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# EFFECTIVE INTERVENTION FREQUENCY OF NEUROMUSCULAR ELECTRICAL STIMULATION FOR PREVENTING JOINT CONTRACTURE CAUSED BY JOINT FIXATION: A NON-WEIGHT-BEARING RAT MODEL

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## ABSTRACT

*Background:* Non-weight-bearing joint fixation causes severe joint contracture, and neuromuscular electrical stimulation (NMES) is assumed to have a preventive effect. However, the effective intervention frequency of NMES for preventing joint contractures has not yet been clarified.

*Objective:* To clarify the effective intervention frequency of NMES for preventing joint contracture caused by joint fixation in non-weight-bearing rats.

*Methods:* Ten-week-old male Wistar rats underwent right ankle joint fixation in full plantar flexion with non-weight-bearing hindlimbs for 1 week. The rats were divided into three groups: non-weight-bearing joint fixation without NMES (NS group), non-weight-bearing joint fixation with one NMES per day (electrical stimulation one time [1T ES group]), and non-weight-bearing joint fixation with two NMESs per day (electrical stimulation two times [2T ES group]). We evaluated ankle dorsiflexion angle, extensibility, amounts of types I and III collagen, cross-sectional area, and wet weight of the soleus muscle-to-body weight ratio.

*Results:* The ankle dorsiflexion angle and extensibility of the soleus muscle on the last day of the experiment were higher in the 1T ES and 2T ES groups than in the NS group. The ankle dorsiflexion angle on the last day of the experiment was higher in the 2T ES group than in the 1T ES group.

*Conclusion:* NMES twice daily had a greater preventive effect on joint contracture caused by joint fixation with non-weight-bearing than NMES once daily.

## KEYWORDS

Intervention, joint fixation, neuromuscular electrical stimulation, non-weight-bearing, muscle

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## INTRODUCTION

Joint contracture occurs when normally stretchable tissues, such as the skin, muscles, tendons, and ligaments are replaced by non-stretchable tissues due to the absence of joint movement[1]. The prevention and treatment of joint contractures are important, because those in the lower limb joints may adversely affect activities of daily living, e.g., walking[2,3].

In several previous animal studies[4-11], joint contracture was simulated by ankle/knee joint fixation for ≥1 week, and is caused by joint fixation for 1 week[4-10]. When joint fixation period is extended, joint contracture progresses[6-10], and becomes more difficult to treatment[11]. Therefore, it is presumed that joint contracture caused by joint fixation for 1 week is the easiest to prevent, since the symptoms are the mildest, it can be considered a target for efficient prevention.

During joint contracture, the tissues around the joints (skin, muscles, tendons, capsules, and ligaments)[1,12] change, and the muscles have increased collagen levels[8-10,12] and decreased extensibility[4]. Muscles are the main cause of joint contracture of the ankle and knee joints after joint fixation for 1 week[5,6]. Particularly, for ankle joint fixation for 1 week, the percentage of muscle contribution to joint contracture (myogenic contracture) was approximately 84.3%[5]. Furthermore, joint contracture caused by joint fixation with non-weight-bearing hindlimbs, such as in patients who have joint fixation for the treatment of lower limb fracture and use crutches and bedridden patients in clinical settings, may be more severe than that caused by joint fixation alone due to the serious decrease in muscle extensibility[4]. Therefore, to investigate clinical interventions for joint contracture, we considered that the most efficient intervention target for those caused by joint fixation with non-weight-bearing for 1 week was the muscles.

Neuromuscular electrical stimulation (NMES) can artificially induce muscle contraction, and suppress collagen production and range of motion limitation[8-10]. When performing NMES, there are many settings, e.g., the intervention frequency, stimulation intensity, and stimulation duration. The intervention frequency for other methods, such as stretching for joint contracture or extended range of motion, has been investigated[13,14]. Stretching of high intervention frequency and duration is more effective for the prevention of joint contracture than stretching of low intervention frequency and duration[14]. However, no previous study has investigated the intervention frequency of NMES for the prevention of joint contracture caused by non-weight-bearing joint fixation. If the intervention loading on the muscle is too high, the recovery of joint contracture is delayed[15]. Interventions for joint contracture caused by non-weight-bearing joint fixation should be evaluated carefully since this symptom is more severe than that caused by joint fixation alone.

