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

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Title

1 Abstract

2 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 3 were divided into four groups that received US irradiation initiated 1, 7, or 14 days after 4 5 injury, or sham stimulation for 4 weeks. Motor function analysis was conducted weekly; 6 however, there was no significant improvement attributed to US treatment. Four weeks after 7 injury, compound muscle action potential amplitude values of the group in which US 8 irradiation was initiated 1 day after the injury showed significant improvement compared to 9 the sham stimulation group. In addition, myelin sheath thickness was significantly greater in 10 the 1-day group than in other groups. These results indicate that US treatment initiated 1 day 11 after peripheral nerve injury promotes maximum regeneration.

12

13 Keywords

14 ultrasound therapy; peripheral nerve injury; functional recovery; nerve regeneration; time15 factors

17 Introduction

Injury to peripheral nerves causes motor function disorders that can adversely affect 18 19 patients' quality of life (Stonner et al. 2017). Although peripheral nerves have an intrinsic 20 regenerative capacity, delayed reinnervation causes degeneration of neuromuscular junction 21 and end-organ atrophy, thereby inhibiting functional recovery (Palispis and Gupta, 2017). 22 Several nerve stimulation methods, such as electric stimulation (Gordon 2016), magnetic 23 stimulation (Zhivolupov et al. 2012), and ultrasonic stimulation (Daeschler et al. 2018a) have 24 been developed to accelerate nerve regeneration. In particular, pulsed ultrasound (US) has 25 been extensively used for several soft tissue and other musculoskeletal applications (Lai et al. 26 2021; Zhang et al. 2017). The effectiveness of US in peripheral nerve regeneration in rats was 27 reported in a meta-analysis study (Daeschler et al. 2018a); however, the optimal treatment 28 methods have not been curated. Akhlaghi et al. (2012) examined several US parameters and 29 demonstrated the need to optimize them.

Intensity is a key parameter in US therapy. Daeschler et al. (2018b) reported that the 30 intensity of 30 mW/cm² (spatial average temporal average, SATA), widely used clinically in 31 32 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 33 (SATA) is the optimal intensity, compared to 30 mW/cm² or 250 mW/cm² (Ito et al. 2020). 34 35 The initiation time of intervention is also an essential factor. Fu et al. (2008) reported that US 36 treatment initiated 1 day, instead of 1 week, after injury promoted tendon healing. Pockett and 37 Gavin (1985) reported that electrical stimulation applied immediately after a crush injury was 38 the most effective. In the early phase after injury, inflammatory responses, including Schwann 39 cell proliferation and Wallerian degeneration, occur successively (Gaudet et al. 2011), and 40 these processes are affected by US treatment (Ito et al. 2020; Raso et al. 2005; Zhang et al.

2009). 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.

50

51 Materials and Methods

52 Animals

53 All procedures were approved by the Institutional Animal Care and Use Committee of 54 Kyoto University (MedKyo19028). Eleven-week-old male Lewis rats weighing 230-280 g 55 were purchased and dual-housed in standardized cages with water and food ad libitum on a 56 12:12-h light-dark cycle. Thirty-six rats received sciatic nerve crush injury and were 57 subsequently randomly divided into four groups based on the initiation time of the US 58 treatment: 1 day after surgery (1D group), 7 days after surgery (7D group), 14 days after 59 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 60 61 for a week prior to surgery. The sample size was determined in our previous study (Wang et 62 al. 2021), and this experiment was divided into three replicates. No adverse events were 63 observed in the present study.

65 Surgery

66 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 67 intraperitoneal injection of mixed anesthetic comprising 0.15 mg/kg medetomidine, 2 mg/kg 68 69 midazolam, and butorphanol (2.5 mg/kg). The left sciatic nerve was exposed through a lateral 70 longitudinal incision along the left thigh. After the nerve was detached from the surrounding 71 tissues, a 2-mm-long nerve at the site below the gluteal tuberosity was crushed for 10 s using 72 a needle holder (No. 12501-13, Fine Science Tools Inc., North Vancouver, Canada). The 73 proximal end of the crush site was marked with a 9-0 nylon epineural stitch (T06A09N20-25, 74 Bear Medic Corporation), and the incision was closed with 4-0 nylon sutures (S15G04N-45, 75 Bear Medic Corporation). Following surgery, 0.375 mg/kg atipamezole was administered 76 intraperitoneally to reverse the anesthesia.

