Original Article

Low-Intensity Pulsed Ultrasound Ameliorates Neuropathic Pain Induced by Partial Sciatic Nerve Ligation Via Regulating Macrophage Polarization

Yao LIU[1, 2], Linze XIA[1, 2], Fumiya KANO[2], Noboru HASHIMOTO[2],

Yoshizo MATSUKA[3], Akihito YAMAMOTO[2], Eiji TANAKA[4]

Abstract：Inflammatory (M1-polarized) macrophages cause neuropathic pain (NP) after nerve injury through non-resolving neuroinflammation. However, increasing evidence suggests that converting M1 to anti-inflammatory M2 macrophages may rescue NP. In the present study, the therapeutic potential of low-intensity pulsed ultrasound (LIPUS) was investigated in a partial sciatic nerve ligation (PSL)-induced NP model.

Materials and Methods: Abnormal pain sensation, such as tactile allodynia, was caused by PSL. Immediately after PSL induction, the mice were subjected to LIPUS treatment for 20 min/day for 7 days. LIPUS was used at an average intensity of 60 mW/cm^2 and a frequency of 1.5 MHz.

Results: In the behavioral test, the LIPUS group showed a significant improvement in the PSL-induced hypersensitivity compared to the PSL group not exposed to LIPUS. We found an increasing number of M2 macrophages in the injured sciatic nerves after LIPUS exposure. LIPUS treatment decreased expression of pro-inflammatory microglial markers in spinal cord.

Conclusions: Our data suggest that LIPUS has an anti-nociceptive effect by increasing anti-inflammatory M2 macrophage and may be a suitable therapeutic candidate for NP.

Introduction

Growing evidence suggests that injury-induced non-resolving neuroinflammation in the central and peripheral nervous systems controls neuropathic pain (NP)[1, 2]. After peripheral nerve injury, Schwann cells are activated and secrete pro-inflammatory cytokines. These inflammatory mediators cause the immune response of injured and uninjured sensory neurons in the dorsal root ganglion (DRG) and the proliferation of satellite glial cells. Simultaneously, the recruited immune cells in the DRG secrete pro-inflammatory cytokines, which activate satellite glial cells and the DRG neurons. Finally, microglia are activated in the central nervous system (CNS) through neuroimmune interactions. These cells work in a cytokine-chemokine network establishing non-resolving neuroinflammation, which leads to NP[1, 3]. Hence, interventions targeting neuroinflammation are considered promising candidates for NP treatment[3].

Macrophages and glial cells are considered central players in NP because of their phagocytic function and the secretion of cytokines and chemokines[4, 5]. It has been suggested that the

[1] Department of Orthodontics and Dentofacial Orthopedics, Tokushima University Graduate School of Oral Sciences
[2] Department of Tissue Regeneration, Tokushima University Graduate School of Biomedical Sciences
[3] Stomatognathic Function and Occlusal Reconstruction, Tokushima University Graduate School of Biomedical Sciences
[4] Department of Orthodontics and Dentofacial Orthopedics, Tokushima University Graduate School of Biomedical Sciences

different activation states of macrophage-monocyte lineages are crucial for inflammation and tissue homeostasis[6-8]. Macrophages are classified as M1 and M2, according to their phenotype: M1 macrophages are related to proinflammatory cytokines and chemokines, while M2 macrophages suppress inflammation. The balance between M1 and M2 macrophages is thought to play an important role in ameliorating NP[9].

Clinically, NP has several forms, including trigeminal neuralgia and diabetic neuropathy[10]. To date, several therapies have shown great potential in clinical applications, such as mesenchymal stem cell treatment[11, 12]. Nevertheless, this type of cell administration is associated with certain risks, such as arrhythmia[13], ossification, and calcification[14]. On the contrary, it has been recognized that low-intensity pulsed ultrasound (LIPUS) is an operative, diagnostic, therapeutic, and safe tool in the medical field. Previous reports have shown that LIPUS can accelerate the regeneration of the sciatic nerve (SCN) after neurotomy[15]. LIPUS also promotes spinal fusion by regulating macrophage polarization[16]. Meanwhile, LIPUS has no side effects, such as deleterious, carcinogenic, and thermal effects, which may degrade living tissues. In our previous study, we showed the anti-inflammatory and regenerative ability of LIPUS in many diseases, such as sialadenitis, skeletal muscle injury, and knee joint synovitis[17-19].

