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Research article

Acupuncture promotes neurological recovery and regulates lymphatic function after acute inflammatory nerve root injury

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ABSTRACT

Aims: To investigate the therapeutic effect of acupuncture on acute inflammatory nerve root injury by regulating lymphatic function.
Main methods: A mouse model of L5 nerve root compression was used to simulate acute nerve root injury. After modeling, acupuncture treatment was given each day for one week. Pain thresholds were assessed before and after modeling and treatment. Immunofluorescence staining was performed to observe the distribution astrocytes and neurons in the lumbar spinal cord, the innervation rate of neuromuscular junctions (NMJs), lymphatic endothelial cells (LECs) of lumbar aortic lymph nodes, and the percentage of M1 macrophages. The number of each type of immune cells in the lumbar aortic lymph nodes (LALNs) was measured by flow cytometry.
Key findings: The model group showed a significant decrease in pain threshold in the affected lower limb, while acupuncture treatment was able to significantly increase it. Acupuncture significantly repaired astrocytes and neurons in the lumbar spinal cord of the compressed segment, increased the innervation rate of nerve endings at NMJs, reduced LECs in the LALNs, reduced the proportion of M1 macrophages in the LALNs, and significantly reduced mononuclear neutrophils and monocytic neutrophils.
Significance: Acupuncture can reduce pain, promote nerve repair in mice with acute nerve root injury, and suppress immune responses in lumbar aortic lymph nodes.

1. Introduction

Lumbar disc herniation is one of the most common clinical conditions of low back pain, it occurs 5 to 20 cases among 1000 adults

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per year. Approximately 80 % of people experience low back pain during their life time, with the highest incidence in the 3rd to 5th decade [1–3]. The disease results from a local inflammatory response caused by nerve roots injury, leading patients to develop pain, paresthesia, numbness and weakness in the lower limbs, muscle wasting, and other typical symptoms [4–6]. It has a severe impact on patients' bodily function and quality of life, as well as a burden on the society and the economy. Currently, the main clinical treatments are surgery, physiotherapy and drug therapy, but there is still a lack of safer and more effective treatment with fewer side effects [7].

Lymphatic vessels are responsible for regulating fluid balance and immune surveillance. Impaired lymphatic function leads to a weakened immune response, tissue swelling and, consequently, inflammatory diseases and tumors [8]. Damage to the lymphatic structure or increased lymphatic production can lead to lymphatic dysfunction, which can exacerbate inflammatory responses and disease symptoms [9,10]. Vertebral lymphatic vessels are present at the level of each vertebral body, beneath the intervertebral tissues and ligaments, and are densely packed around nerve roots and dorsal root ganglia [11,12]. It has been reported in the literature that the LALNs are lumbar reflux lymph nodes [13]. Therefore, vertebral lymphatics and LALNs may be a potential target for the treatment of diseases around spine. Studies have shown that induction of lymphatic dilatation promotes the removal of inflammatory factors and improves arthritic symptoms, whereas inhibition of lymphatic function exacerbates joint inflammation [14]. Therefore, promoting lymphatic function can potentially ameliorate the inflammatory response caused by nerve root injury.

Acupuncture, an important part of traditional Chinese medicine, has been used to treat a wide range of conditions. Studies have shown that acupuncture has an analgesic effect, reducing the use of analgesics and effectively inhibiting the inflammatory response [15–17]. Currently, acupuncture has focused on the regulation of the nervous system, inflammation-related pathways and immune cells in the treatment of diseases [18–20]. However, less research has been conducted on the effects of acupuncture on the lymphatic system. In this study, we investigated whether acupuncture modulates lymphatic vessel function and inhibits the inflammatory response to ameliorate inflammatory injury and promote neurological recovery after acute inflammatory nerve root injury.

2. Materials and methods

2.1. Animal model

All experimental animals were provided by the Animal Experiment Center of Shanghai University of Traditional Chinese Medicine (Animal Qualification Certificate No. 20180006019387). All animal experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (Ethics No. PZSHUTCM190614011.). Sixty male C57BL/6 SPF mice aged six weeks, weighing 19–26 g, were kept in a 24 °C temperature-maintained room with a 12 h light/dark cycle and continuously supplied with water and standard laboratory chows. Then randomly divided into three groups with 20 mice in each group, respectively sham operation group, model group, and acupuncture treatment group.

