



Proteomics Reveals the Effect of Low-Intensity Focused Ultrasound on Spasticity After Spinal Cord Injury

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ABSTRACT

AIM: To explore the efficacy and possible mechanisms of Low-intensity focused ultrasound (LIFU) in alleviating spasticity caused by Spinal cord injury (SCI).

MATERIAL and METHODS: We selected male Sprague–Dawley rats as subjects and performed transverse injuries on the T9 vertebra of their spinal cord (SC) to build SCI. On the 7th day after SCI, LIFU treatment was performed below the SCI segment once a day for 20 min, for 4 consecutive weeks. During treatment, a pressure sensor was used to assess the degree of spasticity. After treatment, the SC tissues from the treatment sites of the SCI+LIFU(-) and SCI+LIFU(+) groups were extracted, and high-throughput sequencing was performed to identify the changes in proteomics. In addition, expression of the growth associated protein 43 (Gap43) was validated by western blotting.

RESULTS: The behavioral results suggested that after 2 weeks of SCI, the rats were significantly induced to have a spastic reaction ($p < 0.05$), while after 4 weeks of LIFU treatment, the spastic response of rats was significantly improved ($p < 0.05$). Western blot analysis showed a significant increase in Gap43 expression in the SCI+LIFU(-) group compared with the sham group, whereas after 4 weeks of LIFU treatment, Gap43 protein expression was significantly decreased ($p < 0.05$).

CONCLUSION: The results of this study showed that LIFU is an alternative treatment that can effectively relieve spastic reactions caused by SCI, possibly by reducing abnormal neuroplasticity or axon regeneration below the SCI segment.

KEYWORDS: Low-intensity focused ultrasound, Spinal cord injury, Spasticity, Neuroplasticity, Proteomics

ABBREVIATIONS: Aif1: Allograft inflammatory factor 1, Arg1: Arginase 1, cAMP: Cyclic adenosine monophosphate, DC: Duty cycle, DEPs: Differentially expressed proteins, FC: fold change, Gap43: Growth associated protein 43, GO: Gene ontology, GABA: Gamma-aminobutyric acid, Grn: Granulin, IL-1: Interleukin-1, IgG: Immunoglobulin G, LIFU: Low-intensity focused ultrasound, LPS: Lipopolysaccharides, NP: Neuropathic pain, NF- κ B: Nuclear factor kappa B, PRF: Pulse repetition frequency, PPI: Protein-protein interaction, rTMS: Repetitive trans-spinal magnetic stimulation, SCI: Spinal cord injury, SC: Spinal cord, TEAB: Triethylammonium bicarbonate, TNF: Tumor necrosis factor, TRAF6: TNF receptor associated factor 6, TBST: Tris-buffered saline with 0.1% Tween® 20 Detergent.

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INTRODUCTION

Spinal cord injury (SCI) can cause motor, sensory, and autonomic neurological disorders (4). The imbalance in the inhibition and promotion of the SC below the injury section is an important cause of spasticity (31). Spasticity is a characteristic of upper motor neuron syndrome, defined as velocity-dependent, strong, consensual enhancement and is accompanied by muscle twitching, reflexes, and cramps (8,31). More than 80% of SCI patients worldwide have spastic reactions, many of whom are disabled as a result (1). Moderate and severe spasticity not only affect the recovery of patients' limb function but also cause complications such as muscle fibrosis and joint contraction, seriously affecting patients' ability to perform daily activities as well as their quality of life and causing significant economic and health burdens to the family and society (20,30). Currently, spasticity is still subject to reliance on drugs, botulinum toxin injection, local neuro-muscular blockade, or surgery (6,18). However, the drug is systemic and long-term use can have toxic side effects throughout the body (27). Botulinum toxin injection, local neuromuscular blockade, and surgery are invasive and require repeated treatment (9,11). Previous studies have shown that neuroplasticity or regeneration is one cause of spasticity. After SCI, the SC above the injured section weakens the information input and control of the neural circuits below the section, but neurons in the SC below the injury section are remodeled and adapted to exhibit hypersensitivity reactions in the form of regeneration at the end of the neuronal axon (10,14,19,21,29). Therefore, the appearance of neuroplasticity or axon regeneration in the following sections of SCI is an important target for spastic onset.