Thus, we hypothesized that LIPUS may suppress NP at the early stage due to its anti-inflammatory ability. To test this hypothesis, we evaluated the effect of LIPUS exposure on NP in a partial sciatic nerve ligation (PSL) mouse model. The macrophage phenotypes in SCN and their effects on PSL-induced glial activation and neuroinflammation were also examined.

Materials and Methods

1. Animals

All animal experiments were approved by the Animal Research Committees of Tokushima University (Permit No: T2020-09) and performed in accordance with the ethical guidelines of the International Association for the Study of Pain. Male ICR mice (Charles River, Yokohama, Japan) aged 7－11 weeks were used in the experiments. All mice were kept in plastic cages under standard laboratory conditions (12-h dark/light cycle, at a temperature of 23℃－24℃) and provided with water and solid diet pellets *ad libitum*. The animals were randomly divided into two experimental groups: the PSL group and the LIPUS group, of five animals each.

2. Partial sciatic nerve ligation (PSL)

PSL was performed according to a previously described method[20]. A mixture of 5.0 mg/mL Vetorphale (Meiji Seika, Tokyo, Japan), 1.0 mg/mL Domitor (Zenoaq, Fukushima, Japan), and 5.0 mg/mL midazolam (Sandoz, Yamagata, Japan) was diluted with distilled water for injection (79% of the total volume) and used for anesthesia (0.1 mL/10 g). The right SCN was exposed in all mice. A 3/8 curved needle with a silk suture was inserted into the nerve, and approximately 1/3－1/2 of the nerve was tightly ligated. The incision was then closed using four skin sutures (4－0). As a sham control, the left SCN was exposed with a small incision but was not ligated.

3. LIPUS exposure

In the LIPUS group, LIPUS (Osteotron V, ITO Co., Tokyo, Japan) was applied immediately after PSL. The ipsilateral SCNs were exposed to LIPUS for 20 min/day for 7 days. The ultrasound exposure system used had a circular surface transducer with a cross-sectional area of 5.0 cm^2. The ultrasound head exhibited an effective radiating area of 4.1 cm^2 and a beam non-uniformity ratio average of 3.6. A pulsed ultrasound signal was transmitted at a spatial averaged intensity of 60 mW/cm^2 at a frequency of 1.5 MHz and a pulse rate of 1:4 (2 ms "on" and 8 ms "off").

4. Behavioral testing

Mice were individually placed on a 5×5 mm metal mesh floor with small plastic containers and allowed to habituate to the environment 2 days before the baseline testing. Before testing, animals were allowed to habituate for approximately 2－3 h before the examination.

PSL-induced tactile allodynia was evaluated as described previously[21]. The investigator was blinded to the treatment groups. Mechanical hypersensitivity was measured using an electronic von Frey device (Ugo Basile S.R.L, Varese, IT). The von Frey filament was applied to the middle of the plantar surface of hind paw, and a positive reaction was recorded when the mice showed a brisk paw withdrawal reaction upon stimulation.

5. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the SCN or the ipsilateral side of L3-4 spinal cord using Isogen (Nippon Gene, Tokyo, Japan), respectively. The contralateral side of SCN or L3-4 spinal cord was used as sham control. The purified total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Thermo Fisher, Carlsbad, CA, USA). THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) was used to perform qRT-PCR, and amplification was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used are listed (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for normalization.

Table 1　Primer sequences used for real-time RT-PCR.

