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Electrical Stimulation-Based Twitch Exercise Suppresses Progression of Immobilization-Induced Muscle Fibrosis via Downregulation of PGC-1α/VEGF Pathway

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Summary

This study aimed to determine whether electrical stimulation-based twitch exercise is effective in inhibiting the progression of immobilization-induced muscle fibrosis. 19 Wistar rats were randomly divided into a control group (n=6), an immobilization group (n=6; with immobilization only), and a Belt group (n=7; with immobilization and twitch exercise through the belt electrode device, beginning 2 weeks after immobilization). The bilateral soleus muscles were harvested after the experimental period. The right soleus muscles were used for histological analysis, and the left soleus muscles were used for biochemical and molecular biological analysis. As a result, in the picrosirius red images, the perimysium and endomysium were thicker in both the immobilization and Belt groups compared to the control group. However, the perimysium and endomysium thickening were suppressed in the Belt group. The hydroxyproline content and α-SMA, TGF-β1, and HIF-1α mRNA expressions were significantly higher in the immobilization and belt groups than in the control group. These expressions were significantly lower in the Belt group than in the immobilization group. The capillary-to-myofiber ratio and the mRNA expressions of VEGF and PGC-1α were significantly lower in the immobilization and belt groups than in the control group, these were significantly higher in the Belt group than in the immobilization group. From these results, Electrical stimulation-based twitch exercise using the belt electrode device may prevent the progression of immobilization-induced muscle fibrosis caused by downregulating PGC-1α/VEGF pathway, we surmised that this intervention strategy might be effective against the progression of muscle contracture.

Keywords

Immobilization • Skeletal muscle • Fibrosis • Electrical stimulation-based twitch exercise • PGC-1α/VEGF pathway

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Introduction

In clinical practice, patients whose procedures require joint immobilization can be at risk of suffering muscle contractures caused by muscular fibrosis. When possible, rehabilitation providers begin their interventions immediately or even before immobilization begins to mitigate this contracture. However, some patients cannot undergo early rehabilitation due to their poor general condition, and in other cases are delayed from rehabilitation due to physician-ordered cast immobilization or bed rest. Thus, these patients often reach an advanced stage of muscle contracture before rehabilitation can be started. However, current intervention strategies against advanced contractures are rarely effective, and it is necessary to develop effective alternate intervention strategies. Our laboratory findings previously revealed immobilization-induced muscle fibrosis is caused by collagen overexpression [1]. Additionally, hypoxia-inducible factor (HIF)-1α was upregulated in skeletal muscles immobilized for 4 weeks, thus we surmise that hypoxic conditions affected the progression of fibrotic lesions [2].

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These hypoxic changes are induced by a decrease in the blood flow volume due to a decrease in the number of capillaries [3,4]. Peroxisome proliferator-activated receptor γ coactivator (PGC)-1α regulates vascular endothelial growth factor (VEGF), which is associated with capillary homeostasis [5]. Downregulation of PGC-1α causes a decrease in the number of capillaries via downregulation of VEGF expression [6,7]. By contrast, muscle contractile exercise induces the upregulation of PGC-1α, which leads to increased blood flow volume by increasing the number of capillaries in skeletal muscles [5, 8]. Therefore, we hypothesised that increasing blood flow volume with muscle contractile exercise effectively prevents the progression of immobilization-induced muscle fibrosis caused by hypoxia. However, in many cases, it is difficult to perform voluntary muscle contractile exercises immediately after the start of immobilization because of the poor health condition of the patient and medical management of their condition. Alternatively, these patients could undergo muscle contractile exercises induced by external stimuli, and we believe that electrical stimulation therapy can be an effective intervention strategy. Electrical stimulation therapy has previously been utilized as a therapeutic intervention and functional substitute for voluntary muscle contraction [9]. Muscle contractile exercise is important to induce PGC-1α in skeletal muscles [10], and electrical stimulation is expected to prevent the reduction of PGC-1α. However, it is unknown whether muscle contractile exercise suppresses the reduction of PGC-1a expression during the advanced stage of immobilization. In addition, a previous study has shown interventions which can provide contractile exercise to a wide range of skeletal muscles may be effective in increasing PGC-1a expression [11]. Therefore, an electrode was needed that could simultaneously stimulate a wider range of skeletal muscles than conventional monopolar electrodes. Belt electrode-skeletal muscle electrical stimulation (HOMER ION Co., Ltd., Tokyo, Japan) was recently developed as an alternative method of applying electrical stimulation therapy. An advantage of this device is that the entire belt is an electrode that can deliver electricity to the entire lower limb [12], it can simultaneously apply muscle contractile exercise to a wider range of skeletal muscle.