It is important to investigate the more effective intervention frequency of NMES for joint contracture prevention of clinical patients with non-weight-bearing joint fixation. Therefore, this study aimed to clarify the effective intervention frequency of NMES for preventing joint contracture caused by joint fixation in non-weight-bearing rats.

## MATERIALS AND METHODS

### Animals

Forty-two 10-week-old male Wistar rats obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) were divided into three groups: 1) non-weight-bearing joint fixation without NMES (NS group; n = 14), 2) non-weight-bearing joint fixation with one NMES per day (1T ES group; n = 14), and 3) non-weight-bearing joint fixation two NMESs per day (2T ES group; n = 14) (*Fig. 1*). All rats were housed in individual cages in a room with a set room temperature of 23°C. The room lights were turned on and off for 12 hours each daily. The rats were fed ad libitum with water and commercially available solid feed. All rats were acclimatized to the experimental environment for 1 week before the experiment, which occurred over 1 week. The breeding of

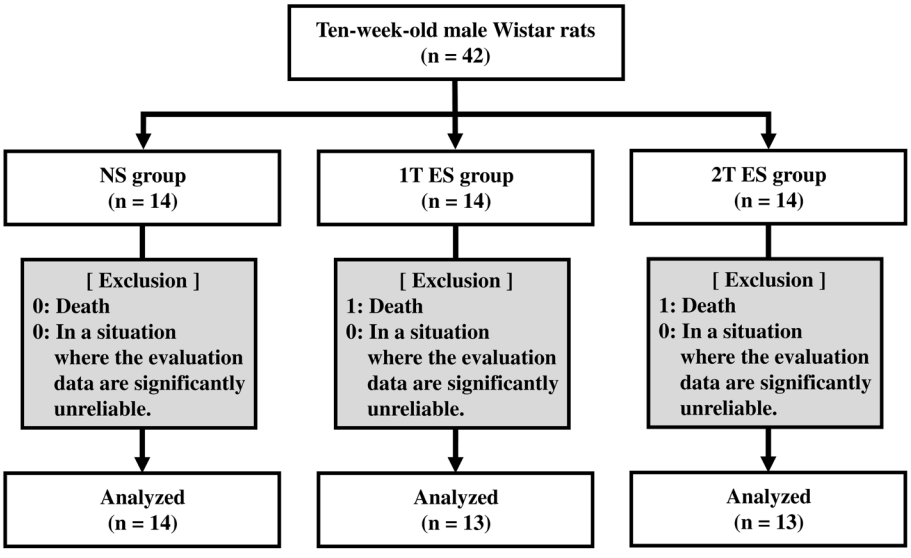



***Figure 1.*** *Study flow diagram. NS group, non-weight-bearing joint fixation without neuromuscular electrical stimulation; 1T ES group, non-weight-bearing joint fixation with one neuromuscular electrical stimulation per day; 2T ES group, non-weight-bearing joint fixation with two neuromuscular electrical stimulations per day.*

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experimental animals and experimental protocols were performed in accordance with the Prefectural University of Hiroshima Animal Experiment Regulations, with approval from the Prefectural University of Hiroshima Animal Experiment Committee (approval number: 18A-029-1).

## Joint fixation

Under anesthesia, all rats had their right ankle joints fixed in full plantar flexion for 1 week using non-elastic tape from the thigh to the foot. Rat toes were exposed to confirm muscle contraction during NMES and the presence or absence of edema. The non-elastic tape was protected against damage using a stainless-steel mesh.

## Non-weight-bearing

Non-weight-bearing was reproduced in all groups using hindlimb suspension[4]. With the rats under anesthesia, a Kirschner wire (diameter, 1.0 mm) was inserted into the tail of the rat to form a triangle and connected to a swivel hook. Hindlimb suspension was performed by attaching a swivel hook to the cage ceiling of wire mesh.

## NMES

NMES (Pulsecure-PRO KR-7; OG Wellness, Okayama, Japan) was applied to the rats once (at approximately 18:00) or twice (at approximately 12:00 and 18:00) a day in the 1T and 2T ES groups, respectively, under anesthesia for 7 days. The hindlimb suspension was temporarily released, and the rats were placed on the left side of a plate on which electrodes were affixed. Additional electrodes were percutaneously applied to the right thigh, and the right soleus muscle was stimulated through the right sciatic nerve (stimulation frequency, 10 Hz; stimulation duration, 30 minutes; stimulation intensity, 30 V; pulse width, 300 μseconds). The same duration of anesthesia used for performing NMES in the 2T ES group was also used in the NS and 1T ES groups. If NMES is properly applied to the sciatic nerve, the soleus and toe flexor muscles contract because the toe flexor muscles are innervated by a branch of the sciatic nerve at the periphery of the soleus muscle[16]. Therefore, the correct performance of NMES was confirmed by observing the right toe flexion.