Gene		Primer sequence
mouse *GAPDH*	Forward	AACTTTGGCATTGTGGAAGG
mouse *GAPDH*	Reverse	GGATGCAGGGATGATGTTCT
mouse *F4/80*	Forward	CCAGAAGGCTCCCAAGGAT
mouse *F4/80*	Reverse	TCTGCTCACTTTGGAGTATCAAGTC
mouse *TNF-α*	Forward	CCCTTTACTCTGACCCCTTTATTGT
mouse *TNF-α*	Reverse	TGTCCCAGCATCTTGTGTTTCT
mouse *IL-1β*	Forward	CCTCTGATGGGCAACCACTT
mouse *IL-1β*	Reverse	TGCTGCCTAATGTCCCCTTG
mouse *iNOS*	Forward	AGCCAAGCCCTCACCTACTTC
mouse *iNOS*	Reverse	GCCTCCAATCTCTGCCTATCC
mouse *Iba1*	Forward	ATGAGGATCTGCCGTCCAA
mouse *Iba1*	Reverse	AAGTTTCTCCAGCATTCGCTTC
mouse *CD206*	Forward	CAGGTGTGGGCTCAGGTAGT
mouse *CD206*	Reverse	TGTGGTGAGCTGAAAGGTGA
mouse *Arginase-1*	Forward	CTCCAAGCCAAAGTCCTTAGAG
mouse *Arginase-1*	Reverse	GGAGCTGTCATTAGGGACATCA
mouse *Ym-1*	Forward	CTCTCCAGAAGCAATCCTGAAGAC
mouse *Ym-1*	Reverse	GCCCAACTGGTATAGTAGCACATC

6. Immunohistochemistry staining

Mice were deeply anesthetized before intracardiac perfusion with PBS, followed by 4% paraformaldehyde. Fixed SCN and L3-L4 spinal cord were collected from mice, post-fixed in 4% paraformaldehyde, and dehydrated overnight in 25% sucrose at 4℃. Frozen tissue embedded in the OCT compound (Sakura Finetek, Torrance, CA, USA) and SCNs were cut longitudinally into 10 μm-thick sections, the spinal cords were cut into 30 μm -thick sections. The sections were permeabilized with 0.3% Triton X-100 in PBS for 15 min, following with 5% normal donkey serum at 25−27℃ for 30 min and incubated overnight with the following primary antibodies: rat anti-F4/80 (1:800, ab6640, Abcam, Cambridge, UK), rabbit anti-CD206 (1:1000, ab64693, Abcam), rabbit anti-Iba1 (1:1000, Wako, Osaka, Japan). The sections were washed with 0.3% Triton X-100 in PBS on the following day and incubated with fluorescence-conjugated secondary antibodies (anti-rabbit Alexa Fluor 488, Thermo Fisher, Eugene, OR, USA; anti-rat Alexa Fluor 555, ab150166, Abcam) around 25℃ for 30 min. The sections were washed with 0.3% Triton X-100 in PBS and incubated with DAPI (1:500, Sigma) at room temperature for 5 min. Finally, the sections were mounted on a slide using a mounting medium and a covered with a cover slip. CD206/F4/80- positive cells 2 mm distal to the injury site in proximal SCN were counted at 400x magnification. All the positive cells were counted from at least three non-overlapping sections obtained from 6 animals per group. Arrows indicate counted CD206^{+}F4/80^{+} cells. Size is around 20 μm to 30 μm. The images of the tissues were captured using a universal fluorescence microscope (BZX800; Keyence, Osaka, Japan).

7. Statistical analysis

Means and standard deviations were calculated from the data and then analyzed using an unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to examine the mean differences at a level of significance of 5%.

