TITLE:
Investigating the Optimal Initiation Time of Ultrasound Therapy for Peripheral Nerve Regeneration after Axonotmesis in Rats

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Title

Investigating the optimal initiation time of ultrasound therapy for peripheral nerve regeneration after axonotmesis in rats

Author name

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Abstract

This study aimed to identify the optimal initiation time of ultrasound (US) therapy for peripheral nerve regeneration after axonotmesis. Thirty-six rats with sciatic nerve crush injury were divided into four groups that received US irradiation initiated 1, 7, or 14 days after injury, or sham stimulation for 4 weeks. Motor function analysis was conducted weekly; however, there was no significant improvement attributed to US treatment. Four weeks after injury, compound muscle action potential amplitude values of the group in which US irradiation was initiated 1 day after the injury showed significant improvement compared to the sham stimulation group. In addition, myelin sheath thickness was significantly greater in the 1-day group than in other groups. These results indicate that US treatment initiated 1 day after peripheral nerve injury promotes maximum regeneration.

Keywords

ultrasound therapy; peripheral nerve injury; functional recovery; nerve regeneration; time factors
Introduction

Injury to peripheral nerves causes motor function disorders that can adversely affect patients’ quality of life (Stonner et al. 2017). Although peripheral nerves have an intrinsic regenerative capacity, delayed reinnervation causes degeneration of neuromuscular junction and end-organ atrophy, thereby inhibiting functional recovery (Palispis and Gupta, 2017). Several nerve stimulation methods, such as electric stimulation (Gordon 2016), magnetic stimulation (Zhivolupov et al. 2012), and ultrasonic stimulation (Daeschler et al. 2018a) have been developed to accelerate nerve regeneration. In particular, pulsed ultrasound (US) has been extensively used for several soft tissue and other musculoskeletal applications (Lai et al. 2021; Zhang et al. 2017). The effectiveness of US in peripheral nerve regeneration in rats was reported in a meta-analysis study (Daeschler et al. 2018a); however, the optimal treatment methods have not been curated. Akhlaghi et al. (2012) examined several US parameters and demonstrated the need to optimize them.

Intensity is a key parameter in US therapy. Daeschler et al. (2018b) reported that the intensity of 30 mW/cm² (spatial average temporal average, SATA), widely used clinically in bone healing, was insufficient for peripheral nerve injuries. We have previously reported that intensity is an essential factor for peripheral nerve regeneration, and that 140 mW/cm² (SATA) is the optimal intensity, compared to 30 mW/cm² or 250 mW/cm² (Ito et al. 2020).

The initiation time of intervention is also an essential factor. Fu et al. (2008) reported that US treatment initiated 1 day, instead of 1 week, after injury promoted tendon healing. Pockett and Gavin (1985) reported that electrical stimulation applied immediately after a crush injury was the most effective. In the early phase after injury, inflammatory responses, including Schwann cell proliferation and Wallerian degeneration, occur successively (Gaudet et al. 2011), and these processes are affected by US treatment (Ito et al. 2020; Raso et al. 2005; Zhang et al. 2017).
This indicates that the initiation time of treatment may impact the therapeutic effects. However, the effects of initiation time of US treatment following peripheral nerve injury remain unclear. Thus, it is crucial to optimize the initiation time of US treatment for clinical applications to achieve the greatest extent of peripheral nerve regeneration.

To optimize the initiation time of US treatment, we used the rat model of sciatic nerve crush injury. This model is widely used in pre-clinical studies as a reproducible model of axonotmesis and is suitable for investigating time-course changes after injury (Geuna 2015). The purpose of this study was to determine the optimal initiation time of US treatment after sciatic nerve crush injury in rats.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (MedKyo19028). Eleven-week-old male Lewis rats weighing 230-280 g were purchased and dual-housed in standardized cages with water and food *ad libitum* on a 12:12-h light-dark cycle. Thirty-six rats received sciatic nerve crush injury and were subsequently randomly divided into four groups based on the initiation time of the US treatment: 1 day after surgery (1D group), 7 days after surgery (7D group), 14 days after surgery (14D group), and sham stimulation throughout the intervention period (sham group) (Fig. 1). Rats were habituated to an experimental environment that included treadmill walking for a week prior to surgery. The sample size was determined in our previous study (Wang et al. 2021), and this experiment was divided into three replicates. No adverse events were observed in the present study.
Surgery