## Measurement of the ankle dorsiflexion angle

The ankle dorsiflexion angle was measured on the first and last days of the experiment. Under anesthesia, the rat was placed in the left lateral decubitus position and the right knee joint was fixed in full flexion with the trunk fixed to the fixation equipment using a cable tie, as described previously[4]. The hindlimbs were supported using an acrylic plate to prevent hip adduction. The right lateral malleolus and the fibular head were used as landmarks. The right ankle joint of each rat was passively dorsiflexed by using a digital tension meter (LTS-1KA; Kyowa Electronic Instruments Co., Ltd., Tokyo, Japan) on the metatarsal bone. The movement was recorded using a digital camera. A still image was captured from this video at the moment when a dorsiflexion force of 0.3 N was applied, as described in a previous study[4]. The ankle dorsiflexion angle was defined as the angle formed by the line connecting the lateral malleolus and fibular head, with a line parallel to the bottom of the calcaneus. This angle was measured three times from one still image using image analysis software (ImageJ, version 1.52a; National Institutes of Health, Bethesda, MD, USA). The mean values from the three measurements were used.

## Extensibility of the soleus muscle

After measuring the ankle dorsiflexion angle on the last day of the experiment, a tensile test was performed on approximately half of the rats in each group to evaluate right soleus muscle extensibility. With the rat under anesthesia, Kirschner wires (diameter, 0.7 mm) were inserted into the right calcaneus and tibia to fix the ankle joint in full plantar flexion. After sacrificing the rat, the skin of the right hindlimb was excised. The right hindlimb was cut from the femur, and a wire was inserted into the tarsal bone. The right hindlimb was attached to the tensile tester via tarsal wires and the femur. All tissues, except for the soleus muscle, were cut. The soleus muscle tensile test was performed using a tensile tester (Autograph, AGS-X 5kN; Shimadzu Corporation, Kyoto, Japan) at an extension rate of 10 mm/min as described previously[4]. The tension torque was measured at a 10 mm extension as described in a previous study[4]. Tensile tests were performed within 20 minutes of sacrifice.

## Tissue sampling and preparation

After measuring the ankle dorsiflexion angle on the last day of the experiment, the rats that be not underwent tensile testing were sacrificed, and the right soleus muscle was excised. After measuring the wet weight, the excised soleus muscle was frozen in cold acetone with dry ice and stored in a freezer at −80°C until analysis. The soleus muscle specimens were thinly sliced (thicknesses, 10 μm) using a cryostat to prepare cross-sections and were placed on glass slides for immunofluorescence staining and hematoxylin and eosin staining.

## Measurement of the cross-sectional area (CSA)

The cross-sections of the specimens were stained with hematoxylin and eosin to measure the CSA of the soleus muscle. The stained cross-sections were photographed at 200× magnification using an all-in-one light microscope (BZ-X710; Keyence Corporation, Osaka, Japan). After photography, the CSA of the soleus muscle fibers was measured using image analysis software (ImageJ, version 1.52a; National Institutes of Health, Bethesda, USA) on 100 fibers per muscle, as described previously[8,9].

## Immunofluorescence staining

Cross-sections of the soleus muscle were fixed in 4%

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paraformaldehyde. After tissue fixation, the sections were washed three times for 5 min with 0.01 M phosphate buffer in saline (PBS; pH 7.4), immersed in mouse monoclonal anti–type I collagen primary antibodies (1:200 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit polyclonal anti–type III collagen (1:200 dilution; Life Science Laboratories, Osaka, Japan) was diluted with 0.01 M PBS and 0.4% Triton X-100. The sections with primary antibodies were incubated at 4°C during overnight. After being rinsed in PBS, the sections were immersed in fluorescent-labeled secondary antibodies Alexa Fluor 488-conjugated anti-mouse IgG (1:1000 dilution; Cell Signaling Technology, Denvers, USA) for type I collagen and Alexa Fluor 555-conjugated anti-rabbit IgG (1:1000 dilution; Cell Signaling Technology, Denvers, USA) for type III collagen were diluted with 0.01 M PBS. The sections were then incubated at room temperature.