77

78 US treatment

79 US irradiation was performed using an ultrasonic treatment apparatus (UST-770, ITO 80 Physiotherapy & Rehabilitation, Tokyo, Japan), as previously reported (Ito et al. 2020). The 81 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 82 83 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 84 85 time, 5 min/day. All rats received US or sham stimulation (0 mW/cm²) daily from the next 86 day following surgery to the 7 days post-surgery, and then 5 days/week until sacrifice 4 weeks 87 later.

89 Motor functional analysis

90 Sciatic functional index

91 Functional recovery was assessed preoperatively and at 1, 2, 3, and 4 weeks after 92 surgery. The sciatic functional index (SFI) was assessed according to a previous report (Wang 93 et al. 2018). The rats' footprints were obtained while walking through a wooden walking alley 94 $(9 \times 10 \times 60 \text{ cm})$. 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 95 96 to the fifth toe (TS: toe spread), and distance from the second toe to the fourth toe (ITS: 97 intermediate toe spread). The SFI value was calculated according to the formula: SFI = -98 38.3((EPL - NPL) / NPL) + 109.5 ((ETS - NTS) / NTS) + 13.3 ((EITS - NITS) / NITS) - 8.8, 99 where the injured side was denoted as E and the non-injured side was denoted as N (Bain et 100 al. 1989).

101

102 Three-dimensional motion analysis

103 Following the SFI measurement, a three-dimensional motion analysis was conducted 104 according to our previous study (Wang et al. 2018). Rats were anesthetized with 2% 105 isoflurane, and colored hemispheric markers were attached to landmarks on the shaved skin as 106 follows: anterior superior iliac spine, greater trochanter, knee joint, lateral malleolus, and fifth 107 metatarsophalangeal joint. The fourth toe was colored with pink ink. After the rats recovered 108 from anesthesia, treadmill walking at a speed of 12 m/min was captured and analyzed using a 109 three-dimensional motion capture apparatus (Kinema Tracer System, Kissei Comtec, Nagano, 110 Japan). Ten steps consisting of at least five consecutive steps were recorded for each rat, and 111 ankle angles and toe angles in the toe-off phases were measured.

113 Electrophysiological analysis

114 Four weeks after surgery, the rats were anesthetized with mixed anesthetics and placed 115 in the prone position. Electrophysiological analysis was conducted using an electromyogram 116 measuring system (Neuropack S1 MEB-9404, NIHON KOHDEN, Tokyo, Japan). Disposable 117 subdermal needle electrodes (NE-115B, NIHON KOHDEN, Tokyo, Japan) were set up as 118 follows: anode stimulating electrodes, the proximal side of the injured site; cathode 119 stimulating electrodes, piriformis muscle; recording electrode, gastrocnemius muscle belly; 120 reference electrode, Achilles tendon; and grounding electrode, subcutaneous on the side of the 121 rat. The distance between the anode and recording electrode was set to 40 mm. An electrical 122 stimulus (frequency: 1 Hz, duration: 0.1 ms) was applied to obtain compound muscle 123 potential amplitude measurements (baseline to the maximal negative peak), and latencies 124 were recorded. The amplitudes and latencies were expressed as the ratio of the injured side to 125 the non-injured side.

126

127 Histomorphometric analysis

128 Following electrophysiological analysis, the rats were sacrificed, and a 5-mm-long 129 specimen of the sciatic nerve was dissected from the proximal end of the injury site. Ultrathin 130 transverse sections, 5 mm distal to the injury site, were prepared as previously described 131 (Wang et al. 2018). The images were examined using transmission electron microscopy 132 (TEM) (Model H-7000, Hitachi High-Technologies, Tokyo, Japan). Ten areas of each section 133 were randomly obtained at a magnification of 2000×. The shortest diameter of the myelinated 134 nerve fibers (α) and axon diameter (β) were measured using ImageJ software (National 135 Institutes of Health, Bethesda, MD, USA). The myelin sheath thickness of each fiber (γ) was 136 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.