Results

1. PSL-induced hypersensitivity was ameliorated by LIPUS

To observe the effect of LIPUS treatment on PSL-induced NP, mechanical pain hypersensitivity (allodynia) was evaluated using a von Frey test on days 3 and 7 after the PSL procedure (Fig. 1a). In all mice, mechanical allodynia was obviously induced by PSL and lasted for 1 week. Conversely, in the LIPUS group mice, hypersensitivity was significantly ($P < 0.01$) ameliorated compared to the PSL group mice on day
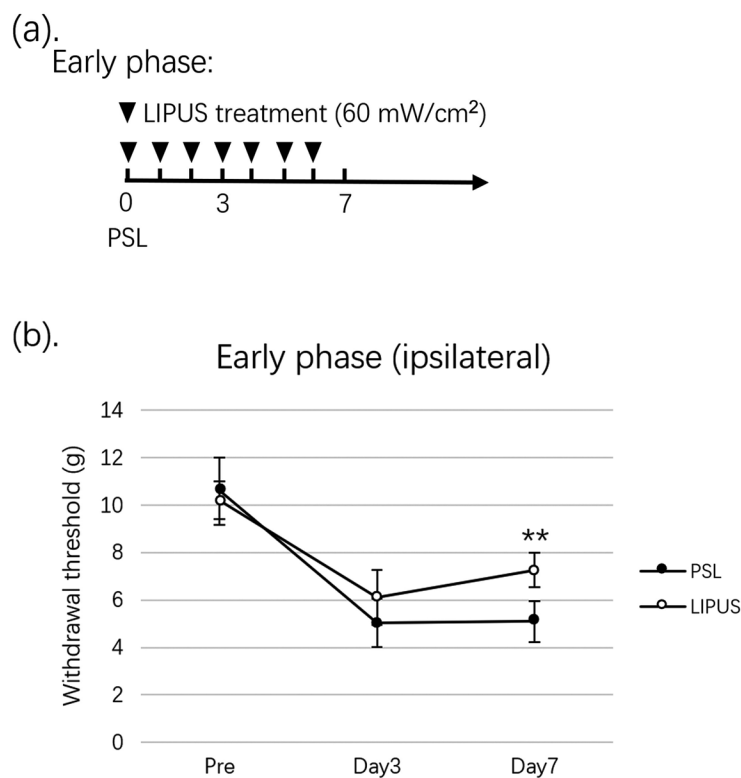
(a).
Early phase:

(b).

Fig. 1 LIPUS treatment ameliorates PSL-induced NP.
(a) Time course of LIPUS treatment in the PSL model. (b) The paw withdrawal thresholds
of the ipsilateral side. The hypersensitivity was determined using a von Frey test. Student's
t-test (n = 5 in each). Data represent the mean ± standard deviation. **$P < 0.01$.

7 (Fig. 1b), we found there is no difference about anti-pain activity of LIPUS at intensity 30 mW/cm^2 and 60 mW/cm^2 (Supplemental figure 1).

2. The number of M2 macrophages was increased in the SCN and peripheral pro-inflammatory cytokines were suppressed by LIPUS

Next, to identify how LIPUS ameliorates PSL-induced NP, we evaluated M2 macrophages in the SCN using qRT-PCR. Seven days after PSL, the gene expression of M2 macrophages, which was assessed by measuring the expression levels of *CD206*, *arginase-1 (Arg-1)*, and *Ym-1*, was significantly increased in the SCN of experimental mice compared to the sham controls (Fig. 2a). Furthermore, the LIPUS treatment downregulated the expression of pan macrophages (F4/80), M1 macrophage (iNOS), pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β (Fig. 2a and b). In the LIPUS group, the expression of TNF-α and IL-1β was significantly reduced compared to that of the PSL group ($P < 0.05$ and $P < 0.01$, respectively). In immunofluorescent staining, the number of CD206$^+$F4/80$^+$ cells significantly increased in LIPUS group compared to PSL group (Fig. 2c and d).

3. Expressions of pro-inflammatory microglial markers in spinal cord were suppressed by LIPUS

Nerve injury can activate glial cells in the spinal cord [1]. To determine the pro-inflammatory microglial markers in the CNS, we evaluated the gene expression in the spinal cord. Iba1, which represents microglia, was upregulated on day 7 after PSL surgery (Fig. 3; Supplemental figure 2). In the LIPUS group, the gene expression of Iba1 significantly ($P < 0.05$) decreased compared to that in the PSL group. Furthermore, the levels of pro-inflammatory cytokines TNF-α and IL-1β in the CNS were significantly ($P < 0.01$ and $P < 0.05$, respectively) reduced in the LIPUS group compared to the PSL group.

Discussion

In PSL-induced NP, several inflammatory mediators are produced by rapidly activated tissue-resident macrophages and Schwann cells, which activate glial cells in the CNS. Once the complex inflammation between the peripheral and central glial cells is established, NP is subjected to long-term treatment and is often difficult to shut down[3].

