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Low-intensity pulsed ultrasound modifies effects of a myelin-related growth inhibitor and enhances neurite re-growth

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***Abstract*—*Axon regeneration is crucial for the functional recovery after spinal cord injury. Myelin-related growth inhibitors, such as Nogo-A, inhibit neurites regrowth and elongation. Low-intensity pulsed ultrasound (LIPUS) is known to show neuroprotective effects in neurodegenerative diseases. In the present study, we evaluated the feasibility of LIPUS for enhancing axon regeneration in the presence of Nogo-A. Low-intensity pulsed ultrasound was exposed to rat cortical neurons in vitro after Nogo-A treatment. The total length of neurites per cell in LIPUS group was significantly larger than that of non-LIPUS group. There was no significant difference in the number of neurites. The results suggest that LIPUS may enhance the elongation of neurites in the presence of Nogo-A while may not affect on neurites sprouting and branching. This preliminary study implies the feasibility of LIPUS for axonal regeneration.*

Keywords*—*axon regeneration, low-intensity pulsed ultrasound, myelin-related growth inhibitor, Nogo-A

I. Introduction

Axonal regeneration after spinal cord injury remains a challenge facing neuroscience. Injured neurons in central nervous system (CNS) have a limited capacity for neurite outgrowth and axonal regeneration. Extracellular matrix, myelin-related growth inhibitors, and astrocyte scars cause regeneration failure [1]. CNS myelin associated proteins, such as Nogo-A, inhibit neurite growth [2]. Extensive efforts have been conducted to develop strategies that enable injured neurons to re-grow. Low intensity pulsed ultrasound (LIPUS) improved peripheral nerve regeneration in rats [3-5] and enhanced neurite outgrowth in tumor cell lines [6,7]. LIPUS may have a potential for axonal regeneration after spinal cord injury. Our ultimate goal is to develop ultrasound-assisted spinal regeneration after spinal cord injury. In this study, we evaluated effects of single LIPUS exposure on primary neurons treated with Nogo-A.

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II. Experimental Methods

A. In vitro model

Primary rat [Fisher 344] cortex neurons (Thermo Fischer Scientific, Waltham, MA, USA) were seeded to 35 mm culture dish coated with rat collagen type-I gel (Corning Inc., Corning, NY, USA). Neurons were maintained in Neurobasal® Medium (Thermo Fischer Scientific) supplemented with B-27® Supplement (Thermo Fischer Scientific) and GlutaMax™-1 (Thermo Fischer Scientific). After culturing for 3 days, neurons were treated with 100 ng/mL Nogo-A (R&D Systems, Minneapolis, MN, USA) for 5 minutes at 37°C. Neurons were culture overnight before sonication.

B. Ultrasound exposure

Ultrasound was exposed to neurons for 10 min at 37°C in a water bath. Ultrasound was generated with a 50 mm-diameter single element non-focused transducer (Fuji Ceramic, Shizuoka, Japan). A sinusoidal electrical signal was generated by an arbitrary function generator (AFG1022, Tektoronics Inc., Beaverton, OR, USA), which was amplified by a power amplifier (T145-5315A, Thamway, Shizuoka, Japan). Ultrasound parameters were as follows; 1 MHz center frequency, 10% duty cycle (100 μsec pulse width, 1 kHz pulse repetition frequency). In this setup, the spatial-average temporal-average ultrasound intensity was 20 mW/cm^2.

C. Microscopic observation

Neurons in the same field of view were observed with a phase-contrast microscope (Eclipse TS100, Nikon, Tokyo, Japan) in 6 consecutive days after the seeding. Three days after the sonication, neurons were stained with calcein-AM (Dojindo, Kumamoto, Japan). Fluorescent images of neurons were captured by a fluorescent microscope (BIOREVO BZ-9000, KEYENCE, Osaka, Japan). Fifteen fields of view were captured

in each group, and experiments were repeated three times. Using Image J®, neurite re-growth was assessed by calculating the total neurite length per neuron, the number of neurites per neuron, and the number of neurites branching per neuron.

III. Results

Neurites transiently became shorter after the Nogo-A treatment, although neurites started elongation without LIPUS (Fig. 1). Three days after the sonication, neurites elongated both in LIPUS group and in without LIPUS group (Fig. 2). The total neurite length per one neuron was 289.6 ± 108 μm in the LIPUS group, while that was 243.6 ± 28.4 μm in without LIPUS group (Fig. 3). LIPUS did not increase the number of neurite or neurite branch.