A sciatic nerve crush injury was induced in all rats after one week of raising, according to a protocol we previously reported (Wang et al. 2018). Rats were anesthetized with an intraperitoneal injection of mixed anesthetic comprising 0.15 mg/kg medetomidine, 2 mg/kg midazolam, and butorphanol (2.5 mg/kg). The left sciatic nerve was exposed through a lateral longitudinal incision along the left thigh. After the nerve was detached from the surrounding tissues, a 2-mm-long nerve at the site below the gluteal tuberosity was crushed for 10 s using a needle holder (No. 12501-13, Fine Science Tools Inc., North Vancouver, Canada). The proximal end of the crush site was marked with a 9-0 nylon epineural stitch (T06A09N20-25, Bear Medic Corporation), and the incision was closed with 4-0 nylon sutures (S15G04N-45, Bear Medic Corporation). Following surgery, 0.375 mg/kg atipamezole was administered intraperitoneally to reverse the anesthesia.

US treatment

US irradiation was performed using an ultrasonic treatment apparatus (UST-770, ITO Physiotherapy & Rehabilitation, Tokyo, Japan), as previously reported (Ito et al. 2020). The rats were anesthetized with 2% isoflurane during US irradiation. An US transducer (effective radiation area: 0.9 mm², beam non-uniformity: 2.9) was placed on the skin above the injury site through a coupling gel. The US parameters were as follows: acoustic frequency, 1 MHz; repetition frequency, 1 kHz; intensity, 140 mW/cm² (SATA); duty cycle, 20%; irradiation time, 5 min/day. All rats received US or sham stimulation (0 mW/cm²) daily from the next day following surgery to the 7 days post-surgery, and then 5 days/week until sacrifice 4 weeks later.
Motor functional analysis

Sciatic functional index

Functional recovery was assessed preoperatively and at 1, 2, 3, and 4 weeks after surgery. The sciatic functional index (SFI) was assessed according to a previous report (Wang et al. 2018). The rats’ footprints were obtained while walking through a wooden walking alley (9 × 10 × 60 cm). Three pairs of footprints were selected, and the following parameters were measured: distance from the heel to the third toe (PL: print length), distance from the first toe to the fifth toe (TS: toe spread), and distance from the second toe to the fourth toe (ITS: intermediate toe spread). The SFI value was calculated according to the formula: SFI = -38.3((EPL - NPL) / NPL) + 109.5 ((ETS - NTS) / NTS) + 13.3 ((EITS - NITS) / NITS) - 8.8, where the injured side was denoted as E and the non-injured side was denoted as N (Bain et al. 1989).

Three-dimensional motion analysis

Following the SFI measurement, a three-dimensional motion analysis was conducted according to our previous study (Wang et al. 2018). Rats were anesthetized with 2% isoflurane, and colored hemispheric markers were attached to landmarks on the shaved skin as follows: anterior superior iliac spine, greater trochanter, knee joint, lateral malleolus, and fifth metatarsophalangeal joint. The fourth toe was colored with pink ink. After the rats recovered from anesthesia, treadmill walking at a speed of 12 m/min was captured and analyzed using a three-dimensional motion capture apparatus (Kinema Tracer System, Kissei Comtec, Nagano, Japan). Ten steps consisting of at least five consecutive steps were recorded for each rat, and ankle angles and toe angles in the toe-off phases were measured.
Electrophysiological analysis

Four weeks after surgery, the rats were anesthetized with mixed anesthetics and placed in the prone position. Electrophysiological analysis was conducted using an electromyogram measuring system (Neuropack S1 MEB-9404, NIHON KOH DEN, Tokyo, Japan). Disposable subdermal needle electrodes (NE-115B, NIHON KOH DEN, Tokyo, Japan) were set up as follows: anode stimulating electrodes, the proximal side of the injured site; cathode stimulating electrodes, piriformis muscle; recording electrode, gastrocnemius muscle belly; reference electrode, Achilles tendon; and grounding electrode, subcutaneous on the side of the rat. The distance between the anode and recording electrode was set to 40 mm. An electrical stimulus (frequency: 1 Hz, duration: 0.1 ms) was applied to obtain compound muscle potential amplitude measurements (baseline to the maximal negative peak), and latencies were recorded. The amplitudes and latencies were expressed as the ratio of the injured side to the non-injured side.