Low-intensity focused ultrasound (LIFU) is a type of mechanical sound wave with energy of 1–100 mW/cm² (2,40), which, as a new and efficient physical stimulus factor, has gradually become a topic of interest in the field of medical ultrasound. LIFU has the advantages of being non-invasive, targeted, and penetrating; so, it has more advantages in nerve regulation (22) LIFU potentially plays a protective role in the treatment of neuropathic pain (NP) (17), epilepsy (38), by central or peripheral neuromodulation and inhibition of neuronal excitability of the SC or cortex. Recently, our team found that LIFU can relieve neuropathological pain caused by sciatic nerve ligation by suppressing SC excitement (24). However, the signaling pathway corresponding to the underlying neuromodulation mechanism of LIFU is unknown; there are few reports on the treatment of spasms with LIFU, and the efficacy is unclear.

Thus, we used proteomics to evaluate the efficacy of LIFU in treating spasticity and the possible underlying mechanisms in a rat model of SCI.

MATERIAL and METHODS

Animals

Eighteen specific pathogen free-grade adult male Sprague–Dawley rats (200–300 g) were selected and purchased from the Department of Experimental Animals of Kunming Medical University (Kunming, China). All rats were placed in a room

with a temperature of 23°C ± 2°C, humidity of 50%–60%, and a light/dark cycle of 12 h, with free access to food and water. The selected animals were approved by the Animal Ethics Committee of Kunming Medical University (No. KMMU2020252).

Grouping and Experiment Protocol

After 1 week of acclimatization, all rats were randomly divided into three groups (six per group): the sham group, only the SC tissue was exposed without injury; the SCI+LIFU(+) group and the SCI+LIFU(-) group, rats that received SCI surgery. Among them, the SCI+LIFU(+) group was treated with LIFU, while the SCI+LIFU(-) group was not intervention by LIFU.

SCI Model

We strictly followed the literature (26) to establish the SCI model. We used a completely severed SC in rats to model SCI. The rats were laid flat on the operating table and anesthetized by light anesthesia. Hair from the T7–T10 part of the rat and its surroundings was removed by an electric clipper. After iodophor disinfection, we used a scalpel to make a 3-cm longitudinal incision, bluntly separated the muscles around the spine to expose the spinous process, lifted the T9 vertebra, excised the lamina to expose the SC plane, and completely transected the SC. Gauze was used to stop bleeding. The injured parts of the muscles, fascia, and skin were sutured and disinfected with iodophor.

LIFU Treatment

LIFU treatment started on the 7th day after SCI from 09:00 to 16:00. After the rats were lightly anesthetized with isoflurane, they were fixed on the table, and using an electric hair removal machine and applying hair removal cream, the hair on the treatment site was removed, exposing the area below the damage section. An ultrasonic probe (1 MHz, DOBO; Changzhou, China) was fixed on this segment and covered the skin. The ultrasonic coupling agent (Aquasonic; Parker Laboratory, Fairfield, NJ, USA) was filled between the probe and the skin to eliminate air bubbles. The ultrasound parameters selected during the experiment were as follows: fundamental frequency, 1 MHz; pulse repetition frequency (PRF), 0.8 KHz; irradiation intensity, 0.68 MPa; duty cycle (DC), 20%; treatment time, 20 min/d; treatment period, 4 weeks. See Figure 1A for the entire experimental process.

Assessment of the Degree of Spasticity

The entire behavioral testing process was carried out in a 'blind' manner. Each rat was placed in central square and allowed to adapt to the environment for 20 min before the testing. According to the method in the literature (35), we placed the rat's tail on the pressure sensor and then manually stimulated the back one-third of the rat's tail until the rat's lower extremities developed cramps or convulsions. The pressure value (in g) was recorded by a miniature pressure sensor placed between the finger and the tail. Each rat was tested nine times, and the average value of the calculated values was the pressure value that induced the rat to spasm. In addition, the entire behavioral test was scheduled from 9:00 to 12:00 in the morning