LIPUS is effective in several diseases[17-19]. Recently, it has been suggested that LIPUS ameliorates the gait patterns
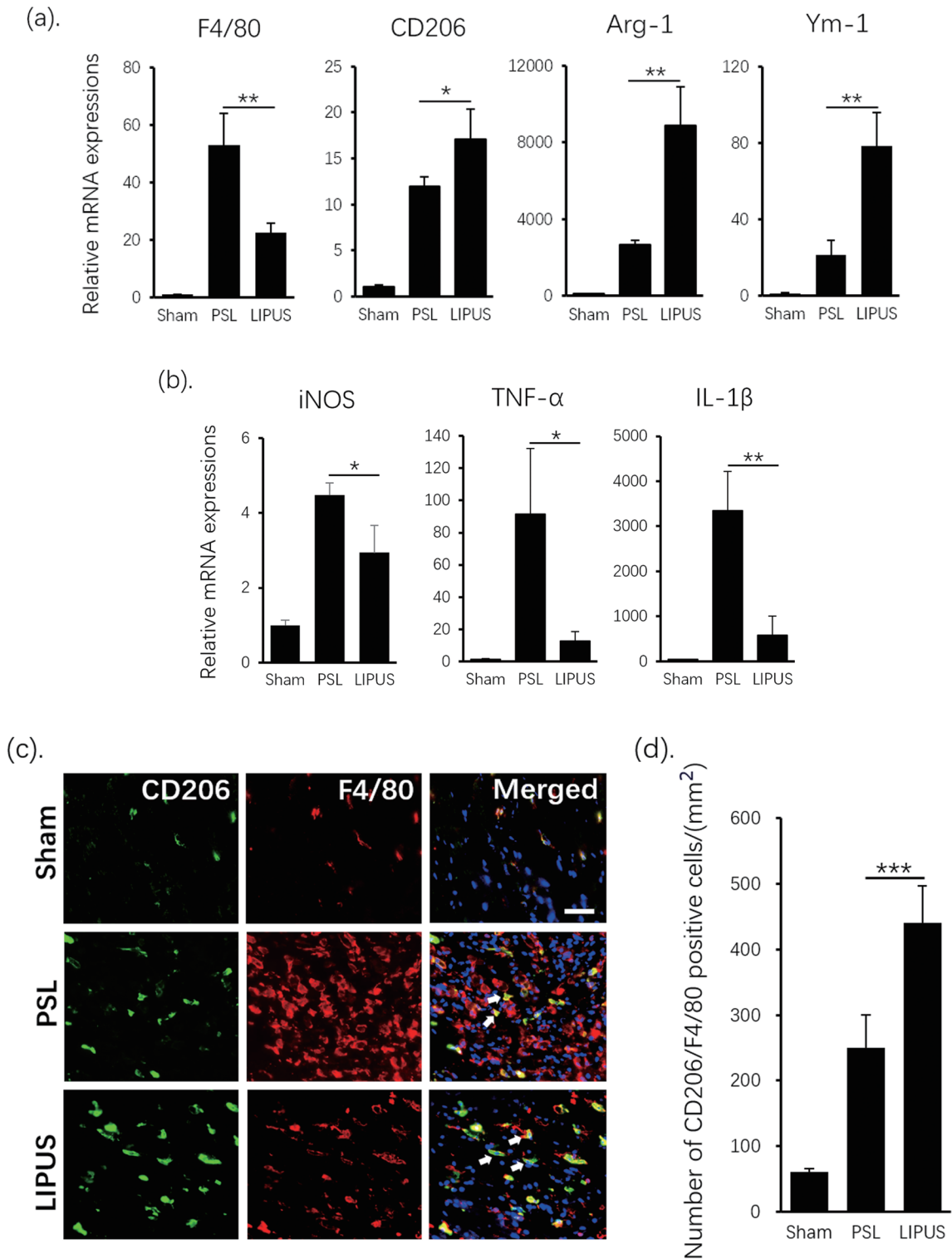
Fig. 2 LIPUS treatment increased the number of M2 macrophages and suppressed the expression of pro-inflammatory cytokines in the ipsilateral sciatic nerve.