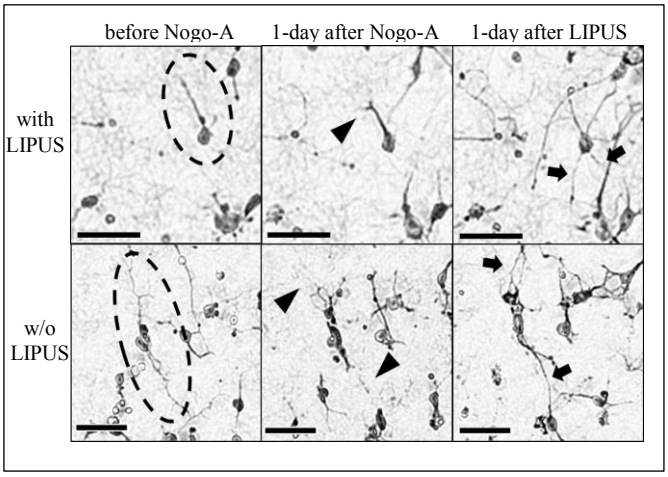

Fig. 1. Blight filed microscopic images of neurons. Neurons in the same field of view (dotted circle) were observed before the Nogo-A treatment, after the Nogo-A treatment, and after LIPUS treatment. Neurites became shorter after the Nogo-A treatment (arrow heads). However, neurites started re-growth in both groups (arrows). Bars, 50 μm.

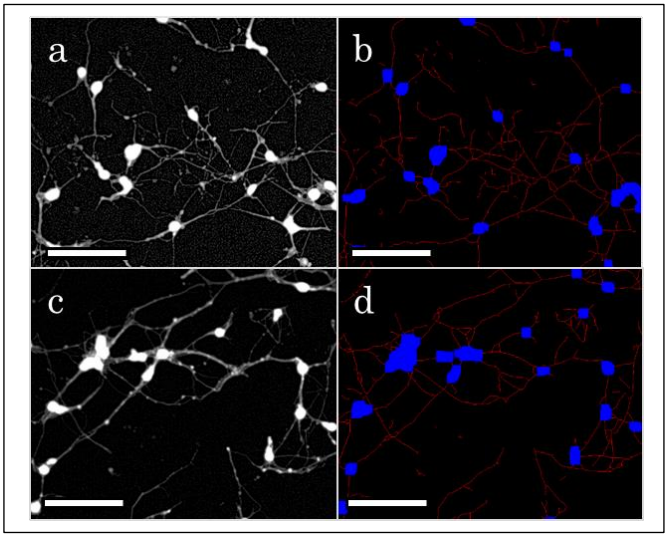

Fig. 2. Fluorescent microscopic images of neurons 3days after the sonication. (a) and (b), LIPUS group. (c) and (d), without LIPUS group. In (b) and (d), neurons (blue) and neurites (red lines) are extracted from the original images (a and c, respectively) using Image J®. Bars, 100 μm.

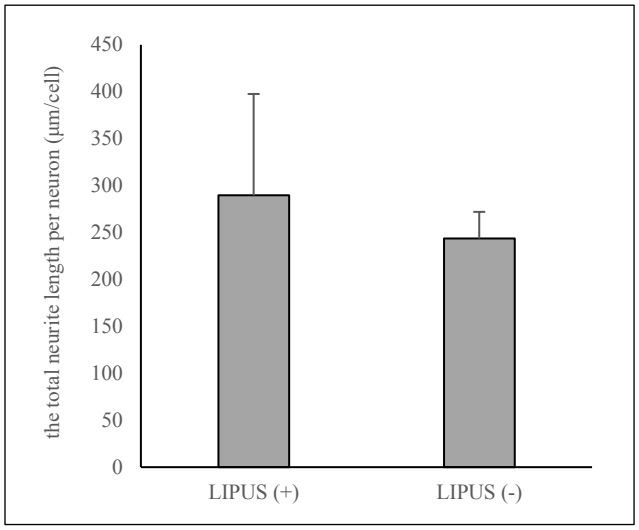

Fig. 3. The total neurite length per neuron. The length was significantly longer in LIPUS group than that of without LIPUS group ($p < 0.05$).

IV. Discussion

This preliminary study shows the feasibility of LIPUS for enhancing neurite re-growth. The total neurite length of each neuron became longer by LIPUS exposure, although neurite spurting or branching was not affected by LIPUS exposure. The results suggest that LIPUS modify the inhibitory effect of Nogo-A on neurites. Nogo-A activates NgR1/RhoA/Rho kinase signaling pathway, which results in changing actin cytoskeleton dynamics in neurites [8]. Previous studies showed that LIPUS activated stretch-activated ion channel and increased the neurite length [6,7]. Further studies will evaluate LIPUS effects on cytoskeleton morphology in neurites.

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