The common muscle contraction patterns using electrical stimulation that have been tested in rat skeletal muscle are twitch and tetanic exercise, with stimulus frequencies of 1–10 Hz and 50–100 Hz respectively [13, 14]. Of these modalities, twitch exercise may be more effective than tetanic exercise in increasing blood flow because it can stimulate muscle contraction at a higher frequency during a given period.

Therefore, the purpose of this investigation was to test the efficacy of belt electrode stimulation as a method of applying twitch exercise in a rat model of immobilization, and to examine the belt's ability to mitigate against immobilization-induced muscle fibrosis.

Methods

Animals

Eight-week-old male Wistar rats (CLEA Japan Inc., Tokyo, Japan) were maintained at the Centre for Frontier Life Sciences of Nagasaki University. The rats were placed in 30 × 40 × 20-cm cages (2 rats/cage) and exposed to a 12-h light-dark cycle at an ambient temperature of 25 °C. Food and water were provided *ad libitum*. In this investigation, 19 rats (263.6 ± 15.6 g) were randomly divided into an experimental group (n=13) and a control group (n=6). In the control group, no treatment or intervention was given for the duration of the study. In the experimental group, both of the animal's ankle joints were immobilized using the procedure described in our previous studies [1]. Briefly, the animals in the experimental group were anesthetized with the combination of the following anesthetic agents: 0.375 mg/kg medetomidine (Kyoritu Pharma, Tokyo, Japan), 2.0 mg/kg midazolam (Sandoz Pharma Co., Ltd., Tokyo, Japan), and 2.5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan). Thereafter, both ankle joints of each rat were fixed in full plantar flexion with plaster casts to immobilize the soleus muscle in a shortened position for 4 weeks. The plaster cast, which was fitted from above the knee joint to the distal foot, was changed weekly to ensure a tight fit and offset loosening due to muscle atrophy. The experimental groups were further divided into an immobilization group (n=6; with the above immobilization only) and the Belt group (n=7; with the immobilization as well as muscle contractile exercise through the belt electrode device starting 2 weeks after immobilization) (Fig. 1). The Ethics Review Committee approved the experimental protocol for Animal Experimentation of Nagasaki University (approval no. 1903281524). All experimental procedures were performed under anesthesia, and efforts were made to minimize distress and/or suffering. All experiments were performed under relevant guidelines and regulations.

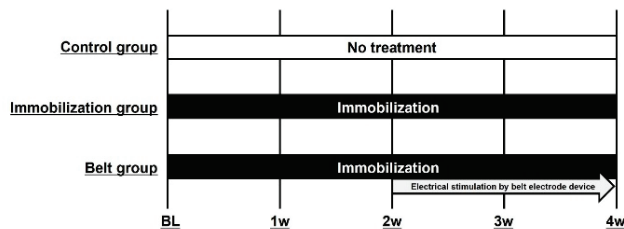

Fig. 1. Experimental protocol for the present study. The control group was normally maintained without treatment and intervention. The experimental groups were divided into the immobilization group and the Belt group with immobilization and muscle contractile exercise through a belt electrode device two weeks after immobilization.

