammatory role in various diseases (Guan et al., 2019). TNF- α is an inflammatory factor elevated in a variety of inflammatory diseases (Blaser et al., 2016). Cell chemotaxis promoting factors include CINC-1, which promotes neutrophil migration by regulating the process of cell surface adhesion, and its expression at the injury site may be a major factor affecting neutrophil infiltration (Bhatia et al., 2000). Neutrophils are important effectors in acute inflammation (Xu et al., 2019). L-Selectin is a cell adhesion molecule expressed by lymphocytes and is a homing receptor for lymphocytes to reach the peripheral lymph nodes (Rosen, 2004). Chronic inflammation promotes tumor growth by inducing the expression of L-selectin (Perfilyeva et al., 2019). PDGF-AA may be involved in the transfer of activated monocytes to inflammation and injury sites (Krettek et al., 2001). Cell generation factor GM-CSF promotes the differentiation of bone marrow precursor cells into macrophages and granulocytes. This factor functions in both the active and stationary phases of inflammatory diseases and has a significant pro-inflammatory effect (Shiomi and Usui, 2015).

In our study, the expression of CNTF, a neurotrophic factor, gradually increased in the early stage of nerve repair after sciatic nerve injury. Natural variation in several important factors involved in the early stage of nerve repair was observed after sciatic nerve injury in Sprague-Dawley rats. The inflammatory proteins MCP-1, RAGE, TNF- α , and ICAM-1 showed different expression trends in the early stage after sciatic nerve injury in rats. These proteins aggravate inflammatory reactions to varying extents after sciatic nerve injury. CINC-1, L-selectin, and PDGF-AA exacerbate inflammation by causing neutrophil aggregation in the injured nerve, with changes in protein expression also influencing the severity of inflammation. GM-CSF stimulates granulocyte differentiation and promotes inflammation through neutrophil aggregation. Ultimately, the expression of neurotrophic factors, such as CNTF, is increased in the early stage after nerve injury, triggering nerve repair.

In this study, significant changes in the expression of MCP-1, RAGE (protein factors directly related to inflammation), CINC-1, PDGF-AA (e.g., protein factors related to chemotaxis

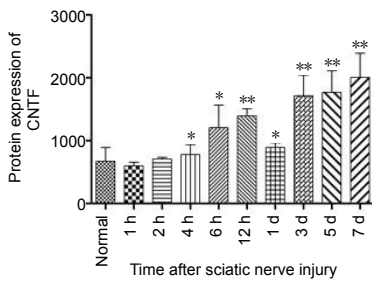


Figure 1 | Changes in the expression levels of neurotrophic-related protein factors in injured sciatic nerve detected by protein chip assay.

Data are expressed as the mean \pm SD. * Indicates up-regulation by over 15% compared with normal, ** Indicates up-regulation by over 100% compared with normal. CNTF: Ciliary neurotrophic factor.

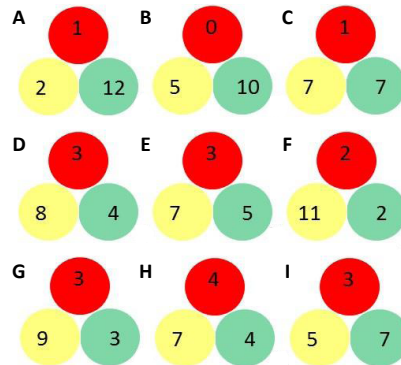


Figure 3 | Changes in protein expression levels at various time points after sciatic nerve injury.

(A-I) 1 (A), 2 (B), 4 (C), 6 (D), and 12 hours (E) and 1 (F), 3 (G), 5 (H), and 7 days (I) after nerve injury. Green indicates the number of protein factors up-regulated by over 100% or down-regulated by over 50% compared with normal group. Yellow indicates the number of protein factors up-regulated or down-regulated by over 15%. Red indicates the number of protein factors showing insignificant changes. Numbers indicate proteins that have changed accordingly.