140

141 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.

146

147 Statistical analysis

148 Data are shown as mean \pm standard error. In the results of motor function analysis,

149 mixed-design repeated-measures one-way analysis of variance (ANOVA) was performed.

150 One-way ANOVA was used to assess the significance of differences in the results of

151 electrophysiological, histomorphometric, and wet muscle weight analyses. *Post-hoc* analysis

152 was conducted using Tukey's HSD test. Statistical significance was set at p < 0.05. All

153 statistical analyses were performed using the JMP Pro 15 software (SAS Institute, Cary, NC,

154 USA).

155

156 **Results**

157 Motor functional recovery

158 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

160 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.

167

168 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 $(0.24 \pm 0.02, p < 0.05)$ (Fig. 3A). There were no significant differences in latency between the groups (Fig. 3B).

173

174 Histomorphometry

175 Representative TEM images and their results are shown in Fig. 4. The mean myelin 176 sheath thickness in the 1D group $(0.64 \pm 0.01 \ \mu\text{m})$ was greater than that in the sham group 177 $(0.60 \pm 0.01 \ \mu\text{m}, p < 0.05)$, 7D group $(0.60 \pm 0.01 \ \mu\text{m}, p < 0.05)$, and 14D group $(0.59 \pm 0.01 \ \mu\text{m}, p < 0.01)$. There were no significant differences in axon diameter or myelinated nerve 179 diameter among the groups.

180

181 Wet muscle weight

182 The low ratio of wet muscle weight 4 weeks after injury indicated muscle atrophy in the 183 injured limb, but none of the analyzed muscles showed significant differences among the 184 groups (Fig. 5).

185

186 **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.

193 Our electrophysiological and histomorphometric results demonstrated that treatment 194 initiated 1 day after the injury, rather than delayed treatment, promoted maximum nerve 195 regeneration. This indicates that US therapy promotes nerve regeneration through the effect of 196 US irradiation on cellular events occurring at the site in the first week of injury. After 197 peripheral nerve injury, Schwann cells proliferate and upregulate cytokines and neurotrophic 198 factors (Jessen et al. 2015). Subsequently, macrophages are recruited by cytokines such as 199 interleukin-1 β (IL-1 β) and monocyte chemoattractant protein 1 (MCP1) to phagocytose 200 myelin debris during Wallerian degeneration jointly with Schwann cells (Martini et al. 2008). 201 Myelin clearance enables the regrowth of injured axons by removing inhibitor molecules, 202 such as myelin-associated glycoprotein (MAG) (Shen et al. 1998). Zhang et al. (2009) 203 reported that US irradiation promoted Schwann cell proliferation and modulated the 204 expression of neurotrophic factors in cultured Schwann cells, although Schwann cell 205 proliferation is not necessary for nerve regeneration (Yang et al. 2008). Similar to Schwann 206 cells, infiltrated macrophages contribute to myelin clearance, which is then polarized to an 207 anti-inflammatory phenotype and promotes nerve regeneration by secreting growth factors 208 and cytokines (Chen et al. 2015). Macrophage polarization occurs within 1 week after injury

209 (Nadeau et al. 2011), and US can modulate macrophage phenotype polarization (da Silva 210 Junior et al. 2017; Zhang et al. 2019). Mokarram et al. (2012) reported that the ratio of pro-211 inflammatory to anti-inflammatory phenotypes correlated with axonal regeneration. US also 212 regulates the expression of inflammatory cytokines and neurotrophic factors in vivo (Ito et al. 213 2020; Wang et al. 2021). A mechanical force can be transduced into intracellular signaling by 214 integrins, a kind of cell adhesion molecules expressed on the cell membranes (Lawson and 215 Burridge, 2014). Previous studies have reported that mechanical stimuli caused by US 216 irradiation can modulate the activation of Schwann cells and macrophages via integrin-217 mediated signaling (Ren et al. 2018; Zhou et al. 2008). As mentioned above, US may affect 218 the activation of Schwann cells and macrophages in the early phase of injury, especially 219 within the first week, and provide an environment for axonal regeneration.