We improved the modeling of nerve root compression based on previous studies. We used mice as the model animal in this experiment and silicone tubing as the apparatus for compression [21,22]. Mice were anesthetized by inhalation of isoflurane, and the entire procedure was performed under a body microscope (Cat#MZ61). The mice were secured in the prone position, and the scalpel made a right incision about 4 cm along the spinous process of L3-L6 to the median line. The paraspinal muscles were dissected and separated to the articular eminence. The right L5 lamina was exposed until the foraminal was fully exposed (located below the joint eminence). A polypropylene cannula was inserted into the anterior wall of the foraminal (Fig. 1). The right L5 nerve root was compressed, and the right posterior limb was constricted and twitched, indicating a successful model was made. Finally, the skin tissue was sutured. In the control group, sham surgery was performed to peel the paraspinal muscle to the joint eminence, expose the right L5 lamina and foramina, and suture the skin tissue without inserting a polypropylene cannula.

2.2. Acupuncture treatment

On the first postoperative day, the mice in the acupuncture group were anesthetized with isoflurane gas and fixed. The L5 Jiaji acupuncture point (EX-B2) was selected. The size of the acupuncture needles we use: 0.16*7 mm. Details of the acupuncture operation include 45-degree oblique stabbing at a depth of 0.1 inch. Acupuncture needle was performed, twirled the needles every 5 min, and the acupuncture needles were removed after 20 min of retention, continuous intervention for seven days. The remaining mice in each group were anesthetized for 20 min only.

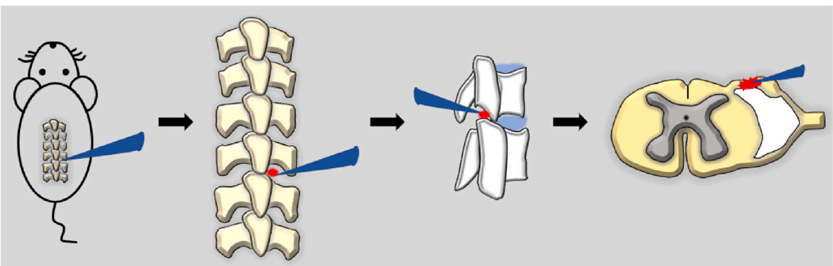


Fig. 1. Schematic diagram of the nerve root injury model.

2.3. Pain threshold measurement

2.3.1. Determination of pain threshold for mechanical pain

The Von Frey Hair Test Kit was used, and tests were performed in order from smallest to largest according to the number of grams on the test pen. The plantar center of the affected hind limb was stimulated with a fiber wire, and the order of stimulation was from small to large, and each intensity was stimulated 3 times. When the mouse lifts the affected hind limb or licks its paw within 5 s, the value on the fiberwire pen was recorded as the mechanical pain threshold of that mouse.

2.3.2. Determination of thermal pain threshold

The thermal pain threshold (Infrared thermal pain tester for rats and mice, Cat#37370) of the experimental mice in each group was measured with a plantar thermal pain tester, and the infrared light was focused on the plantar. The mice were tested with fixed parameters (IR = 40) in the infrared heat pain tester, and the recording range of pain threshold was from 0 to 30s. Mice were placed individually in a bottomed mailing glass box compartment for acclimatization (15–30 min). The mice were allowed to acclimatize to the environment in advance and the test is performed in a quiet state. Record the corresponding time (in seconds) when the affected hind limb lifts or licks the foot. The test was performed every 10 min and each mouse is evaluated 3 times and the average value is taken. During the test, the mice should be carefully observed to determine whether the foot is lifted due to pain or the number of tests should be increased appropriately, and the average of the last three thermal pain threshold values should be calculated.

2.4. Immunofluorescence staining

LALNs and spinal segments from mice were harvested at the end of the needle treatment protocol, frozen, and sectioned using cryostat microtome (10 μm); anterior tibialis muscle from mice was harvested, and myofilaments were torn using ophthalmic forceps. After sealing in blocking solution, sections were double stained with primary immunostaining antibodies diluted with Ki67 (1:500, eBioscience, Cat#So1A15) and LYVE1 (1:500, Abcam, Cat#Ab14917) for lymph nodes, CD11b (1:500, Arigo, Cat#ARG22000) and iNOS (1:500, Novus Biologicals, Cat#NBPI-3380) for lymph nodes, NeuN (1:500, Abcam, Cat#Ab177487) and GFAP (1:500, Arigo, Cat#ARG64066) for spinal segments, and synapsin (1:500, CST, Cat#D12G5) and neurofilament (1:500, CST, Cat#C28E10) for motor nerve and presynaptic membrane with primary antibodies incubated overnight at 4 °C. Primary antibodies were retrieved and incubated with secondary antibodies of the appropriate species, and myofibrils were incubated with the appropriate secondary antibodies (Alexa Fluor 488, 1:1000, Beyotime, Cat#A0423) and α-bungarotoxin (1:1000, Biotium, Cat#CF®594) for postsynaptic