### Measurement of fluorescence intensity

The stained sections were photographed at 400× magnification using an all-in-one fluorescence microscope (BZ-X710; Keyence Corporation, Osaka, Japan). Ten fields in the captured images per muscle were selected using image analysis software (BZ-X analyzer; Keyence Corporation, Osaka, Japan). These 10 fields were used to measure the positive area of the fluorescence set under the same conditions using image software to determine the relative amount of collagen for each individual section. Positive areas of types I and III collagen ($\mu m^2$) were normalized to whole fields ($\mu m^2$). Data were calculated as the positive area of type I or III collagen / whole field × 100 (%).

### Statistical analysis

Repeated two-way analysis of variance was used to compare the ankle dorsiflexion angles (dependent variable) with the independent variables of time (first day of the experiment and last day of the experiment) and group (NS, 1T ES, and 2T ES groups). When there was a time × group interaction, a simple main effect was examined for each independent variable using the post-hoc Bonferroni test. To compare the CSA, wet weight of the soleus muscle-to-body weight ratio, extensibility, and amount of types I and III collagen in the soleus muscle between the groups, normality was verified using the Shapiro–Wilk test, one-way analysis of variance (normal distribution) and post-hoc Bonferroni test or Kruskal–Wallis test (non-normal distribution), and post hoc Steel–Dwass test.

The Spearman test was performed to analyze the correlation between the ankle dorsiflexion angle and extensibility of the soleus muscle, and the correlation between ankle dorsiflexion angle and type I or III collagen. The Bell Curve for Excel software (version 3.21; Social Survey Research Information Co., Ltd., Tokyo, Japan) was used to perform statistical analysis, and statistical significance was set at $p < 0.05$.

## RESULTS

### Ankle dorsiflexion angles

The ankle dorsiflexion angles in each group are presented in *Table 1* and *Figure 2*. There was no significant difference in the ankle dorsiflexion angle between the groups on the first day of the experiment. However, a significant difference was observed on the last day of the experiment. The ankle dorsiflexion angle in the NS group was significantly lower than that in the 1T and 2T ES groups, and it was significantly lower in the 1T ES group than in the 2T ES group. The ankle

| Measured outcome | | Group | | |
|---|---|---|---|---|
| | | NS | 1T ES | 2T ES |
| Ankle dorsiflexion angle (°) | First day | 131.1 ± 3.4 | 132.5 ± 3.4 | 129.9 ± 2.8 |
| | Last day | 63.0 ± 8.8 * | 111.8 ± 12.4 *,† | 121.4 ± 4.6 *,†,¶ |
| Soleus muscle | Wet weight of the soleus muscle-to-body weight ratio (mg/g b.wt.) | 0.49 ± 0.03 | 0.52 ± 0.04 | 0.53 ± 0.05 |
| | CSA ($\mu m^2$) | 3052.3 (2932.1 - 3159.3) | 2178.7 (1954.4 - 2185.5) † | 2672.8 (2484.7 - 2717.7) ¶ |
| | Tension torque (N) | 3.01 (2.83 - 3.15) | 0.20 (0.15 - 0.82) † | 0.16 (0.15 - 0.28) † |
| | Type I collagen (%) | 41.5 ±22.2 | 26.9 ± 12.2 | 26.2 ± 13.2 |
| | Type III collagen (%) | 17.2 ± 3.2 | 17.4 ± 3.9 | 17.5 ± 8.1 |

***Table 1.*** *Ankle dorsiflexion angle and assessment of the soleus muscle.*

*Data of the ankle dorsiflexion angle, wet weight of the soleus muscle-to-body weight ratio, and types I and III collagen are shown as mean ± standard deviation. Data of the CSA and tension torque are shown as median (first to third quartiles). NS group: non-weight-bearing joint fixation without neuromuscular electrical stimulation; 1T ES group: non-weight-bearing joint fixation with NMES once per day; 2T ES group: non-weight-bearing joint fixation with NMES twice per day. * p <0.05 compared with the first day; † p <0.05 compared with the NS group; and ¶ p <0.05 compared with the 1T ES group.*