Protocol for belt electrode device

Cyclic muscle twitch contraction was performed using an electrical stimulator for small animals (Homer Ion), as described in our previous research [15]. The electrical stimulator consisted of a control unit (for setting the stimulus cycle, frequency, and intensity) and a belt electrode. Animals in the Belt groups were anesthetized and the casts of both hind limbs were removed. The electrical stimulator then was connected to the belt electrodes, these electrodes were then wrapped around the proximal thigh and distal lower leg. Electrical stimulation was administered bilaterally, with the cast removed, with an amplitude of 3 mA or less (continuous energization), at 10 Hz frequency, for 30 minutes. This treatment was given once daily, 6-days per week, for two weeks. After each electrical stimulation, immobilization via casts on both hind limbs was resumed immediately.

ROM of the ankle joint dorsiflexion

Each week after immobilization, the rats were anesthetized, and the range of motion (ROM) of ankle joint dorsiflexion was determined using a goniometer. The ROM was measured as the angle (0–180°) between the line connecting the fifth metatarsal to the malleolus lateralis of the fibula and the line connecting the malleolus lateralis of the fibula to the canter of the knee joint. The ankle was passively dorsiflexed with a tension of 0.3 N using a tension gauge (Shiro Industry, Osaka, Japan) [2].

Tissue sampling and preparation

The left and right soleus muscles of all the rats were harvested premortem at the conclusion of the experimental period. The right soleus muscles were embedded in tragacanth, and the samples were frozen in liquid nitrogen and cryo-sectioned. Serial frozen cross-sections of the muscles were mounted on glass slides for histological and immunohistochemical analyses. A part of the left soleus muscle was rapidly frozen in liquid nitrogen for biochemical analysis. The remaining left soleus muscles were treated with Ranelates® (Ambion, CA, USA) immediately after excision for molecular biological analysis.

Histological analysis

Cross sections were stained with Haematoxylin and Eosin (H&E) stain, picrosirius red stain, and alkaline phosphatase stain following previous protocols [16]. The stained cross-sections of the muscles were then evaluated under an optical microscope (BZ-X800, KEYENCE, Osaka, Japan). H&E-stained cross-sections were used to identify myofiber morphological characteristics and signs of previous muscle injury, such as centralized nuclei. Subsequently, picrosirius red-stained cross-sections were used to identify the perimysium and endomysium of the soleus muscle. Additionally, alkaline phosphatase-stained cross-sections were used to calculate the capillary-to-myofiber ratio and CSA using a BZ-X Analyser (KEYENCE).

Molecular biological analysis

Segments of the left soleus muscles were used for this analysis. Total RNA was extracted from the muscle samples using a RNeasy Fibrous Tissue Mini Kit (Qiagen, CA, USA). It was then used as a template with a QuantiTect® Reverse Transcription Kit (Qiagen) to prepare cDNA, and real-time RT-PCR was performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, CA, USA). The cDNA concentration of all samples was unified to 25 ng/μl, and 0.2 μl cDNA was applied to each well. The synthetic gene-specific primers that were used are listed in Table 1. The threshold cycle (Ct) was determined using an Mx3005P Real-Time qPCR System (Agilent Technologies). The mRNA expression of target genes was calculated using the ΔΔCT method.

Biochemical analysis

The soleus muscles were assessed for hydroxyproline expression following our previously described method [1]. Briefly, muscle samples were immersed in 1.0 M PBS (pH 7.4) and homogenized using Micro Smash^{TM} (MS-100R; Tomy, Tokyo, Japan). Subsequently, the samples were hydrolyzed in 6 N HCl for 15 h at 110 °C and then dried using an evaporator (EZ-2 HCL-resistant model; Ikeda Scientific, Tokyo, Japan).