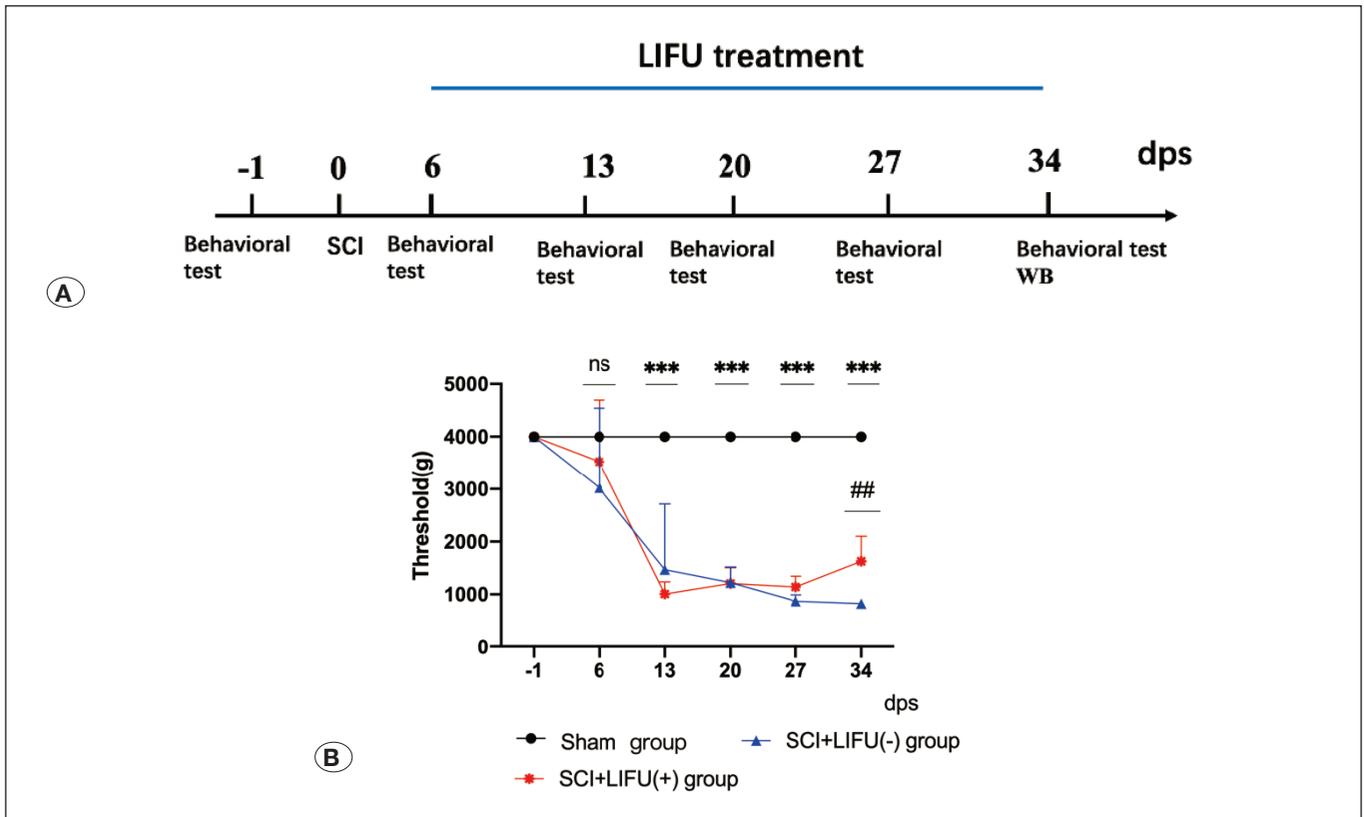


Figure 1: A) The flowchart of this experiment. **B)** Compared with the sham group, the SCI+LIFU(-) group and the SCI+LIFU(+) group did not show obvious spasticity on the 6th day after surgery, and the pressure threshold was higher ($p > 0.05$). At 13 days after surgery, compared with the sham group, the SCI+LIFU(-) group and the SCI+LIFU(+) group exhibited more spasticity induction and a lower pressure threshold ($p < 0.05$). After 4 weeks of LIFU treatment, compared with the SCI+LIFU(-) group, the pressure threshold of SCI+LIFU(+) group induced spastic reaction increased significantly ($p < 0.05$), but the pressure threshold of SCI+LIFU(+) group was still lower than that the sham group ($p > 0.05$). The data are expressed as mean \pm standard error mean (SEM), ns $p > 0.05$ against the SCI+LIFU(-) group and the SCI+LIFU(+) group. *** $p < 0.05$ against the SCI+LIFU(-) group and the SCI+LIFU(+) group; ## $p < 0.05$ against the SCI+LIFU(-) group. One-way analysis of variance (ANOVA); $n = 6$ per group

Proteomics Analysis

Each protein sample was dissolved in 8 M urea and 100 mM triethylammonium bicarbonate (TEAB) buffer (pH, 8.5), followed by the addition of trypsin and 100 mM TEAB buffer. After incubation for 4 h at 37°C, the sample was incubated overnight in trypsin and CaCl₂ enzymes. Then, the sample was centrifuged at 12000 \times g for 5 min, and the supernatant was filtered through a C18 column. The column was washed three times with 0.1% acetic acid and 3% acetylene and then eluted with 0.1% acetic acid and 70% acetylene. The filtered fluid was collected and freeze-dried.