Histomorphometric analysis

Following electrophysiological analysis, the rats were sacrificed, and a 5-mm-long specimen of the sciatic nerve was dissected from the proximal end of the injury site. Ultrathin transverse sections, 5 mm distal to the injury site, were prepared as previously described (Wang et al. 2018). The images were examined using transmission electron microscopy (TEM) (Model H-7000, Hitachi High-Technologies, Tokyo, Japan). Ten areas of each section were randomly obtained at a magnification of 2000×. The shortest diameter of the myelinated nerve fibers (α) and axon diameter (β) were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The myelin sheath thickness of each fiber (γ) was obtained using the formula \( \gamma = (\alpha - \beta) / 2 \). Since the results of histomorphometry depend on
distance from the injury site (Raso et al. 2005), specimens were excluded if the ultrathin
sections were prepared from unspecified sites. We analyzed five sciatic nerves from each
group.

**Wet muscle weight measurement**

Immediately after the sciatic nerve dissection, tibialis anterior (TA), extensor digitorum
longus (EDL), gastrocnemius (GA), and soleus (Sol) muscles were harvested bilaterally and
weighed using a digital scale (AE200, Mettler-Toledo, Columbus, OH, USA). The results are
expressed as the ratio of the injured side to the non-injured side.

**Statistical analysis**

Data are shown as mean ± standard error. In the results of motor function analysis,
mixed-design repeated-measures one-way analysis of variance (ANOVA) was performed.
One-way ANOVA was used to assess the significance of differences in the results of
electrophysiological, histomorphometric, and wet muscle weight analyses. *Post-hoc* analysis
was conducted using Tukey’s HSD test. Statistical significance was set at $p < 0.05$. All
statistical analyses were performed using the JMP Pro 15 software (SAS Institute, Cary, NC,
USA).

**Results**

**Motor functional recovery**

SFI values decreased to approximately -90 after injuries and returned to -20 ($p < 0.01$),
but no significant differences were observed between the intervention groups or interaction
between the evaluation time and intervention group (Fig. 2A). Three-dimensional analysis
also revealed functional impairment and recovery after injury ($p < 0.01$). The toe angle analysis showed a significant difference between the intervention groups ($p < 0.05$), but the interaction between the intervention groups and evaluation times was not significant (Fig. 2B). The ankle angle analysis showed similar results to that of the toe angle, excluding the intervention groups ($p = 0.06$) (Fig. 2C). These results indicate that the US treatment did not cause significant functional recovery under these experimental conditions.

**Electrophysiology**

Fig. 3 shows the results of electrophysiology at four weeks after injury. The mean amplitude in the 1D group (0.36 ± 0.04) was significantly improved compared to the sham group (0.24 ± 0.02, $p < 0.05$) (Fig. 3A). There were no significant differences in latency between the groups (Fig. 3B).

**Histomorphometry**

Representative TEM images and their results are shown in Fig. 4. The mean myelin sheath thickness in the 1D group (0.64 ± 0.01 μm) was greater than that in the sham group (0.60 ± 0.01 μm, $p < 0.05$), 7D group (0.60 ± 0.01 μm, $p < 0.05$), and 14D group (0.59 ± 0.01 μm, $p < 0.01$). There were no significant differences in axon diameter or myelinated nerve diameter among the groups.

**Wet muscle weight**

The low ratio of wet muscle weight 4 weeks after injury indicated muscle atrophy in the injured limb, but none of the analyzed muscles showed significant differences among the groups (Fig. 5).
Discussion and Conclusion

US has received attention as a treatment option for peripheral nerve injury (Daeschler et al. 2018a). US parameters are essential for accelerating nerve regeneration (Akhlaghi et al. 2012), but most of the existing studies comparing US parameters have focused only on intensity. Other US therapeutic conditions should also be optimized for peripheral nerve regeneration. In this study, we investigated the impact of US initiation time on the treatment of sciatic nerve crush injury in rats.