Table 1. Arrangement of synthetic gene-specific primers

Object gene	Arrangement Forward	Reverse	Gene Bank No.
HIF-1α	5'-CGAGCTGCCTCTTCGACAAG-3'	5'-CCCAGCCGCTGGAGCTA-3'	AF057308.1
PGC-1α	5'-CAAGCCAAACCAACAACTTTATCTCT -3'	5'- CACACTTAAGGTTCGCTCAATAGT -3'	AY237127.1
VEGF	5'-ATCATGCGGATCAAACCTCACC-3'	5'- GGTCTGCATTCACATCTGCTATGC-3'	AY702972.1
TGF-β1	5'-AGAAGTCACCCGCGTGCTAAT-3'	5'-CACTGCTTCCCGAATGTCTGA-3'	BC076380.1
α-SMA	5'-CGGGCTTTGCTGGTGATG-3'	5'-GGTCAGGATCCCTCTCTTGCT-3'	BC158550.1
β-actin	5'-GTGCTATGTTGCCCTAGACTTCG-3'	5'-GATGCCACAGGATTCCATACCC-3'	BC063166.1

HIF, hypoxia-inducible factor; PGC, peroxisome proliferator-activated receptor γ coactivator; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; SMA, smooth muscle actin

Table 2. Measurements and standard deviations in all parameters

	Con	Im	Belt
ROM on ankle joint dorsiflexion (°)			
after 1 week	160.0	119.2 ± 5.1*	120.0 ± 5.2*
after 2 weeks	160.0	101.7 ± 3.8*	101.1 ± 5.9*
after 3 weeks	160.0	79.2 ± 2.9*	87.5 ± 6.4*#
after 4 weeks	160.0	67.9 ± 3.3*	78.6 ± 6.6*#
Hydroxyproline content (µg/mg dry weight)	3.9 ± 0.4	10.9 ± 1.7*	8.0 ± 1.9*#
α-SMA mRNA expression	1.1 ± 0.4	6.5 ± 2.3*	3.6 ± 1.9*#
TGF-β1 mRNA expression	0.9 ± 0.3	3.4 ± 1.2*	1.8 ± 0.5*#
HIF-1α mRNA expression	0.9 ± 0.4	5.1 ± 1.5*	2.7 ± 0.9*#
Cross-sectional area (µm^2)	4035.1 ± 365.5	1632.9 ± 221.3*	1777.8 ± 279.6*
Capillary-to-myofiber ratio	2.6 ± 0.1	1.7 ± 0.1*	1.9 ± 0.1*#
VEGF mRNA expression	0.9 ± 0.3	0.4 ± 0.2*	0.7 ± 0.2*#
PGC-1α mRNA expression	1.0 ± 0.2	0.4 ± 0.2*	0.7 ± 0.2*#

ROM: Range of motion; α-SMA: α-smooth muscle actin; TGF-β1: transforming growth factor-β1; HIF-1α: hypoxia-inducible factor-1α; VEGF: vascular endothelial growth factor; PGC-1α: Peroxisome proliferator-activated receptor γ coactivator-1α. * Significant difference ($p<0.05$) compared with the Con group. # Significant difference ($p<0.05$) compared with the Im group.

Muscle samples were hydrolyzed in NAOH for 1 h at 90 °C. The hydrolyzed specimens were then mixed with buffered chloramine-T reagent and then oxidized at 20 °C. The chromophore was developed by adding Ehrlich's aldehyde reagent. The absorbance of each sample was measured at 540 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, San Jose, CA, USA). The absorbance values were plotted against the concentration of the hydroxyproline standard. A known standard curve facilitated the conversion between absorbance and hydroxyproline content in each of the samples. The hydroxyproline content of the samples was normalized as content per dry weight (µg/mg, dry weight).

Statistical analysis

All data is presented as mean ± standard deviation. Differences among groups were assessed using one-way analysis of variance (ANOVA), followed by Scheffé's method as a *post hoc* test. An *a priori* level of significance (α) was set at 0.05 for all statistical tests.

Results

Measurements and standard deviations for all parameters are listed in Table 2.

ROM on ankle joint dorsiflexion

The dorsiflexion ROM was significantly lower in the experimental group than in the control group.

However, these magnitudes were significantly higher in the Belt group than in the immobilization group (Table 2 and Fig. 2A).