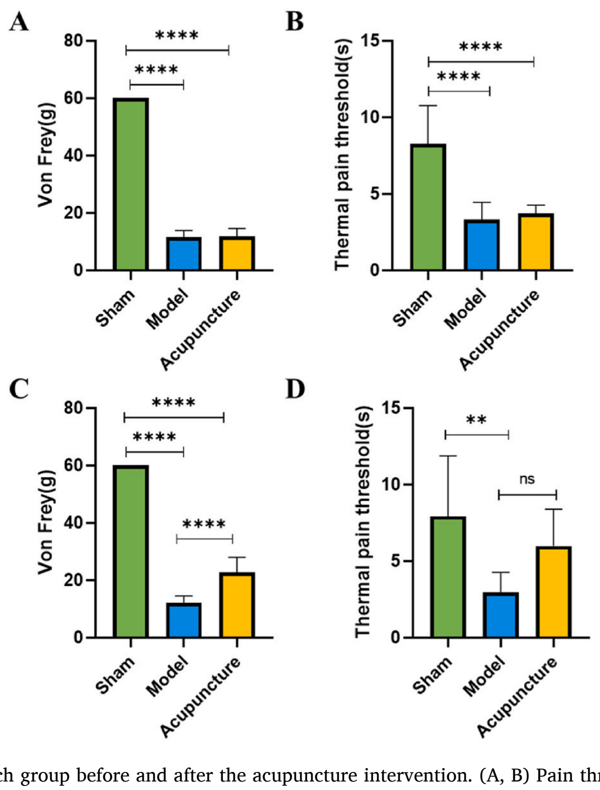


Fig. 2. Pain thresholds of mice in each group before and after the acupuncture intervention. (A, B) Pain thresholds of mice in each group before acupuncture intervention, n = 10, ****$p < 0.0001$. (C, D) Pain thresholds of mice in each group after acupuncture intervention, n = 10, **$p < 0.01$, ****$p < 0.0001$.

membrane, with fluorescence for 1 h at room temperature and protected from light. Finally, the sections were blocked with DAPI-containing blocking solution and imaged under a fluorescence microscope.

2.5. Flow cytometry

The fresh mouse lumbar aortic lymph nodes were ground to separate the cells to make a cell suspension, which was filtered, centrifuged, and resuspended with 1640 medium. The supernatant was discarded by centrifugation. Single-stained tubes and blank control tubes were set up and stained with cell fluorescently labeled antibodies in the dark at room temperature (including APC-coupled anti-F4/80, BV421-coupled anti-Ly6G, PE-coupled anti-Ly6C, FITC-coupled anti-CD11b, Percpcy5.5 Conjugated anti-CD3, Pe-cy7 conjugated anti-B220, FVS510 conjugated anti-FVS). After staining for 30 min, add staining Buffer to each tube and centrifuge to wash away unbound antibodies. Then add 300 μL of staining Buffer to each tube to resuspend the cell pellet. Finally, data were obtained on a quantitative imaging analysis flow cytometer (Amnis ImageStream MK II, Luminex) and analyzed using IDEAS software.