Our electrophysiological and histomorphometric results demonstrated that treatment initiated 1 day after the injury, rather than delayed treatment, promoted maximum nerve regeneration. This indicates that US therapy promotes nerve regeneration through the effect of US irradiation on cellular events occurring at the site in the first week of injury. After peripheral nerve injury, Schwann cells proliferate and upregulate cytokines and neurotrophic factors (Jessen et al. 2015). Subsequently, macrophages are recruited by cytokines such as interleukin-1β (IL-1β) and monocyte chemoattractant protein 1 (MCP1) to phagocytose myelin debris during Wallerian degeneration jointly with Schwann cells (Martini et al. 2008). Myelin clearance enables the regrowth of injured axons by removing inhibitor molecules, such as myelin-associated glycoprotein (MAG) (Shen et al. 1998). Zhang et al. (2009) reported that US irradiation promoted Schwann cell proliferation and modulated the expression of neurotrophic factors in cultured Schwann cells, although Schwann cell proliferation is not necessary for nerve regeneration (Yang et al. 2008). Similar to Schwann cells, infiltrated macrophages contribute to myelin clearance, which is then polarized to an anti-inflammatory phenotype and promotes nerve regeneration by secreting growth factors and cytokines (Chen et al. 2015). Macrophage polarization occurs within 1 week after injury.
Nadeau et al. 2011, and US can modulate macrophage phenotype polarization (da Silva Junior et al. 2017; Zhang et al. 2019). Mokarram et al. (2012) reported that the ratio of pro-inflammatory to anti-inflammatory phenotypes correlated with axonal regeneration. US also regulates the expression of inflammatory cytokines and neurotrophic factors in vivo (Ito et al. 2020; Wang et al. 2021). A mechanical force can be transduced into intracellular signaling by integrins, a kind of cell adhesion molecules expressed on the cell membranes (Lawson and Burridge, 2014). Previous studies have reported that mechanical stimuli caused by US irradiation can modulate the activation of Schwann cells and macrophages via integrin-mediated signaling (Ren et al. 2018; Zhou et al. 2008). As mentioned above, US may affect the activation of Schwann cells and macrophages in the early phase of injury, especially within the first week, and provide an environment for axonal regeneration.

Inconsistent with a previous study (Wang et al. 2021), US did not improve motor function recovery. We observed muscle atrophy in the injured leg, but there was no significant difference in motor function between the intervention groups. The toe angle of the 1D group was higher than that of the 7D group at 3 weeks after injury. There was a significant difference in the intervention groups, but not in the interaction between evaluation time and intervention group, indicating that a difference in pre-surgical factors may have affected the results despite the randomized distribution. Moreover, additional conditions for US irradiation should be considered. US has high directivity, especially in the near field, calculated by the formula: 

$$Z = \frac{a^2 f}{c}$$

where $Z$ is the near-field length, $a$ is the radius of the transducer, $f$ is the frequency, and $c$ is the velocity of sound in the tissue (Harrison et al. 2016). We used a transducer with an area of 0.9 cm$^2$, and the frequency was set to 1 MHz. US propagates through tissues at approximately 1540 ms$^{-1}$ (Evans 2006). The calculated $Z$ value is approximately 19 mm, which indicates that the US reaches the nerves in the near field, and
the irradiated area is restricted to below the transducer aperture. Therefore, it might be
important to select an irradiation site based on the nerve’s anatomy or regeneration phase, as
Bergmeister et al. (2018) suggested. However, the optimal irradiation site is as yet unknown.
Optimization of the irradiation site is crucial to establish US treatment for peripheral nerve
regeneration in clinical applications.