H&E imaging, picrosirius red imaging, and hydroxyproline content

H&E-stained cross-sections showed no abnormal findings in the experimental group except for atrophic changes. Evaluation of the picrosirius red images demonstrated that the perimysium and endomysium were thicker in the experimental groups than in the control group. However, in comparison between the experimental groups, the perimysium and endomysium thickening were reduced in the Belt group compared to the immobilization group (Fig. 2B). The differences in level of hydroxyproline contents were statistically significant between the control and experimental groups, and between the immobilization and Belt groups (Table 2 and Fig. 2C).

α-SMA, TGF-β1, and HIF-1α mRNA expression

The mRNA expressions of α-smooth muscle actin (SMA), transforming growth factor (TGF)-β1, and HIF-1α were significantly higher in the experimental groups than in the control group, and these were significantly lower in the Belt group compared to the immobilization group (Table 2 and Fig. 3A-C).

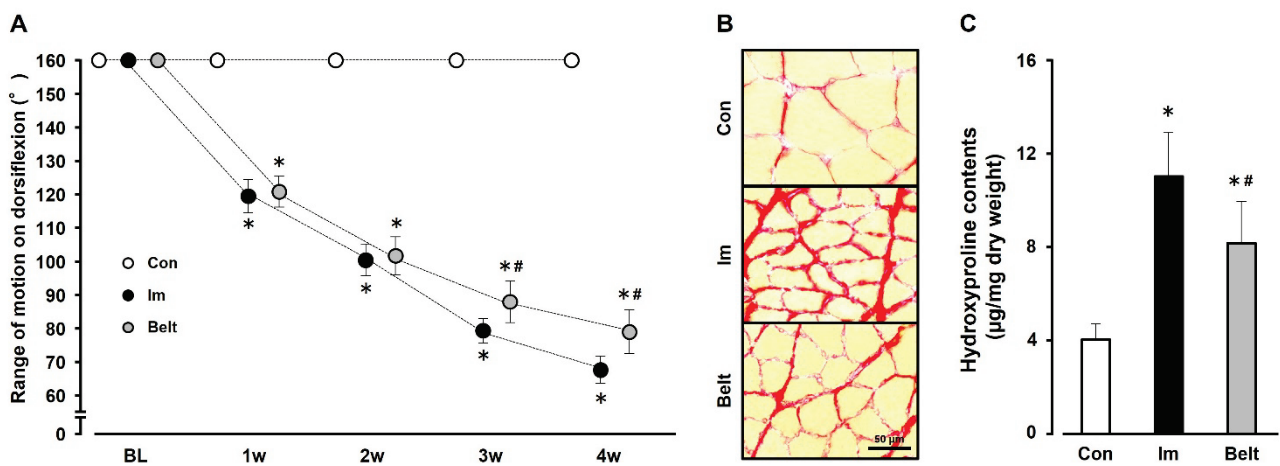

Fig. 2. Range of motion of the ankle joint on dorsiflexion (**A**), picrosirius red imaging (**B**), and hydroxyproline contents (**C**) in soleus muscles. White circles and bar represent the control group. Black circles and bar represent the immobilization group. Gray circles and bar represent the Belt group. Data presented as mean ± standard deviation. * Significant difference (p<0.05) compared with the control group. # Significant difference (p<0.05) compared with the immobilization group.