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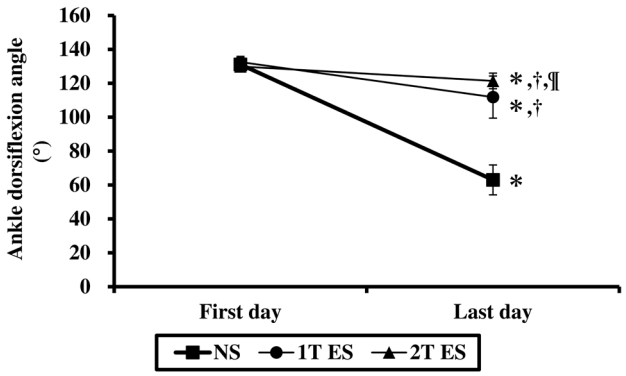



***Figure 2.*** *Ankle dorsiflexion angle value on the first and last days of the experiment.*
** p <0.05 compared with the first day; † p <0.05 compared with the NS group; and ¶ p <0.05 compared with the 1T ES group. NS group, non-weight-bearing joint fixation without neuromuscular electrical stimulation; 1T ES group, non-weight-bearing joint fixation with one neuromuscular electrical stimulation per day; 2T ES group, non-weight-bearing joint fixation with two neuromuscular electrical stimulation per day.*

| | Ankle dorsiflexion angle on the last day of the experiment (°) | Tension torque (N) |
|---|---|---|
| Ankle dorsiflexion angle on the last day of the experiment (°) | - | -0.722 |
| Tension torque (N) | * | - |

***Table 2.*** *Correlation between the ankle dorsiflexion angle and extensibility of the soleus muscle.*
*The value is the correlation coefficient. Cells where the same variables were compared were omitted and shown as "-". * p <0.05.*

dorsiflexion angles on the last day of the experiment in all groups were significantly lower than those on the first day.

## Wet weight of the soleus muscle-to-body weight ratio

The wet weights of the soleus muscle-to-body weight ratios in all groups are shown in *Table 1* and *Figure 3*. No significant differences were observed between the groups.

## CSA of the soleus muscle

The CSAs of the soleus muscles in each group are presented in *Table 1* and *Figure 3*. The CSA of the soleus muscle was significantly lower in the 1T ES group than in the NS and 2T ES groups.

## Extensibility of the soleus muscle

The tensile test results of the soleus muscle in each group are presented in *Table 1* and *Figure 3*. Extensibility of the soleus muscle was significantly lower in the NS group than in the 1T and 2T ES groups. No significant differences were observed between the 1T and 2T ES groups. The correlation between the ankle dorsiflexion angle and extensibility of the soleus muscle is shown in *Table 2* and *Figure 3*. There was a significant negative correlation between the ankle dorsiflexion angle and extensibility of the soleus muscle.

## Amounts of types I and III collagen of the solus muscle

The amounts of types I and III collagen in the soleus muscle of each group are shown in *Table 1* and *Figure 3*. No significant differences were observed in the amounts of types I and III collagen in the soleus muscle between the groups. The correlation between the ankle dorsiflexion angle and type I or III collagen in the soleus muscle is shown in *Table 3* and *Figure 3*. There was no significant correlation between the ankle dorsiflexion angle and type I or III collagen in the soleus muscle.

## DISCUSSION

The present study sought to clarify the effective intervention frequency of NMES for preventing joint contracture caused by joint fixation in non-weight-bearing rats. In this study, the ankle dorsiflexion angle in all groups on the last day of the experiment was significantly lower than that on the first day. Furthermore, there was a negative correlation between the ankle dorsiflexion angle and soleus extensibility. Therefore, joint contracture occurred in all groups, and we considered decreased muscle extensibility to be related to joint contracture. In contrast, the ankle dorsiflexion angles in the two groups that underwent NMES on the last day of the experiment were significantly higher than those in the NS group. These findings demonstrate that NMES had a preventive effect on joint contracture caused by non-weight-bearing joint fixation.

The main components of the muscle are the muscle fibers and fascia that surround the fibers, e.g., the endomysium

| | Ankle dorsiflexion angle on the last day of the experiment (°) | Amount of type I collagen (%) | Amount of type III collagen (%) |
|---|---|---|---|
| Ankle dorsiflexion angle on the last day of the experiment (°) | - | -0.220 | 0.106 |
| Amount of type I collagen (%) | N.S. | - | -0.069 |
| Amount of type III collagen (%) | N.S. | N.S. | - |

***Table 3.*** *Correlation between the ankle dorsiflexion angle on the last day of the experiment and collagen of the soleus muscle.*
*The value is the correlation coefficient. Cells where the same variables were compared were omitted and shown as "-". N.S.: no significant.*