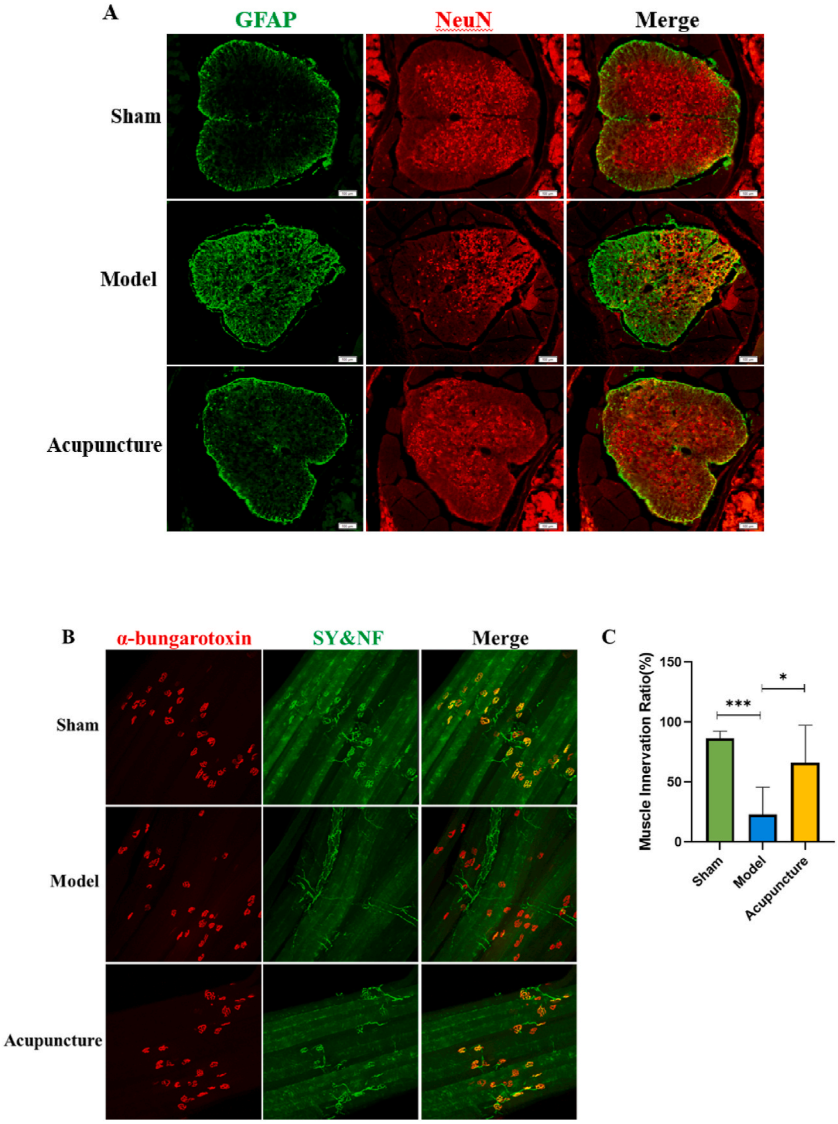


Fig. 3. Acupuncture promotes recovery of spinal astrocytes and neurons and effectively improves loss of innervation after nerve root injury. (A) Immunofluorescence staining of spinal cord astrocytes and neurons. Astrocytes in green, neurons in red. n = 9, Scale bar: 100 μm. (B) Immunofluorescence staining of the neuromuscular junction of the anterior tibial muscle. Green represents the postsynaptic membrane (motor neuron terminal). Red represents the postsynaptic membrane (acetylcholine receptors). n = 6, Scale bar: 20 μm. (C) Nerve terminal innervation rates were derived by calculating the ratio of the postsynaptic membrane to the presynaptic membrane. ***$p < 0.001$, *$p < 0.05$.

2.6. Data statistics

Data were expressed as mean ± standard deviation. The results were analyzed using IBM SPSS Statistics 26 software, and mapping was used GraphPad Prism 8 software. ANOVA analysis of variance was used to compare multiple sets of data with the post hoc comparison. In all analyses, a value of $p < 0.05$ was considered statistically different. The results were presented in a column plot.

3. Results

3.1. Acupuncture effectively increases mechanical pain thresholds in model mice

To investigate the effect of acupuncture on the pain threshold of mice with nerve root injury, the pain threshold was assessed before and after treatment. The results showed that acupuncture could increase the mechanical pain threshold but not the thermal pain threshold compared to the model group (Fig. 2A–D).

3.2. Acupuncture promotes recovery of spinal astrocytes and neurons and effectively improves loss of innervation after nerve root injury

In normal conditions, astrocytes in the spinal cord are mainly distributed in the white matter region. In contrast, astrocytes in the model group infiltrated from the white matter to the gray matter, and were distributed in both the white and gray matter of the spinal cord. After acupuncture treatment, the distribution of astrocytes was again similar to that of the sham operation group (Fig. 3A).

The neuromuscular junction is responsible for the transmission of electrical signals from nerves to muscle fibers, which are responsible for muscle contraction. Impairment of the synaptic transmission of this structure leads to neuromuscular disorders such as muscle atrophy, etc. We observed the structure of the neuromuscular junction by immunofluorescence staining and found that muscle innervation occurred after nerve root injury, while the rate of innervation of nerve endings increased significantly after acupuncture intervention compared to the model group (Fig. 3B and C).

3.3. Acupuncture improves the number of LECs of lumbar aortic lymph nodes

To investigate whether acupuncture has an effect on the lymphatic vasculature to alleviate the inflammatory response caused by nerve root injury, we examined the changes in the lymphatic vessels in the LALNs. The results of immunofluorescence staining showed that the proliferation of endothelial cells in the lymphatic vessels of the LALNs was significantly reduced in the acupuncture group compared to the model group (Fig. 4A and B).