Mobile phase A (2% acetylene, 98% water, ammonia to pH = 10) and mobile phase B (98% acetylene, 2% water) were prepared as follows. The mixture of freeze-dried powder with mobile phase A was centrifuged at 12000 \times g for 10 min at room temperature. The L-3000 HPLC system and Waters BEH C18 column (4.6 mm \times 250 mm, 5 μ m) were used, and the column temperature was set to 45°C. One tube was collected every minute, combined into 10 fractions, and the combined tissue was frozen for subsequent use. The sample protein was dissolved in mobile phase A (100% water, 0.1% formic acid)

and mobile phase B (80% acetylene, 0.1% formic acid) and was separated using the EASY-nLCTM 1200 ultra-efficient liquid phase system. The peptide segment was separated by an ultra-efficient liquid phase system and injected into the Nanospray Flex™ ion source for ionization and then into the Q Exactive™ HF-X mass spectrometer for analysis. The data acquisition mode uses a data-dependent scanning program, in which the female ions of the top 40 with the highest ion strength are selected after full scanning enter the high-energy collision pool in turn and break up with 32% of the fractured energy, followed by secondary mass spectrometry analysis.

Bioinformatics Analysis

The t-test was used for statistical analyses to quantify the significant differences between the SCI+LIFU(+) group and SCI+LIFU(-) group ($p < 0.05$), and fold change (FC) > 1.2 or < 0.83 was defined as a differentially expressed proteins (DEPs) (42). To better understand the function of proteins, ClusterProfiler package (version: 4.0.) in R was employed to analyze the Gene Ontology (GO) function of potential targets and enrich the Reactome pathway (41). The heat map is displayed by the R software package pheatmap. All the above

analysis methods and R package were implemented by R foundation for statistical computing (2021) version 4.1.1. The STRING database (37) was used to analyze protein-protein interactions (<https://string-db.org/>).

Western Blot Analysis

We placed the injured part of the rat SC tissue on ice, extracted the relevant protein, and quantified the protein concentration using the bicinchoninic acid assay kit (Biomed, Beijing, China). Proteins were resolved by electrophoresis, electrotransferred to a membrane, and blocked in 5% skimmed milk powder for 2 h. After three washes with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) for 15 min each, the membranes were incubated overnight at 4°C with primary antibodies including growth associated protein 43 (Gap43) (1:1000; Cell Signaling Technology [CST], Danvers, MA, USA) and β -tubulin (1:1000; Abcam, Cambridge, MA, USA). The next day, the membranes were washed three times with TBST for 15 min each, followed by incubation with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG) antibody (1:5000; CST), and incubated for 2 h at room temperature. Proteins were detected by using enhanced chemiluminescence (ECL; Tanon, Shanghai, China) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses

The data are expressed as the mean \pm standard error of the mean. SPSS 26.0 (IBM Co., Armonk, NY, USA) was used for the statistical analyses, and GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to plot the graphs. Analysis of variance was used to analyze the behavioral and Western blot results. The significance level was denoted as $\alpha = 0.05$ and $p < 0.05$.

RESULTS

Behavioral Test Verifies the Effect of LIFU on Spasticity in Rats with SCI

We evaluated the changes in the degree of spasticity of rats in each group at different periods in Figure 1B. From the SCI animal model to LIFU treatment the day before, neither the SCI+LIFU(-) group nor the SCI+LIFU(+) group had significant spastic reactions in the lower extremities, but the pressure threshold was reduced from 4000 g to (3023 \pm 1517 g) and (3521 \pm 1173 g), respectively. The thresholds were not statistically significant compared to pre-treatment ($p > 0.05$), and there was no statistical significance between the two groups ($p > 0.05$). On the 13th day after surgery, the tails of the rats in the SCI+LIFU(-) group and of those in the SCI+LIFU(+) group were significantly induced by pressure stimulation to have lower limb spasticity and increased sensitivity, at which point the pressure thresholds were (1468 \pm 1245 g) and (1007 \pm 230.2 g), respectively. The differences were not statistically significant between the two groups ($p > 0.05$). After treatment with LIFU, the spasticity of the SCI+LIFU(+) group gradually improved, and the pressure threshold for inducing spastic reactions through stress stimulation gradually increased, at which point the pressure thresholds of the SCI+LIFU(+) group

and SCI+LIFU(-) group were (1629 \pm 192.5 g) and (824 \pm 38.9 g), respectively, with statistical significance ($p < 0.05$). However, the pressure threshold for the SCI+LIFU(+) group was below that of the sham group ($p < 0.05$). Behavioral cues suggested that LIFU could effectively relieve the spastic reaction caused by SCI after 4 weeks of treatment. Therefore, we chose the LIFU-treated SC tissue from the SCI+LIFU(+) and SCI+LIFU(-) groups for proteomic analysis to explore the possible molecular mechanisms by which LIFU relieves spasticity.