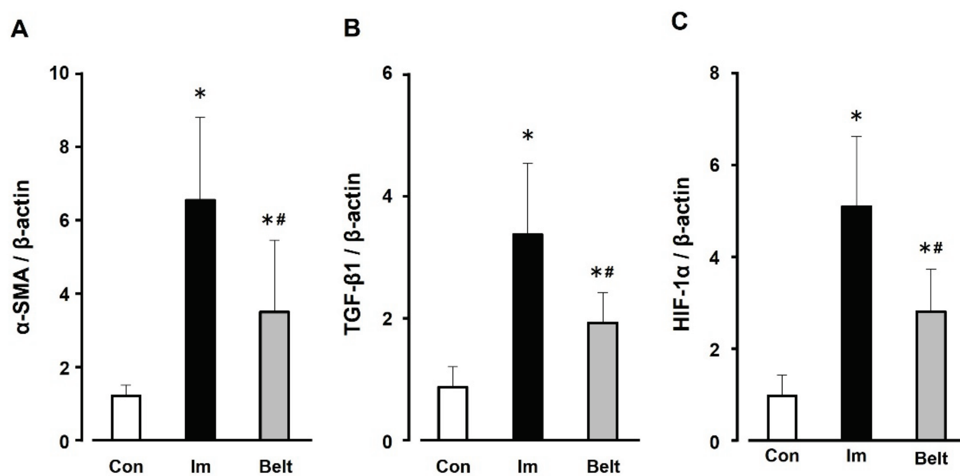

Fig. 3. mRNA expression of α-SMA (**A**), TGF-β1 (**B**), and HIF-1α (**C**) in soleus muscles. White bars represent the control group. Black bars represent the immobilization group. Gray bars represent the Belt group. Data presented as mean ± standard deviation. * Significant difference (p<0.05) compared with the control group. # Significant difference (p<0.05) compared with the immobilization group.

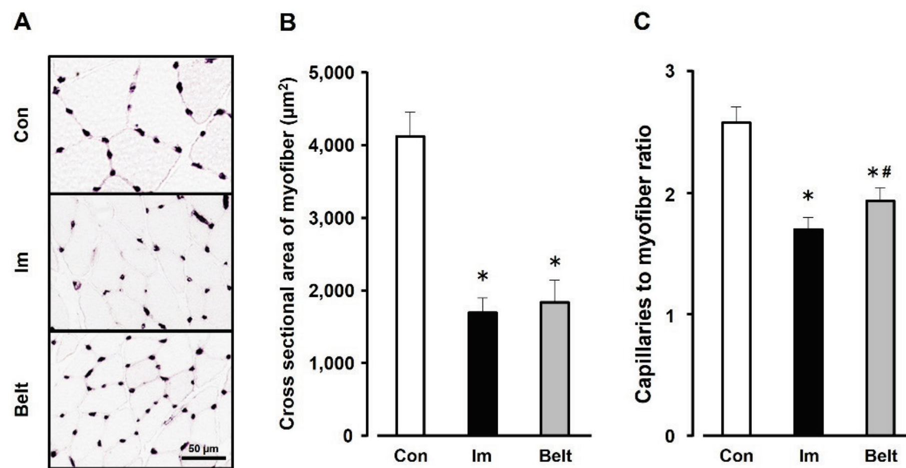

Fig. 4. Cross-sectional area (CSA) of myofiber and capillaries to myofiber ratio in soleus muscles. (**A**) Alkaline phosphatase-staining of soleus muscles. Dark purple indicates the capillaries. (**B**) CSA of the myofiber. (**C**) Capillaries to myofiber ratio. White bars represent the control group. Black bars represent the immobilization group. Gray bars represent the Belt group. Data presented as mean ± standard deviation. * Significant difference ($p<0.05$) compared with the control group. # Significant difference ($p<0.05$) compared with the immobilization group.

Cross-sectional area and capillary-to-myofiber ratio

On the alkaline phosphatase-stained cross sections (Fig. 4A). Although the capillary-to-myofiber ratio and CSA were significantly lower in the experimental groups than in the control group, the ratio was significantly higher in the Belt group than in the immobilization group (Table 2 and Fig. 4B-C).

VEGF and PGC-1α mRNA expression

The VEGF and PGC-1α mRNA expressions were significantly lower in the experimental groups than in the control group, and the expression was significantly higher in the Belt group than in the immobilization group (Table 2 and Fig. 5A-B).