3.4. Acupuncture reduced the proportion of monocytes, monocytic neutrophils and M1 macrophages in the LALNs

Further changes in immune cells within the LALNs in each group were examined by quantitative imaging flow cytometry, which showed that the proportion of mononuclear neutrophils in the lymph nodes was significantly increased in the model and acupuncture

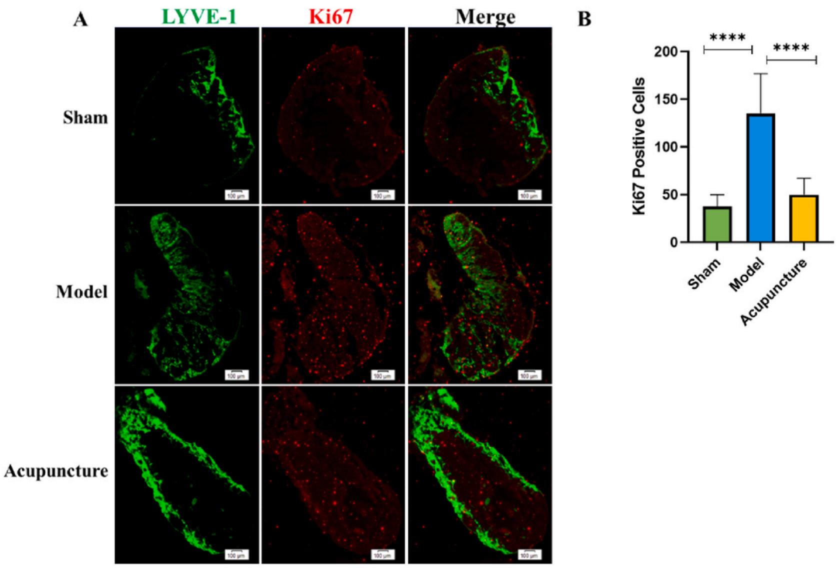


Fig. 4. Acupuncture improves the number of LECs of LALNs. (A) Endothelial cell and proliferation indicator staining of lymphatic vessels in LALNs. (B) Proliferation statistics of lymphatic endothelial cells in LALNs. n = 9, ****$p < 0.0001$. scale bar: 100 μm.

treatment groups compared with the sham-operated group. The proportion of mononuclear neutrophils was significantly lower in the acupuncture group than in the model group (Fig. 5A, B, G). The results of immunofluorescence staining show that the proportion of M1 macrophages was significantly lower in the acupuncture group (Fig. 5F and G). While the proportions of B cells, T cells and neutrophils were not statistically different between the groups (Fig. 5C–E, G).

4. Discussion

Our findings of the present study are summarized as follows: (1) Acupuncture increased the pain threshold of nerve root injury