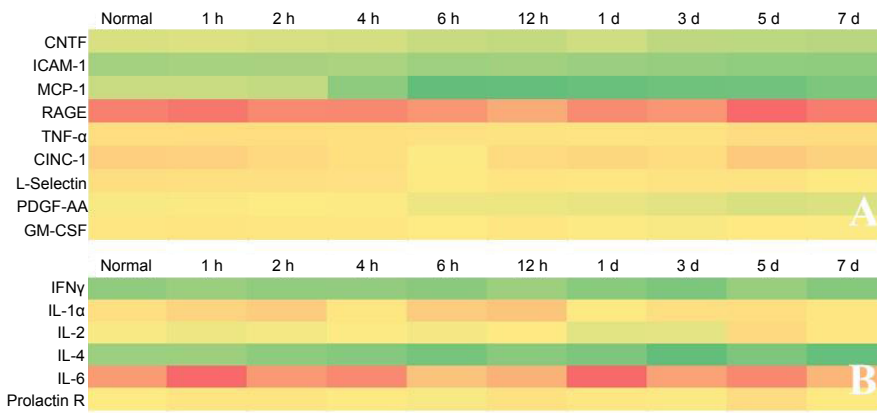


Figure 2 | Heat map of the levels of protein factors in injured sciatic nerve.

The average expression level of each protein factor is shown as a log value to depict a heat map. (A) Proteins with a changing trend. (B) Proteins with no changing trend. Greener colors indicate higher protein expression and redder colors indicate lower protein expression. CINC-1: Cytokine-induced neutrophil chemoattractant-1; CNTF: ciliary neurotrophic factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; ICAM-1: intercellular cell adhesion molecule-1; IFN γ : interferon γ ; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; PDGF-AA: platelet-derived growth factor-AA; RAGE: receptor of advanced glycation end products; TNF- α : tumor necrosis factor- α .

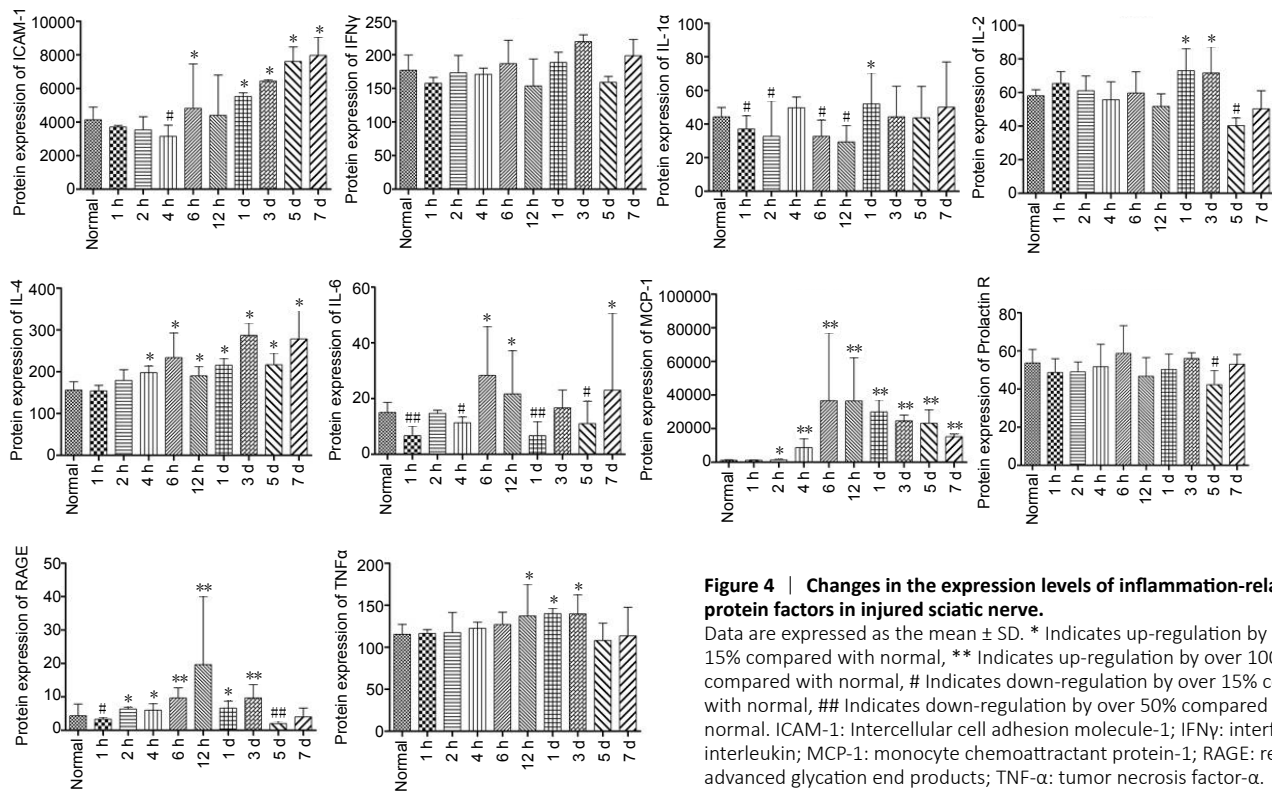


Figure 4 | Changes in the expression levels of inflammation-related protein factors in injured sciatic nerve.