Proteomics Show DEPs in the SC

The ratio of the mean value of all repeated quantitative values of each protein in the comparison sample was used as the different FC. To explore the significance of the difference, the t-test was performed on the relative quantitative value of each protein in the two compared samples, and the corresponding corrected P-value was calculated, which was used as the significance index. The default was $p \leq 0.05$. According to this condition, the upregulated and downregulated DEPs were screened out. Compared with the SCI+LIFU(-) group, a total of 42 differential proteins were screened, of which 13 were upregulated proteins and 29 were downregulated proteins. In addition, the relative content of the differential proteins of the SCI+LIFU(+) group and the SCI+LIFU(-) group was analyzed, and the results are shown in Figure 2.

Reactome Pathway Enrichment Analysis of DEPs

To study the function of the DEPs between the SCI+LIFU(+) group and the SCI+LIFU(-) group, we conducted a Reactome pathway enrichment analysis of the DEPs. Analysis of the enriched pathways showed that they were mainly related to neutrophil degranulation, Toll-like receptor cascades, tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) mediated nuclear factor kappa B (NF- κ B) activation, and interleukin-1 (IL-1) signaling (Figure 3).

Biological Process Enrichment Analysis of DEPs

To study the function of DEPs between the SCI+LIFU(+) group and the SCI+LIFU(-) group, we enriched the biological process of DEPs through the GO database. GO analysis showed that the DEPs were mainly enriched in biological processes such as the cell response to oxidative stress, response to axon damage, internal apoptosis in response to oxidative stress, and cell response to chemical stress (Figure 4). In these biological processes, the response to axon damage was more closely related to this study, and the proteins were mainly related to processes corresponding with allograft inflammatory factor 1 (Aif1), arginase 1 (Arg1), Gap43, and granulysin (Grn) (Table I).

Protein-Protein Interaction Network Construction

To explore the mechanism by which LIFU relieves spasticity, we used the STRING Protein Interoperability Database (<http://string-db.org/>) to construct a protein-protein interaction (PPI) network diagram of the DEPs that were altered by spasticity and reversed by LIFU. Then, we conducted an MCODE analysis of the established PPI network diagram and found a module including the Gap43 protein (Figure 5).