(a) Gene expression of M2-polarized macrophages and (b) of pro-inflammatory cytokines on day 7. Results are expressed relative to the levels observed in the sham control. ANOVA with Tukey's multiple comparisons test (n = 3 in each). (c) Images of immunofluorescent staining of CD206, F4/80 in SCN. Arrows indicate counted CD206+F4/80+ cells. Scale bar: 50 μm. (d) Quantification of CD206+F4/80+ cell numbers in SCN. ANOVA with Tukey's multiple comparisons test (n = 6 in each). Data represent the mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
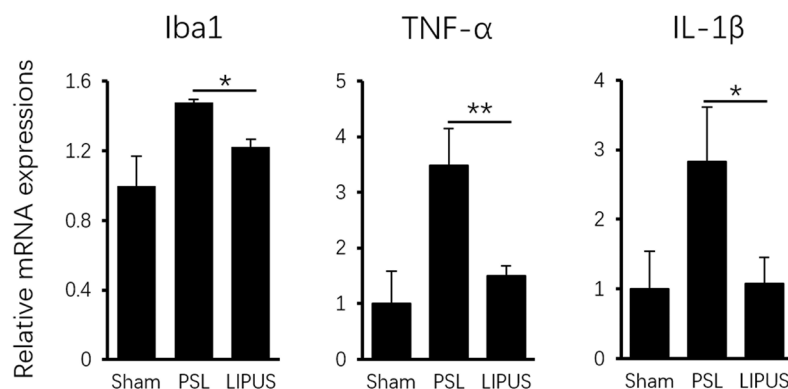
Fig. 3 LIPUS treatment decreased expression of pro-inflammatory microglial markers in the central nervous system.
Gene expression of Iba1, TNF-α, and IL-1β on day 7. Results are expressed relative to the levels of the sham control. ANOVA with Tukey's multiple comparisons test (n = 3 in each). Data represent the mean ± standard deviation. $*P < 0.05$, $**P < 0.01$.

and synovial inflammation, which may be due to its ability to inhibit mature IL-1β[23]. Zhang et al.[16] explored LIPUS in spinal degeneration treatment via spinal fusion promotion and demonstrated that LIPUS accelerated spinal fusion and stimulated the transition of M1 macrophages to M2 macrophages in vivo[16]. They also showed a significant increase in the expression levels of M2-related genes and anti-inflammatory factors, such as Arg-1 and IL-4, after LIPUS treatment[16]. Furthermore, LIPUS stimulation leads to an early reduction in the number of neutrophils and M1 macrophages and an increase in the number of M2 macrophages in injured muscle[24]. However, the anti-inflammatory effects of LIPUS treatment in PSL-induced NP are still unclear. To our knowledge, this is the first study to report the analgesic effects of LIPUS treatment in nerve injury-induced NP.

Here, we hypothesized that the anti-inflammatory effects of LIPUS treatment may suppress the early stage of NP induced by PSL. Therapeutic ultrasound produces vibrational forces when it passes through the cell culture or the tissue, the vibrational forces cause a rise in temperature and hence increased blood flow, decreased muscle pain and so on[25]. In our previous study, we exposed LIPUS to cultured cells, and confirmed that pre-attenuation of the flask material was around 5%, and no or less reduction of vibrational force was observed when the distance between the ultrasound transducer and the cultured cells was approximately 4 mm[26]. In mice, the distance from the skin to target SCN is approximately 3 mm in lateral position. Hence, we hypothesized LIPUS exposure has efficacy on PSL-induced NP in mice model. However, the distance from the skin to target SCN is approximately 6.5 cm in lateral position in human beings. Thus, further study is needed to investigate the efficacy of LIPUS on human NP.