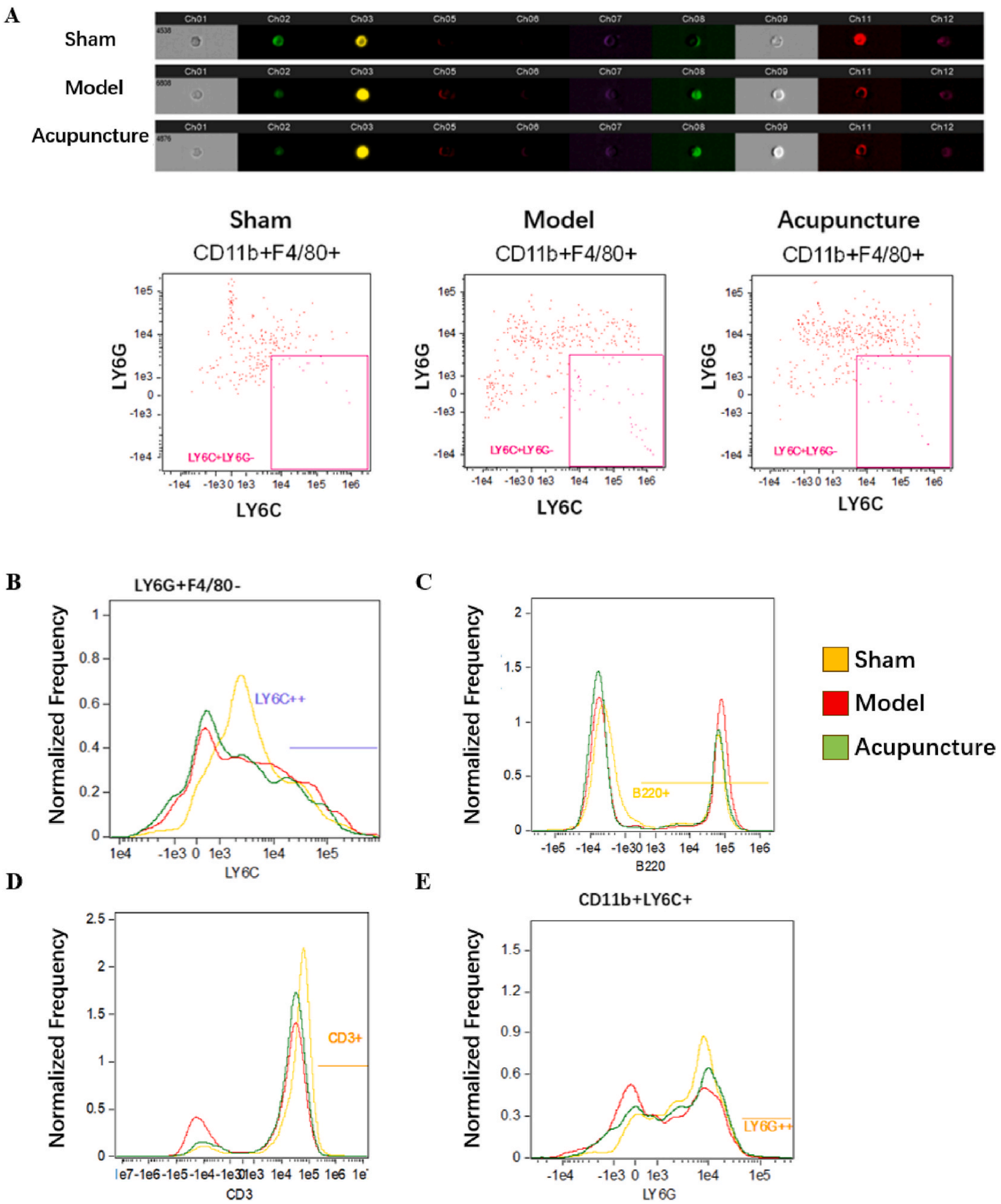
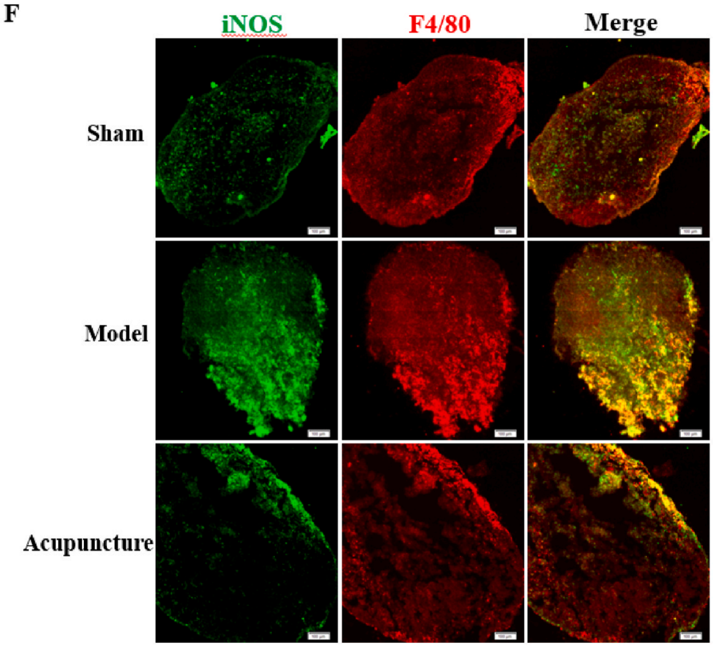


Fig. 5. Acupuncture reduced the proportion of mononuclear neutrophils and M1 macrophages in the lumbar aortic lymph nodes. (A, B) Results of flow cytometry and analysis of mononuclear neutrophils. (C) Results of flow cytometry and analysis of B cells. (D) Results of flow cytometry and analysis of T cells. (E) Results of flow cytometry and analysis of neutrophils. (A–E) n = 4. (F) Immunofluorescence staining of M1 macrophages in LALNs. n = 9 (G) The statistics results of the proportion of each immune cell (from A to F). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Scale bar: 100 μm.