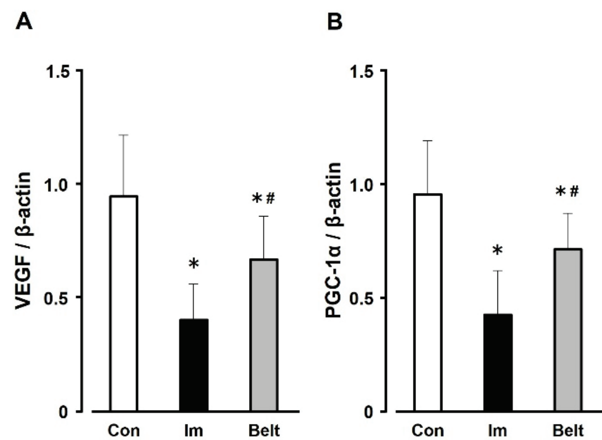

Fig. 5. mRNA expression of VEGF (**A**) and PGC-1α (**B**) in soleus muscles. White bars represent the control group. Black bars represent the immobilization group. Gray bars represent the Belt group. Data presented as mean ± standard deviation. * Significant difference ($p<0.05$) compared with the control group. # Significant difference ($p<0.05$) compared with the immobilization group.

Discussion

This study investigated the biological effects of electrical stimulation-based twitch exercise using belt electrode devices on the progression of immobilization-induced muscle fibrosis. In the immobilization group, the ROM on dorsiflexion decreased to 74.5 % at 1 week, 63.6 % at 2 weeks, 50.0 % at 3 weeks, and finally 42.4 % at 4 weeks of that in the control group following immobilization, which is consistent with the results of previous studies [2]. These results demonstrated the occurrence of muscle contracture in the immobilization group. The CSA was significantly lower in the immobilization group than in the control group, demonstrating that muscle fiber atrophy occurred in the immobilized rat soleus muscles. The present study indicated that CSA decreased to 40.5 % at 4 weeks in the control group, which is similar to the results of our previous studies [2]. Muscle fiber atrophy is caused by myonuclear apoptosis and the loss of muscle proteins [17, 18]. Decreasing the myonuclei via apoptosis was observed in the rat soleus muscles by other studies using the same animal model as this study following 1 week of immobilization [19]. Additionally, the upregulation of ubiquitin-proteasome pathway enzymes leads to the loss of muscle protein, and the expression of these enzymes increases in immobilized rat soleus muscles [19].

Therefore, we surmised that both the change in myonuclear apoptosis and loss of muscle protein were related to muscle fiber atrophy.

The picrosirius red images show that the perimysium and endomysium were thicker in the immobilization group than in the control group. Additionally, the level of hydroxyproline content increased in the immobilization group, suggesting that immobilization-induced muscle fibrosis occurred in the rat soleus muscles. Several reports have shown that TGF-β1 is an important component of muscle fibrosis formation [2, 20]. Moreover, TGF-β1 is a key factor that promotes the conversion of fibroblasts into myofibroblasts, a differentiation process commonly associated with immobilization-induced muscle fibrosis [2]. Myofibroblasts produce large amounts of collagen and play a major role in pathological contracture [21, 22]. In this study, TGF-β1 and α-SMA mRNA expression increased in the rat soleus muscle after 4 weeks of immobilization. These results indicated that TGF-β1 mRNA upregulation affects the differentiation of fibroblasts into myofibroblasts and that these alterations are associated with the incidence of immobilization-induced muscle fibrosis.

TGF-β1 is overexpressed under hypoxic conditions [7]. In the present study, HIF-1α expression in the immobilization group was significantly higher than that in the control group; immobilized rat soleus muscles might therefore be hypoxic. Capillaries play a key role in supplying oxygen to skeletal muscles, and changes in the number of capillaries affect muscular blood flow volume [23, 24]. A reduction in blood flow due to immobilization decreasing the number of capillaries can lead to hypoxic conditions [25]. In the present study, we found that the capillary-to-myofiber ratio was significantly lower in the immobilization group than in the control group. This decrease in capillaries may be responsible for inducing hypoxia in the immobilized rat soleus muscles. VEGF is the most important regulator of capillary homeostasis, and PGC-1α regulates VEGF expression in skeletal muscles [5]. PGC-1α is a transcriptional coactivator, and this factor should be increased for correct angiogenesis by upregulating VEGF expression [5, 26]. In the immobilization group, PGC-1α mRNA expression was downregulated in the rat soleus muscles. Moreover, VEGF mRNA expression was significantly lower in the immobilization group than that in the control group. These results indicated that VEGF expression decreases with PGC-1α downregulation. Therefore, these alterations may result in hypoxia via the reduction of capillaries, and lead to the differentiation of fibroblasts into myofibroblasts *via* TGF-β1 upregulation; leading to immobilization-induced muscle fibrosis.