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and perimysium[12,16]. Collagen is the main component of the endomysium and perimysium and is increased by joint fixation[12]. Particularly, the amount of type I collagen in the soleus muscle increases as the period of joint fixation increases[12]. Therefore, we assumed that the amounts of types I and III collagen in the soleus muscle of the NS group were increased by joint fixation in this study. Although there was no significant difference in the amounts of types I and III collagen in the soleus muscle between the groups that underwent NMES and the NS group, the soleus muscle extensibility and the ankle dorsiflexion angle on the last day of the experiment in the two groups that underwent NMES were higher than those in the NS group. In a previous study[12], many fascial fiber orientations of the normal soleus muscle were parallel to the long axis of the muscle and parallel to the short axis of the soleus muscle after joint fixation for ≥4 weeks. Therefore, when joint contracture occurs, the fascia surrounding the muscle fiber alters types I and III collagen levels as well as, but also the fascia fiber orientation. If a change in fascia fiber orientation occurs from the early phase due to severe joint contracture caused by joint fixation with non-weight-bearing for 1 week, artificial muscle contraction using NMES might prevent muscle extensibility from reducing changes to the fascia fiber orientation.

Muscle atrophy is caused by joint fixation[6,8-10]; therefore, we hypothesized that the soleus muscle in the NS group was atrophied by joint fixation. NMES can prevent muscle atrophy[8,9]. However, in this study, NMES did not prevent a decrease in the CSA or wet weight-to-body weight ratio of the soleus muscle. Therefore, we infer that the NMES setting in this study could not prevent muscle atrophy.

Next, we considered the effect of the intervention frequency (once or twice daily) on joint contracture. The fascia surrounding muscle fibers affects muscle extensibility[12]; therefore, it is assumed that the fascia