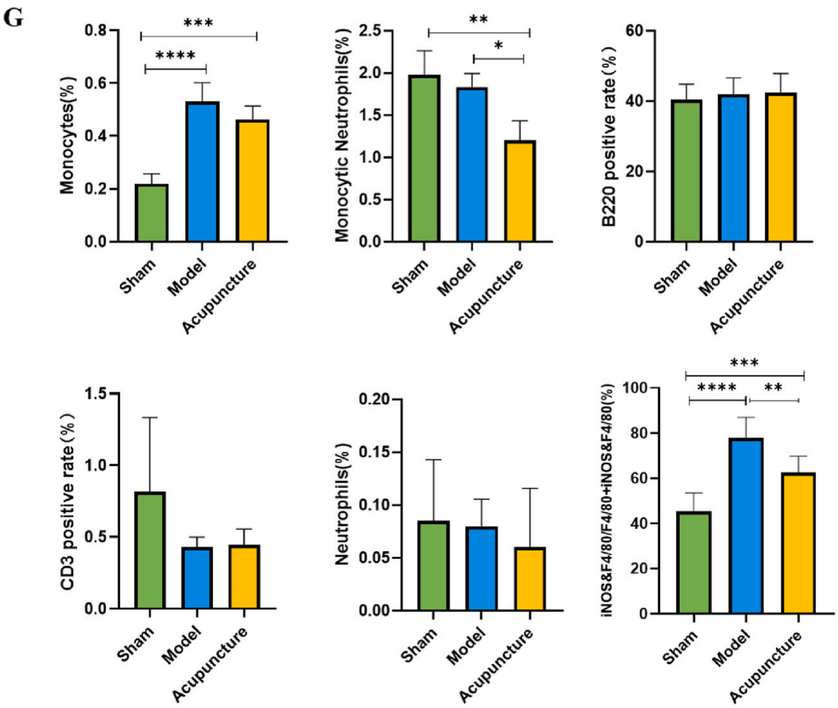


Fig. 5. (*continued*).

mice. (2) Acupuncture inhibited the activity of spinal astrocytes to achieve analgesia. (3) Acupuncture inhibited the proliferation of lymphatic endothelial cells. (4) Acupuncture reduced the proportion of monocytes, monocytic neutrophils and M1 macrophages in the LALNs. To sum up, in our study, we found that acupuncture increased the pain threshold, improved the repair of astrocytes and neurons in the spinal cord of mice with nerve root injury, as well as improving the innervation rate of nerve endings at the neuromuscular junction. Acupuncture reduced the activation of immune cells and the M1-type macrophages in the LALNs (Fig. 6).

In several previous studies, acupuncture is effective in improving neuropathic pain. It can replace commonly used analgesics to reduce side effects from drugs and has focused on the effects of acupuncture on increasing the pain threshold, decreasing pain sensitivity, regulating neurotransmitter, modulating inflammatory signaling pathways, and activating spinal glial cells which are closely related to pain signaling [23–29]. In the present study, acupuncture relieved pain caused by nerve root injury, demonstrating the analgesic effect of acupuncture and providing modulation of spinal astrocyte and neuronal disarray after modeling, which is consistent with the results of other acupuncture treatments for pain. It has been found that in neuropathic pain, activation of astrocytes causes mechanical hyperalgesia. Destruction of primary sensory neurons leads to thermal hyperalgesia. This is the mechanisms of mechanical hyperalgesia and thermal hyperalgesia [30,31]. This study found that acupuncture effectively inhibited the activity of spinal astrocytes and improved mechanical hyperalgesia. We hypothesize that the mechanism of effect of acupuncture may be better for the treatment of mechanical hyperalgesia. However, the specific effect mechanism of acupuncture needs further study. The neuromuscular junction (NMJ) is a highly specific synaptic structure formed by motor neurons (presynaptic membrane) and their target muscle fibers (postsynaptic membrane). It is responsible for converting electrical signals generated by motor neurons into electrical signals for muscle cells [32]. Acupuncture has been reported to promote the expression of acetylcholine receptors on the postsynaptic membrane of the neuromuscular junction and to improve muscle dysfunction caused by neuromuscular disorders, whereas the effect of acupuncture on the presynaptic membrane has been less reported [33–35]. In the present study, loss of innervation was found after acute nerve root injury, while the innervation rate of nerve endings was significantly increased after acupuncture intervention, suggesting that acupuncture also plays a role in improving the presynaptic membrane, and its related mechanism needs further study.