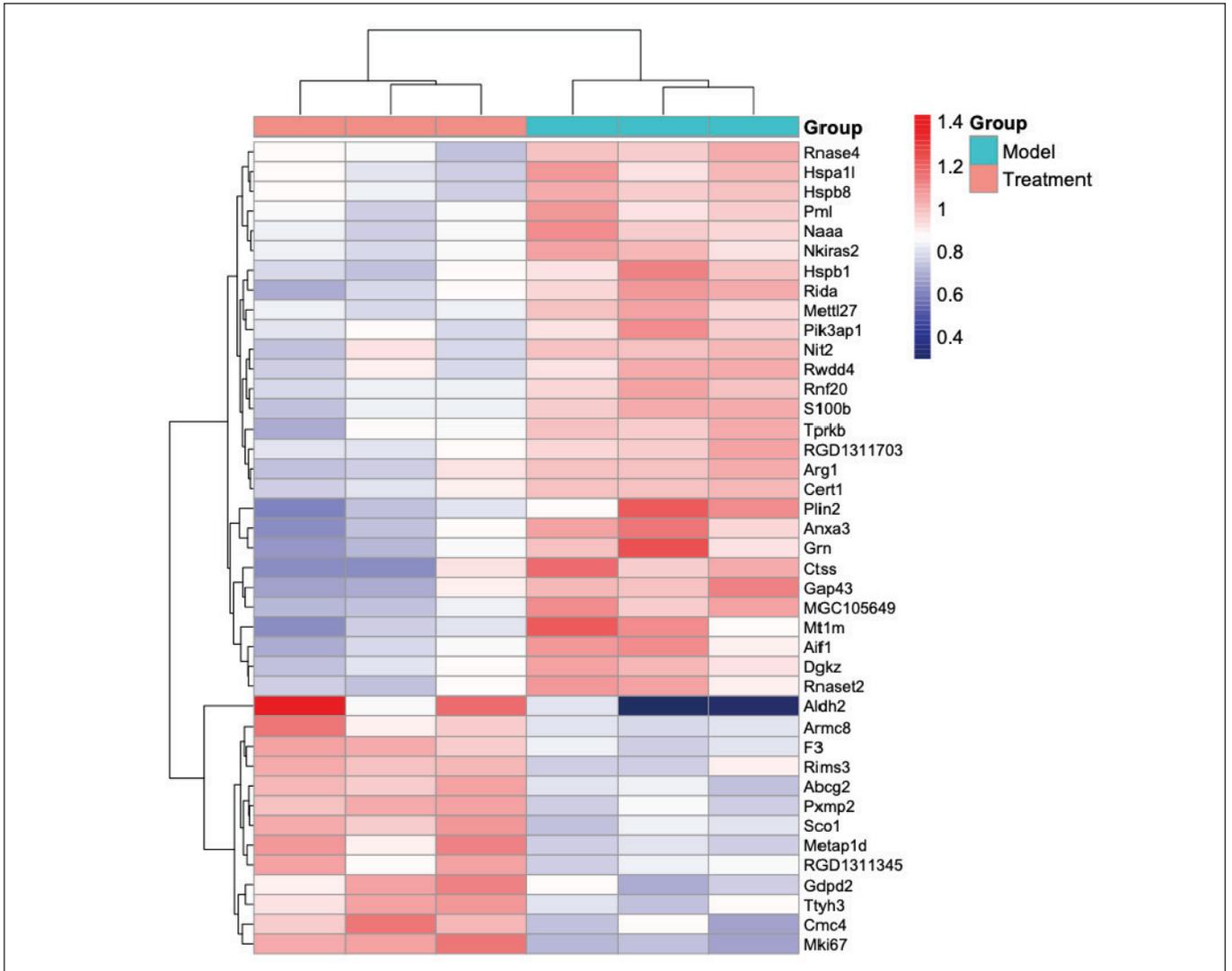


Figure 2: Differential protein expression induced by the SCI+LIFU(+) group and SCI+LIFU(-) group. Heat map of the cluster analysis of differential protein expression between the SCI+LIFU(+) group and the SCI+LIFU(-) group (n=3). The red color in the figure represents the upregulated protein, and the blue color represents the downreg-ulated protein.

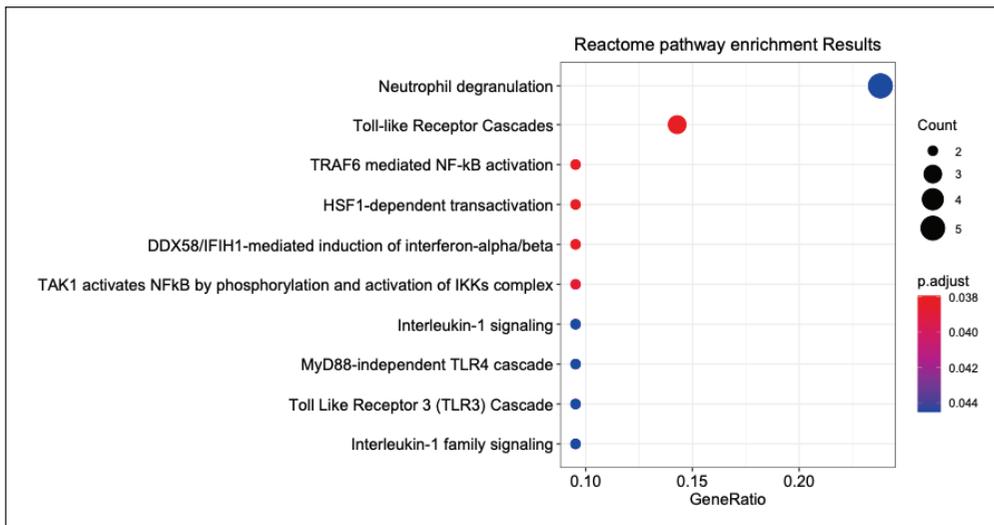


Figure 3: Reactome pathway enrichment analysis.

Validation of Gap43 Protein

Gap43 was selected as the DEP through biological process enrichment, Reactome path enrichment analysis, and evaluation of the functional effects of proteins and PPI. Western blot analysis showed that the expression of Gap43 in the SCI+LIFU(-) group was upregulated, which was reversed after LIFU treatment (Figure 6). Together, the results showed that Gap43 is an important protein during the course of SCI, which may be a molecular target for LIFU-mediated reduction of spasticity after SCI.