Our data revealed that LIPUS increased M2 macrophage in the SCN and reduced PSL-induced inflammation in the peripheral nerves. Moreover, in the CNS, the decreased expression of Iba1 and pro-inflammatory cytokines, together with the restored hypersensitivity, imply that LIPUS exposure may be a promising treatment strategy for nerve injury-induced NP. Additionally, whether LIPUS has a direct effect on suppressing the activation of Schwann cells, satellite glial cells in peripheral nerves, and glial cells in the CNS has been unclear. Therefore, additional studies are necessary to investigate the signaling pathways of these cells after LIPUS exposure. It would be useful to further understand the detailed mechanisms of the effect of LIPUS on injured nerves under inflammation.

Although LIPUS has an anti-inflammatory effect on NP, the recovery of the mechanical pain threshold by LIPUS treatment was not complete, even 7 days after the PSL procedure. A satisfactory amelioration of NP is crucial for functional recovery and the quality of life of the patient. Recently, we evaluated the therapeutic potential of conditioned medium derived from stem cells of human exfoliated deciduous teeth (SHED-CM) to NP induced by PSL and suggested that the systemic administration of SHED-CM led to a considerable recovery of SCN function by increasing the number of anti-inflammatory M2 macrophages (unpublished data). Increasing evidence stands by the individual application of physiotherapy and anti-inflammatory or anabolic medications; however, their effects when applied concurrently have not been reported. In the future, LIPUS treatment together with anti-inflammatory or anabolic medications and SHED-CM administration may become an effective clinical procedure for the treatment of NP.

Conclusions

LIPUS treatment increased M2 macrophage in the SCN and hence reduced PSL-induced inflammation in both the peripheral nervous and central nervous system, downregulated hypersensitivity suggested LIPUS ameliorated PSL-induced NP effectively. All the evidence shows that LIPUS may be a promising candidate for NP.

Acknowledgements

Conflict of interest statement:

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Early phase:

▼ LIPUS treatment (30 or 60 mW/cm²)
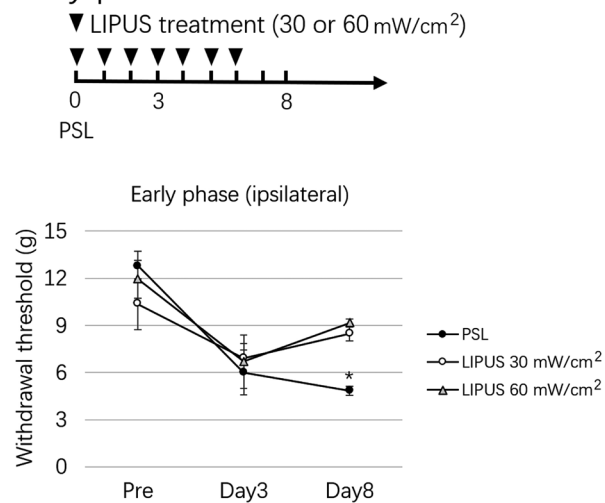
Suppl. Fig 1   Effect of different spatial averaged intensity on PSL model.
ANOVA with Tukey's multiple comparisons test (n = 5). Data represent the mean ± standard deviation. *$P < 0.05$. for the LIPUS 30 mW/cm² or 60 mW/cm² vs. PSL comparisons.
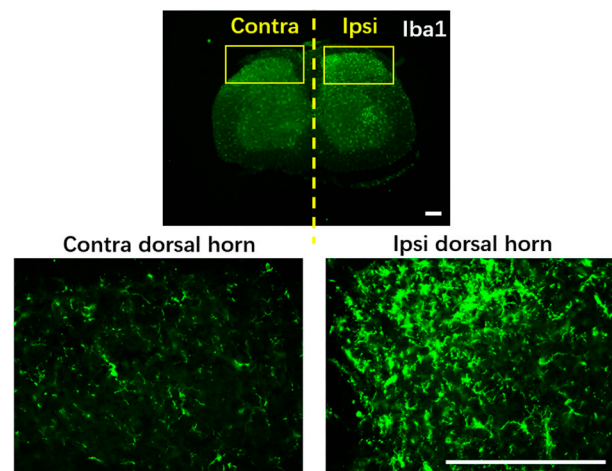
Suppl. Fig 2   Immunofluorescent staining of Iba1 in spinal cord after PSL 7 days (n = 3-4).
Scale bar: 200 μm.