Data are expressed as the mean \pm SD. * Indicates up-regulation by over 15% compared with normal, ** Indicates up-regulation by over 100% compared with normal, # Indicates down-regulation by over 15% compared with normal, ## Indicates down-regulation by over 50% compared with normal. ICAM-1: Intercellular cell adhesion molecule-1; IFN γ : interferon γ ; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; RAGE: receptor of advanced glycation end products; TNF- α : tumor necrosis factor- α .

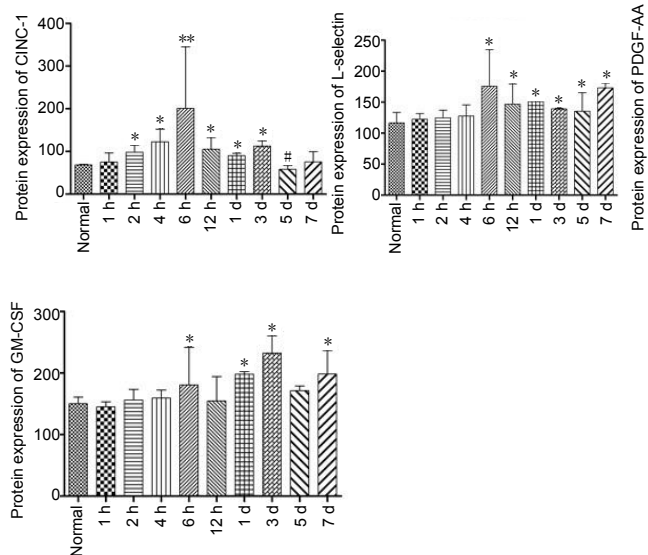


Figure 6 | Changes in the expression levels of cell generation-related protein factors in injured sciatic nerve. Data are expressed as the mean ± SD. * Indicates up-regulation by over 15% compared with normal. GM-CSF: Granulocyte-macrophage colony-stimulating factor.

of granulocytes, and macrophages) and CNTF (neurotrophic related protein) were observed at 4, 6 and 12 hours and 5 days after nerve injury, respectively. However, this study lacked a discussion on the mechanism of changes in the proteins due to the complex regulation nets, only describing the dynamic changes in proteins in the early stages of nerve injury. The certain conclusions that can be drawn from our research were that inflammation and cell chemotaxis led to the secretion of neurotrophic factors and participated in the repair process in the first week of sciatic nerve repair.

The limitation of this study is that the multiple protein changes during Wallerian degeneration, which have been detected by protein chips, have not been verified through western blot or enzyme-linked immunosorbent assay. However, the study has identified changes in particular protein factors related to the repair of peripheral nerve injury in the early stage of nerve repair, which may contribute to further studies of the mechanism underlying the repair of this injury.

Our results will inform our future research, when we will focus on the roles of MCP-1, CINC-1, RAGE, CNTF, PDGF-AA, among other factors, in the early stage of inflammation and the chemotaxis of white blood cells and macrophages following peripheral nerve injury and repair. To further explore the pathways involved during the first week after peripheral nerve injury, high-throughput sequencing combined with different cell models and animal models could be used. This should enhance the detection and verify the changes of these factors to explore the changes in inflammation, immune cell chemotaxis and neurotrophic factors after peripheral nerve injury.

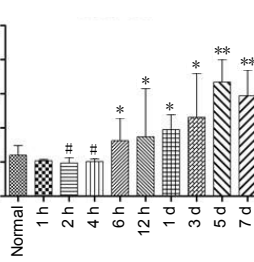
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Figure 5 | Changes in the expression levels of cell chemotaxis-related protein factors in injured sciatic nerve.