DISCUSSION

Spasticity is one of the most common SCI complications (34). It is a movement disorder characterized by hyperreflexia and muscle stiffness, which affects the recovery of motor function and quality of life (13). Although some progress has been made in neurorehabilitation and medications, there is still a lack of effective and non-invasive treatment for spasticity (28). In this study, we demonstrated the efficacy and safety of LIFU in alleviating spasticity after SCI and its effect on neural remodeling below the injury section. Two weeks after the SCI model was established in rats, there were obvious symptoms of spasticity such as increased tension and increased muscle hardness in both lower limbs and tails, and lower pressure stimulation of the tail was given to induce a compass reaction in the lower limbs. However, 4 weeks after LIFU treatment, the tension and muscle stiffness of the lower limbs and tail of the rats were reduced, the threshold for spasm caused by tail pressure stimulation was increased, and the sensitivity was weakened. Therefore, from the clinical symptoms, LIFU can significantly alleviate the spastic reaction induced by SCI.

To obtain insights into the potential mechanism by which LIFU alleviates spasticity, three SC samples below the injured segment from the SCI+LIFU(+) group and the SCI+LIFU(-)

group were used for proteomic analysis. Cluster analysis showed the DEPs by comparing the LIFU-treated SCI group with the untreated SCI group. Forty-two DEPs were identified, including 13 upregulated and 29 downregulated proteins. We further predicted the potential functions of the DEPs using Reactome pathway and the biological process of GO and PPI network analysis. Through the Reactome pathway enrichment analysis, the following pathways were found to be highly correlated with the functions of the DEPs after LIFU treatment: neutrophil degranulation, Toll-like receptor cascades, TRAF6-mediated NF- κ B activation, and IL-1. Based on GO functional analysis, we speculated that the corresponding genes Aif1, Arg1, Gap43, and Grn, which respond to axon injury, might interact with the abnormal neuroplasticity involved in spasticity development. With PPI network analysis, we identified Gap43 as one of the crucial nodes in a module that might be altered by spasticity and reversed by LIFU. Subsequently, Western blotting was used to validate the differentially modified Gap43 expression identified in proteomics analysis.

Based on the results of this study, we can initially conclude that LIFU can effectively relieve spastic symptoms; however, its specific mechanism of action is still unclear. Previous studies have reported that the use of gene and motor combination therapy in the mouse SCI model can promote an increase in the number of Gamma-aminobutyric acid (GABA) inhibitory intermediate neurons, change the ratio of excitatory to inhibitive intermediate neurons, and reduce muscle spasticity after SCI by neural remodeling in the SC or changing the excitability of the central neural circuit in the SC (7). The study by Bendella et al. (5) showed that linking the uninjured parts of the SC to surrounding nerves and muscles increases the patient's spastic response. The cause of this result was a burst of nerve axis buds. These findings show that abnormal neural remodeling may play a significant role in spasticity processing and may potentially reveal a therapeutic target of spasticity.

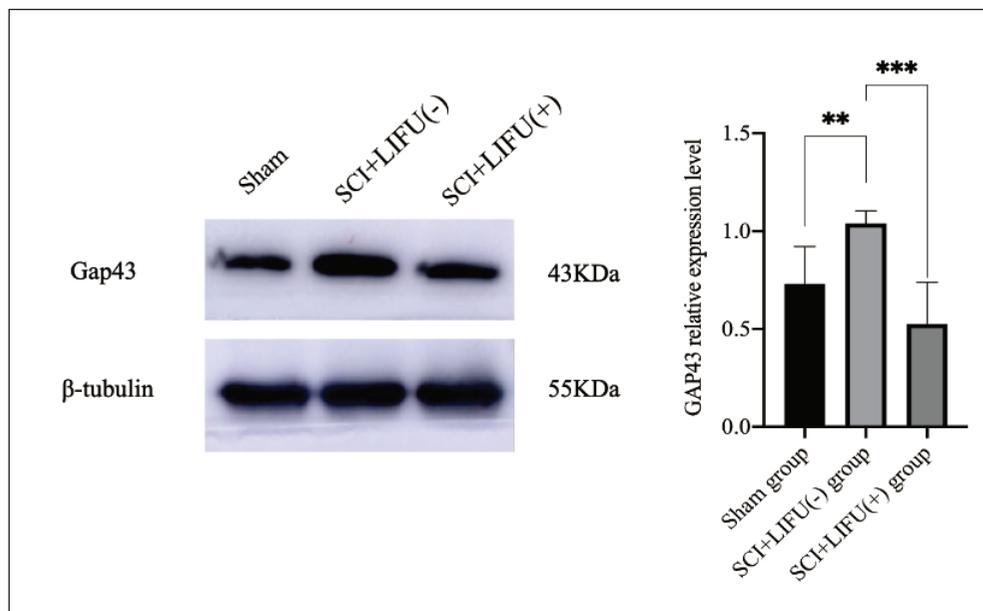


Figure 6: After 4 weeks of LIFU treatment, Gap43 proteins were relatively expressed in each group. All data are represented as the mean \pm SEM; * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA, three per group.