220 Inconsistent with a previous study (Wang et al. 2021), US did not improve motor 221 function recovery. We observed muscle atrophy in the injured leg, but there was no significant 222 difference in motor function between the intervention groups. The toe angle of the 1D group 223 was higher than that of the 7D group at 3 weeks after injury. There was a significant 224 difference in the intervention groups, but not in the interaction between evaluation time and 225 intervention group, indicating that a difference in pre-surgical factors may have affected the 226 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 227 formula: $Z = a^2 f / c$, where Z is the near-field length, a is the radius of the transducer, f is the 228 229 frequency, and c is the velocity of sound in the tissue (Harrison et al. 2016). We used a 230 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 231 232 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.

238 Our study has several limitations. First, it is not known whether long-term intervention 239 or treatment in the early phase after injury is essential. US treatment initiated early after the 240 injury is essential for tendon healing (Fu et al. 2008), while long-term treatment impacts bone 241 healing (Azuma et al. 2001). Persistent expression of neurotrophic factors might interfere with 242 nerve regeneration (Hoyng et al. 2014), and prolonged electrical stimulation does not promote 243 nerve regeneration (Asensio-Pinilla et al. 2009). These reports indicate that optimal intervention 244 conditions might depend on the properties of stimuli or tissues; therefore, the optimal timing of the US 245 treatment for peripheral nerve regeneration benefits the investigation. Second, the crush injury 246 model resulted in the exclusion of misdirected regrowth of regenerating axons. Yeh et al. 2010 247 reported that electrical stimulation exacerbated nerve regeneration when the treatment was continued 248 for 2 weeks after the nerve injury, indicating that interventions may cause misdirection of regrowing 249 axons depending on the treatment timing. Mechanosensitive ion channels on the growth cone, such as 250 transient receptor potential (TRP) channels and Piezo channels, inhibit axon regeneration (Kerstein et 251 al. 2013; Song et al. 2019), indicating that the US itself may have adverse effects. Optimization of the 252 US treatment is necessary its safe application in clinical practice; therefore, optimal timing of the US 253 treatment should also be investigated. Third, although three-dimensional motion analysis is more 254 sensitive than the sciatic functional index (Wang et al. 2018), skin movement causes errors 255 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. 256 257 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.

267

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274

275 **Conflict of Interest**

276 The authors have no conflict of interest to declare.

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395 Figure Caption List

396

397 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.
- 399 Sham stimulations were applied during the sham period. Sham group was applied sham

Fig. 1 Study design and experimental settings. (A) Thirty-six rats were randomly divided into

- 400 stimulation throughout intervention period. Motor functional analysis was conducted on
- 401 pre-operation and each week after injury. Four weeks after injury, electrophysiology,
- 402 histomorphometry, and wet muscle weight were analyzed. (B) Sciatic nerve crush injury
- 403 (arrow) was induced at the site below the gluteal tuberosity (asterisk). (C) An US transducer
- 404 was placed on the skin above the injury site through a coupling gel for US and sham
- 405 stimulations. PID = post-injury day; US = ultrasound. MF = motor functional analysis.
- 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.
- 410 Fig. 3 Electrophysiological analysis. (A) Amplitude and (B) latency were expressed in the 411 ratio of injured to intact leg (n = 9 for each group). * p < 0.05.
- 412 Fig. 4 Histomorphometric analysis. (A) Representative images of (a) sham group, (b) 1D
- 413 group, (c) 7D group, and (d) 14D group obtained by a transmission electron microscopy.
- 414 (B) Mean myelinated nerve diameter, (C) axon diameter, and (D) myelin sheath
- 415 thickness (n = 5 for each group). Scale bars = 2 μ m. * p < 0.05.
- 416 Fig. 5 Wet muscle weight measurement. The ratio of injured leg to intact leg of (A) TA, (B)
- 417 EDL, (C) GA, and (D) Sol (n = 9 for each group). TA = tibialis anterior; EDL = extensor
- 418 digitorum longus; GA = gastrocnemius; Sol = soleus.
 - 18



MF Electrophysiology Histomorphometry Wet muscle weight

В





Fig. 2









14D



A _{0.6}

0

Sham