Data are expressed as the mean ± SD. * Indicates up-regulation by over 15% compared with normal, ** indicates up-regulation by over 100% compared with normal, # indicates down-regulation by over 15% compared with normal. CINC-1: Cytokine-induced neutrophil chemoattractant-1; PDGF-AA: platelet-derived growth factor-AA.



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Additional file:

Additional Table 1: *Changes in neurotrophic-related protein factors in injured sciatic nerve.*

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Additional Table 1 Changes in neurotrophic-related protein factors in injured sciatic nerve

Factor	Normal	Post-operation								
		1 h	2 h	4 h	6 h	12 h	1 d	3 d	5 d	7 d
CNTF	671.67±218.73	598.33±57.00	708.33±25.32	777.67±160.66	1205.00±361.29	1394.67±115.25	892.33±73.87	1712.67±328.81	1769.00±343.87	2006.67±383.88
ICAM-1	4129.67±754.03	3702.33±93.77	3528.67±780.93	3149.33±662.2963	4814.67±2648.58	4398.00±2394.55	5521.00±224.93	6429.00±95.17	7604.67±870.14	7964.33±1091.41
IFN γ	177.00±22.61	157.67±8.74	173.33±25.70	171.00±9.00	186.67±34.82	153.67±40.08	188.67±15.14	219.67±10.02	159.00±8.89	198.67±24.21
IL-1 α	44.33±5.51	37.00±8.00	32.67±21.20	49.67±6.43	32.67±9.72	29.33±9.87	52.00±18.52	44.33±18.18	43.67±18.82	50.00±26.96
IL-2	58.00±3.61	65.33±7.09	61.00±8.882	55.67±10.69	59.67±12.74	51.67±7.57	73.00±13.23	71.67±15.63	40.33±4.62	50.33±10.69
IL-4	156.0000±20.2978	154.33±13.28	179.00±25.71	197.67±16.05	233.67±59.34	190.33±22.37	215.67±16.65	287.00±29.87	216.67±28.04	278.33±68.66
IL-6	15.00±3.61	6.67±3.51	14.67±1.15	11.33±2.08	28.33±17.62	21.67±15.53	6.67±5.13	16.67±6.43	11.00±8.00	23.00±27.73
MCP-1	1114.00±376.40	1144.33±225.72	1396.67±325.68	8728.00±5357.65	36660.33±40501.34	36445.00±25825.71	29938.67±7176.70	24628.33±3489.97	23193.00±8125.88	15184.27±1635.55
Prolactin R	53.67±7.09	48.67±7.23	49.00±5.20	51.67±11.85	58.67±14.57	46.67±9.82	50.33±8.02	56.00±3.00	42.33±7.51	53.00±5.20
RAGE	4.33±3.5119	3.33±0.58	6.33±0.58	6.00±2.00	9.67±3.06	19.67±20.43	6.67±2.08	9.67±4.04	2.00±1.00	4.00±2.65
TNF- α	115.33±11.93	116.33±4.73	117.33±24.01	122.67±7.23	127.00±14.80	137.33±37.98	140.00±6.56	139.67±23.12	108.00±20.81	113.67±33.84
CINC-1	68.33±1.53	75.00±21.38	98.00±16.64	122.67±29.50	200.67±144.92	105.00±27.73	90.00±5.57	112.33±12.42	58.00±9.17	75.67±23.86
L-Selectin	116.67±16.77	122.33±9.24	124.67±12.22	127.67±17.79	175.67±59.28	146.67±32.87	151.00±0.00	139.00±1.00	135.33±30.01	173.00±7.81
PDGF-AA	240.67±56.15	208.00±7.21	194.33±32.87	203.00±16.52	325.33±132.27	347.67±283.67	390.67±87.08	462.00±258.31	668.67±133.01	589.00±150.40
GM-CSF	150.00±10.82	145.00±8.00	156.00±17.35	159.33±13.01	180.67±61.01	154.67±39.37	198.00±4.36	232.33±28.36	171.33±7.51	198.33±38.53

Data are expressed as the mean \pm SD. CINC-1: Cytokine-induced neutrophil chemoattractant-1; CNTF: ciliary neurotrophic factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; ICAM-1: intercellular cell adhesion molecule-1; IFN γ : interferon γ ; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; PDGF-AA: platelet-derived growth factor-AA; RAGE: receptor of advanced glycation end products; TNF- α : tumor necrosis factor- α .