Gap43 is a key protein in the synaptic frontal membrane, which affects the trans-mission of information between synapses (43). Gap43 protein, as an important marker, is involved in the process of neural remodeling or axon regeneration (16). When axial bursts or neurons regenerate new growth cones, Gap43 is expressed more as a marker of the growth cone, mediating the transmission of information between neurons (15). Njoo et al. (32) found in the inflammatory pain model in which neural remodeling appears in the SC of mice, manifested in increased growth cones and dendritic ratchet density of neurons, increased Gap43 expression, and consistent with the behavior of pain hypersensitivity response. It takes longer for patients with spasticity to peak in potential detection after the conflation of excitatory synapses, suggesting that axons sprout at the far end of the α motor neuron dendrites and form more synaptic contact (33). In our study, the rat spasticity response was significant in the SCI+LIFU(-) group, and the level of Gap43 protein expression in the SC in this group was significantly increased compared to that in the sham group, which may illustrate the potential relationship between neural remodeling and spastic development in the SC. Liu et al. (25) found an increase in Gap43 expression in the injured area of SCI rats after the treatment of repetitive trans-spinal magnetic stimulation (rTMS), which seems to contradict our results. It should be noted that, in our study, the increase of Gap43 expression was mainly found in the area of the lumbar and sciatic segment, which is below the injured segment (T10) and is the main innervation segment of the lower limbs of rats. Therefore, the correlation between post-SCI spasticity and neuroplasticity below the injury section needs to be explored.

Accumulating evidence has shown that immune cells can influence the remodeling of neural circuits and that the inflammatory response can promote neural remodeling or regeneration. Pro-inflammatory substances such as lipopolysaccharides (LPS) can remodel or regenerate nerves by activating small glial cells and collecting macrophages to promote the release of growth factors (39), and inflammatory cytokines such as IL-1 β and TNF- α also enhance the phosphorylation of Cyclic adenosine monophosphate (cAMP) response element-binding protein, which promotes increased neuroplasticity and axon growth (3).

The Toll-2 receptor not only protects neurons in the human brain but also promotes an increase in the number of neurons and indirectly regulates neuroplasticity (23). The NF- κ B signaling pathway activation is involved in most inflammatory reactions, but it also plays an important role in neuronal development and synaptic plasticity (12). In animal experiments involving mice, NF- κ B was found to block the forward brain and reduce the growth of synapses and the transmission of information between synapses, thereby reducing abnormal neuroplasticity (36). Therefore, throughout our proteomics and validation of proteins, LIFU may have achieved spasticity relief by reducing the inflammatory response in the SC and by inhibiting abnormal neural remodeling or axon regeneration. Future studies will focus on exploring the correlation of these identified inflammatory pathways and neuroplasticity-related proteins and confirming the regulatory role of neural remodeling involved in spasticity development.

■ CONCLUSION

Our findings suggest that pathways related to the activation and inflammatory response of immune cells and proteins involved in the abnormal neuroplasticity and axonal regeneration are affected most significantly after LIFU therapy of post-SCI spasticity. Proteins of neural remodeling such as Gap43 may be crucial for LIFU therapy of spasticity after SCI in rat models. The relationship and underlying mechanism between the inflammatory response and neuroplasticity could be used as a novel diagnostic and therapeutic target against SCI-induced neuroplasticity. However, the additional mechanisms and interactions need to be further validated by extensive experiments.

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■ AUTHORSHIP CONTRIBUTION

Study conception and design: BW, HZ

Data collection: LA, YL

Analysis and interpretation of results: MC, SC, YL, XT

Draft manuscript preparation: BW, HZ, MC, SC, YL, XT, LA, YL

Critical revision of the article: BW, HZ, MC, SC, YL, XT, LA, YL

All authors (BW, HZ, MC, SC, YL, XT, LA, YL) reviewed the results and approved the final version of the manuscript.

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