Our study has several limitations. First, it is not known whether long-term intervention
or treatment in the early phase after injury is essential. US treatment initiated early after the
injury is essential for tendon healing (Fu et al. 2008), while long-term treatment impacts bone
healing (Azuma et al. 2001). Persistent expression of neurotrophic factors might interfere with
nerve regeneration (Hoyng et al. 2014), and prolonged electrical stimulation does not promote
nerve regeneration (Asensio-Pinilla et al. 2009). These reports indicate that optimal intervention
conditions might depend on the properties of stimuli or tissues; therefore, the optimal timing of the US
treatment for peripheral nerve regeneration benefits the investigation. Second, the crush injury
model resulted in the exclusion of misdirected regrowth of regenerating axons. Yeh et al. 2010
reported that electrical stimulation exacerbated nerve regeneration when the treatment was continued
for 2 weeks after the nerve injury, indicating that interventions may cause misdirection of regrowing
axons depending on the treatment timing. Mechanosensitive ion channels on the growth cone, such as
transient receptor potential (TRP) channels and Piezo channels, inhibit axon regeneration (Kerstein et
al. 2013; Song et al. 2019), indicating that the US itself may have adverse effects. Optimization of the
US treatment is necessary its safe application in clinical practice; therefore, optimal timing of the US
treatment should also be investigated. Third, although three-dimensional motion analysis is more
sensitive than the sciatic functional index (Wang et al. 2018), skin movement causes errors
between bone-derived and skin-derived angles (Bauman and Chang, 2010). Skin movement
errors may have made it difficult to detect differences in motor function in the present study.
Additionally, the amount of activity the rats received in the cages was not regulated. Asensio-
Pinilla et al. (2009) reported that electrical stimulation had a more beneficial effect when combined with exercise, so exercise in the cages may have impacted motor function recovery and muscle enlargement. Further studies are needed to demonstrate the effects of US initiation time on functional recovery. Finally, we did not evaluate the effect of US on Schwann cells or macrophages, which may be impacted by US irradiation. US is known to affect these cells in vitro (Zhang et al. 2009; Zhang et al. 2019), but the effects remain unclear in peripheral nerves. Additional studies should be conducted to address these issues.

In conclusion, we investigated the initiation time of US treatment and found that US treatment initiated 1 day after the injury promoted maximum peripheral nerve regeneration.

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Conflict of Interest

The authors have no conflict of interest to declare.
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Pockett S, Gavin RM. Acceleration of peripheral nerve regeneration after crush injury in rat.


**Figure Caption List**

1. **Fig. 1** Study design and experimental settings. (A) Thirty-six rats were randomly divided into four groups based on the initiation time of the US treatment. US treatments were initiated 1 day (1D group), 7 days (7D group), and 14 days (14D group) after injury. Sham stimulations were applied during the sham period. Sham group was applied sham stimulation throughout intervention period. Motor functional analysis was conducted on pre-operation and each week after injury. Four weeks after injury, electrophysiology, histomorphometry, and wet muscle weight were analyzed. (B) Sciatic nerve crush injury (arrow) was induced at the site below the gluteal tuberosity (asterisk). (C) An US transducer was placed on the skin above the injury site through a coupling gel for US and sham stimulations. PID = post-injury day; US = ultrasound. MF = motor functional analysis.

2. **Fig. 2** Motor function analysis. (A) SFI, (B) Toe angles, and (C) Ankle angles were analyzed in pre-operation and each week after injury (n = 9 for each group). None of analyses showed significant improvement attributed to US intervention. SFI = sciatic functional index.

3. **Fig. 3** Electrophysiological analysis. (A) Amplitude and (B) latency were expressed in the ratio of injured to intact leg (n = 9 for each group). *p < 0.05.

4. **Fig. 4** Histomorphometric analysis. (A) Representative images of (a) sham group, (b) 1D group, (c) 7D group, and (d) 14D group obtained by a transmission electron microscopy. (B) Mean myelinated nerve diameter, (C) axon diameter, and (D) myelin sheath thickness (n = 5 for each group). Scale bars = 2 μm. *p < 0.05.

5. **Fig. 5** Wet muscle weight measurement. The ratio of injured leg to intact leg of (A) TA, (B) EDL, (C) GA, and (D) Sol (n = 9 for each group). TA = tibialis anterior; EDL = extensor digitorum longus; GA = gastrocnemius; Sol = soleus.
A

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PID
0 1 7 14 21 28
MF MF MF MF MF

Electrophysiology
Histomorphometry
Wet muscle weight

B

C

Transducer

*
Fig. 2

A Self-archived copy in Kyoto University Research Information Repository
https://repository.kulib.kyoto-u.ac.jp

A

B

C
Fig. 3

A. Self-archived copy in Kyoto University Research Information Repository
https://repository.kulib.kyoto-u.ac.jp
Fig. 4

A

B

C

D

Myelinated nerve diameter (μm)

Myelin sheath thickness (μm)

Axon diameter (μm)

Sham 1D 7D 14D

Sham 1D 7D 14D

Sham 1D 7D 14D

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