Electrical stimulation therapy with belt electrodes is useful for performing muscle twitch exercises. The dorsiflexion ROM was significantly higher in the Belt group than in the immobilization group. In the Belt group, the thickening of the perimysium and endomysium and the excessive increase in hydroxyproline content via immobilization were mitigated. These results indicate that the electrical stimulation-based twitch exercise prevented the progression of muscle contracture due to immobilization-induced muscle fibrosis. The mRNA expressions of α-SMA, TGF-β1, and HIF-1α were significantly lower in the Belt group than in the immobilization group. Additionally, the capillaries to myofiber ratio in the Belt group was significantly higher than that in the immobilization group. The number of capillaries regulates blood flow in skeletal muscles. Skeletal muscles with a high number of capillaries have a high blood flow volume [23], which mitigates hypoxia in skeletal muscle [4]. Therefore, we surmised that electrical stimulation-based twitch exercise with belt electrode devices mitigated hypoxia by the preventing the loss of capillaries. This might limit the differentiation of fibroblasts into myofibroblasts via TGF-β1 mRNA upregulation. Moreover, in the present study, VEGF and PGC-1α mRNA expression were significantly higher in the Belt group than in the immobilization group, and the decrease in VEGF and PGC-1α mRNA expression was counteracted by electrical stimulation-induced twitch exercise using belt electrode devices. A previous study showed that PGC-1α was an important factor related to angiogenesis, and PGC-1α expression increased only in a wide range of skeletal muscle exercises [6]. The belt electrode device can induce more muscle twitch exercises in many skeletal muscles than the monopolar electrode devices, which may explain its success in affecting PGC-1α regulation [12]. In addition, previous studies showed that PGC-1α regulated VEGF expression *in vivo* [5, 27]. Based on our results and previous research, we surmise that electrical stimulation-based twitch exercise with belt electrode devices prevented the decrease in capillaries via the downregulation of VEGF and PGC-1α; these beneficial effects led to the suppression of hypoxia-induced fibrosis in immobilized rat soleus muscles.

This study has several limitations. It is uncertain

whether the current electrical stimulation protocol is the most effective. Further examination of various frequencies, intensities, duty cycles, duration, intraday sessions, and electrode type (e.g., monopolar electrode) of electrical stimulation protocols is required.

Additionally, this study was unable to determine the blood flow volume in rat soleus muscles. Therefore, we need to perform a non-invasive assessment for muscular blood flow volume in the future. Moreover, the molecular mechanisms underlying the regulation of PGC-1α expression remain unknown. To answer this question, further investigations are needed to search for adenosine monophosphate, sirtuins, and calcium ions. Finally, data related to the cause-and-effect relationship between cellular and molecular events were insufficient. Therefore, further studies using antagonists or inhibitors are warranted to address this limitation.

In summary, electrical stimulation-based twitch exercise using the belt electrode device may mitigate the progression of immobilization-induced muscle fibrosis caused by downregulating PGC-1α/VEGF pathway, and this alteration may suppress the progression of limited dorsiflexion. Therefore, we surmise that this intervention strategy might be effective against the progression of muscle contracture.

Conflict of Interest

This work was supported by the Japanese Society for Electrophysical Agents in Physical Therapy, the Japan Society for the Promotion of Science, and ALCARE Co. Ltd. The funders had no role in study design, data collection, analysis, publication decision, or manuscript preparation.

Acknowledgements

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