Lymphatic vessels play a main role in the removal of inflammatory cytokines from inflamed tissues. Some clinical studies have reported that acupuncture can modulate lymphatic function and improve symptoms including lymphoedema and lymph node enlargement caused by postoperative malignancies and their postoperative complications such as limb dysfunction [36–38]. This suggests that acupuncture has some modulatory effect on the lymphatic vascular system. We observed a significant proliferation of lymphatic endothelial cells in lumbar aortic lymph nodes after nerve root injury, whereas the proliferation of lymphatic endothelial cells was inhibited after acupuncture intervention. However, the current study is limited to the effect of acupuncture on cell proliferation and other phenomena, which is a single indicator and needs to be investigated to determine whether it affects other aspects and the underlying mechanisms. Lymphangiogenesis mediates important processes in inflammatory diseases. Inflammation is accompanied by changes in lymphatic vessel proliferation and lymphatic vessel endothelial cell proliferation in the lymph nodes at the corresponding sites. Lymphangiogenesis is important in the progression of the disease, and inhibition of lymphatic vessel proliferation improves the symptoms of the disease [39,40]. It suggests that lymphatic vessels are involved in the development of this disease, and appropriate immune activation is involved in the development of the disease, which can be used as one of the strategies for treatment and indicators for observation. In the present study, we found lymphatic vessel proliferation in the lumbar aortic lymph nodes after modeling, while the proliferation phenomenon was significantly attenuated after acupuncture treatment.

Recently, the modulatory effects of acupuncture on immune cells have received much attention. Macrophages, monocytes and B cells may all be potential targets for acupuncture to modulate immune function [41,42]. Acupuncture is able to regulate the balance of $CD4^+$ T cell subsets in a mouse model of asthma to alleviate the airway inflammatory response in allergic asthma [43]. Macrophages play an important role in the inflammatory response and can be polarized into M1 phenotype cells by the in vivo microenvironment. M1 polarization of macrophages produces a variety of inflammatory factors as well as cytotoxic factors, which in turn amplify the inflammatory response and promote disease progression [44]. In various animal disease models, acupuncture has been found to inhibit macrophage polarization to the M1 phenotype or promote polarization to the M2 phenotype to relieve inflammation and analgesia [45–47]. In the present study, acupuncture significantly reduced the number of mononuclear neutrophils and decreased the polarization of M1 macrophages, thereby inhibited the inflammatory response and promoted nerve repair. However, one week's treatment may not be enough to produce changes in other immune cells. Future experiments may be focused on setting more time points and setting up different protocols to further explore the regulatory functions of acupuncture on the lymphatic system, immunity and inflammation.

5. Conclusion

The effect of acupuncture in relieving neuropathic pain caused by nerve injury and reducing nociceptive sensitization through different pathways has been investigated [48,49]. And in this study we focused on the finding of the modulating effect of acupuncture on lymphatic function to explore the efficacy of acupuncture on injury after nerve root compression from another perspective. Based on our findings, we could suppose that acupuncture may be one of the effective methods to treat the acute nerve root injury and regulated lymphatic function. Still, more clinical works are needed to clarify the role of acupuncture in regulating lymphatic function after inflammatory nerve injuries.

Funding

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Data availability statement

Data included in article. Material/referenced in article.

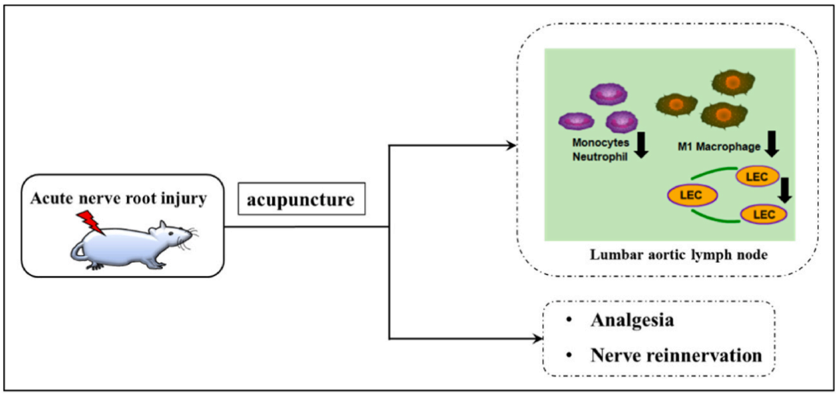

Fig. 6. Effect mechanism of acupuncture on nerve root injury.

CRediT authorship contribution statement

Jie Wang: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Jian-ju Liu:** Writing – original draft, Software, Methodology, Conceptualization. **Zhan-ying Tang:** Methodology, Investigation. **Qian-qian Liang:** Writing – review & editing, Supervision, Funding acquisition. **Jia-wen Cui:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Jie Wang: Conceptualization, Methodology, Formal analysis, Writing-Original draft preparation. Jian-ju Liu: Conceptualization, Methodology, Software, Writing-Original draft preparation. Zhan-ying Tang: Methodology, Investigation. Qian-qian Liang: Writing - Review & Editing, Supervision, Funding acquisition. Jia-wen Cui: Writing - Review & Editing, Project administration, Supervision, Funding acquisition. All authors read and approved the final manuscript.

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