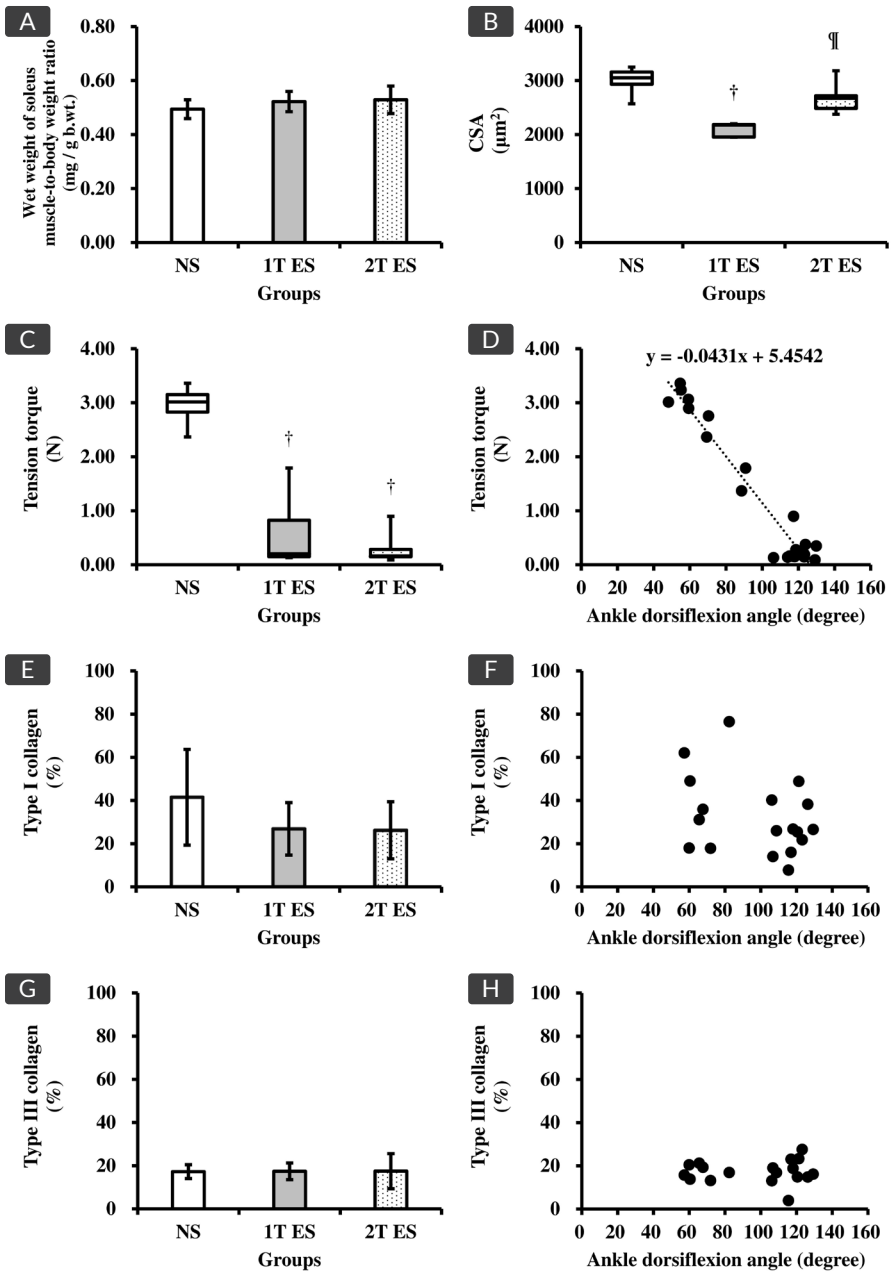



***Figure 3.*** *Assessment outcomes of the soleus muscle on the last day of the experiment.*

*A) The wet weight of the soleus muscle-to-body weight ratio. B) The cross-sectional area (CSA) of the soleus muscle. C) The tension torque of the soleus muscle. D) The correlation of the ankle dorsiflexion angle on the last day of the experiment with the tension torque of the soleus muscle. E, G) Types I and III collagen of the soleus muscle, respectively. F, H) The correlation of the ankle dorsiflexion on the last day of the experiment with type I or III collagen, respectively. † p <0.05 compared with the NS group. ¶ p <0.05 compared with the 1T ES group. NS group, non-weight-bearing joint fixation without neuromuscular electrical stimulation; 1T ES group, non-weight-bearing joint fixation with one neuromuscular electrical stimulation per day; 2T ES group, non-weight-bearing joint fixation with two neuromuscular electrical stimulation per day.*

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surrounding the muscle fibers can move during muscle extension and contraction. In other words, normal fascial movements are maintained through muscle extension and contraction. However, if the ankle joint is fixed in full plantar flexion and non-weight-bearing, the soleus muscle is in a shortened position, and soleus muscle contraction in non-weight-bearing is decreased more than that in weight-bearing[17,18]. Therefore, the soleus muscle of the NS group was affected by joint fixation and non-weight-bearing and could not be moved sufficiently. In contrast, the soleus muscle fascia of the two groups that underwent NMES moved more by artificial muscle contraction using NMES than the NS group. Generally, long-duration interventions for joint contracture are more effective than short-duration interventions[19,20]. However, this depends on the intervention conditions[19,21]. In this study, the total duration of NMES in the 2T ES group per day was 60 minutes, which was longer than that in the 1T ES group (30 minutes). The ankle dorsiflexion angle in the 2T ES group on the last day of the experiment was higher than those in the 1T ES and NS groups. Therefore, we conclude that the total duration of NMES in the 2T ES group was more effective and appropriate for joint contracture than that in the 1T ES group.

The intervention for joint contracture was performed once daily[5,8-10,15]. In a recent study[22], intervention for joint contracture was performed twice daily (morning and evening). It is assumed that this intervention frequency (twice daily) for joint contractures has a shorter interval between interventions than one intervention per day. In this study, the NMES interval in the 1T ES group was approximately 23 hours and 30 minutes, that in the 2T ES group was 5 hours and 30 minutes (from the first session to the second session), and 17 hours and 30 minutes (from the second session to the first session the next day). The NMES interval was shorter in the 2T ES group than in the 1T ES group. Thus, we infer that the short interval may be more effective for preventing joint contracture than the long interval, as NMES twice daily has a shorter interval for lack of muscle fascia movement than NMES once daily.

We found that NMES has a preventive effect on joint contracture caused by non-weight-bearing joint fixation; particularly, NMES performed twice daily had a greater preventive effect than once a day NMES. However, a limitation of this study is that we did not investigate the muscle fiber orientation or the effect of other NMES settings, excluding the intervention frequency. In future studies, we recommend investigating the muscle fiber orientation and the effect of NMES settings, excluding the intervention frequency.

## CONCLUSIONS

This study's results indicated that NMES twice daily has a more preventive effect on joint contracture than NMES once daily because it has a shorter interval for lack of muscle fascia movement.

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