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Effects of electrical stimulation therapy on the blood flow in chronic critical limb ischemia patients following regenerative therapy

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Abstract

Objectives: We investigated the effects of electrical stimulation therapy on cutaneous and muscle blood flow in critical limb ischemia patients following regenerative therapy.

Methods: Three groups were studied: 10 healthy young subjects, 10 elderly subjects, and 7 critical limb ischemia patients after regenerative therapy. After 5 min rest, electrical stimulation was applied at 5 Hz on the tibialis anterior muscle for 10 min. We estimated the relative changes in oxyhemoglobin and total hemoglobin compared to the basal values at rest ($\Delta[HbO_2]$, $\Delta[Hb_{tot}]$), which reflected the blood flow in the skin and muscle layer, and we simultaneously measured the tissue O_2 saturation (S_tO_2) throughout the electrical stimulation and recovery phase by near-infrared spectroscopy.

Results: The $\Delta[HbO_2]$ and $\Delta[Hb_{tot}]$ values of the muscle layer in critical limb ischemia patients increased gradually and remained significantly higher at the 5-min and 10-min recovery periods after the electrical stimulation without reducing the S_tO_2, but there is no significant change in the other two groups. Skin blood flow was not influenced by electrical stimulation in three groups.

Conclusion: This improvement of the peripheral circulation by electrical stimulation would be beneficial as the adjunctive therapy after regenerative cell therapy.

Keywords

Critical limb ischemia, near-infrared spectroscopy, regenerative cell therapy, hemoglobin, electrical stimulation

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Introduction

The number of patients with peripheral arterial disease (PAD) is rapidly increasing, and the number of patients with chronic critical limb ischemia (CLI) classified as Fontaine stage III/IV that is resistant to current therapies is also increasing. TransAtlantic Inter-Society Consensus (TASC) II project[1] recommended the appropriate medications such as vasodilator, antiplatelet, or prostaglandins for the medical treatment, but the pharmacological effects were often limited in the CLI patients because of the severely decreased blood flow in the limbs. The long-term patency and limb salvage rate of bypass surgery or catheter intervention for CLI patients also has been not optimal.[2]

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Table 1. Baseline characteristics in healthy young subjects.

	1	2	3	4	5	6	7	8	9	10
Age (years)	21	21	21	25	24	24	21	20	22	22
Male/female	M	M	M	M	M	M	M	M	M	M
Height (cm)	175.2	168.2	170.5	170.3	164.8	170.4	171.4	177.5	173.5	171.2
Weight (kg)	58.0	55.6	65.5	55.8	54.9	57.6	67.0	61.6	65.3	65.1
Body mass index (%)	18.9	19.7	22.5	19.2	20.2	19.8	22.8	19.6	21.7	22.2

Table 2. Baseline characteristics in elderly subjects.

	1	2	3	4	5	6	7	8	9	10
Age (years)	71	72	72	69	65	60	72	68	62	69
Male/female	M	F	M	F	M	F	M	M	F	M
Height (cm)	165.0	150.5	165.0	153.0	174.0	157.0	154.0	157.0	148.0	160.0
Weight (kg)	70.0	45.0	67.0	43.0	63.0	48.0	55.0	62.0	42.0	70.0
Body mass index (%)	25.7	19.8	24.6	18.4	20.8	19.5	23.2	25.2	19.2	27.3
ABI	1.26/1.25	1.16/1.20	1.26/1.25	1.18/1.24	1.00/1.28	1.25/1.22	1.16/1.14	0.95/1.02	1.05/1.02	1.10/1.13
Medication	None	None	Anti-hypertensive lipid-lowering agent	Lipid-lowering agent	Anti-hypertensive lipid-lowering agent	None	None	None	None	Anti-hypertensive oral hypoglycemic agent

ABI: ankle-brachial pressure index.

Exercise therapy has been established as an effective treatment for PAD patients classified as Fontaine stage II, but it is not recommended for the CLI patients because of the risk of aggravation of ulcer. Exercise therapy in CLI patients after post-regenerative therapy might also be effective to increase the muscle blood flow. However, for individuals with CLI classified as Fontaine stage III/IV, severe pain[3] and the risk of ulcer aggravation[1] often make exercise therapy difficult.

Angiogenic cell therapy for CLI based on intramuscular injections of autologous bone marrow mononuclear cells (BM-MNC) has also been clinically established. In the previous report, BM-MNC transplantation therapy in 115 CLI patients had provided a significantly improved 3-year survival rate, limb amputation rate, and pain-free walking distance.[4]

On the other hand, transcutaneous electrical nerve stimulation (TENS) is one of the classical physical therapies which has been used for the pain relief.[5] There were several reports regarding the alterations of local circulation by TENS. Cramp et al.[6] reported low-frequency TENS (4 Hz); when applied at a "strong but comfortable" intensity to a peripheral nerve, it significantly increased the local cutaneous blood flow compared to a high-frequency electrical stimulation (ES) (110 Hz) application in the healthy subjects. ES might have the potential to be effective for improving peripheral circulation and tissue repair after regenerative therapy. However, there have been few studies of ES therapy for CLI patients after regenerative therapy. We hypothesized that an increase in CLI patients' muscle blood flow after regenerative angiogenic cell therapy could be achieved by applying low-frequency ES (i.e. 5 Hz) in this study.

Otherwise, in the last two decades, the concentrations of deoxyhemoglobin (HHb) and oxyhemoglobin (HbO_2) and total hemoglobin (Hb_{tot}) signals have been measured using near-infrared spectroscopy (NIRS).[7] NIRS was reported to reflect the change in microvascular blood flow into skeletal muscles.[7] Here, we used customized NIRS with two additional detector probes to assess the peripheral blood flow in dissociable skin and muscle of the lower limb. Our second hypothesis was that the application of low-frequency (5-Hz) ES could increase both cutaneous and muscle blood flow in the lower limb.

The purposes of this study were to test these two hypotheses and to determine the effects of low-frequency ES therapy on the blood flow in the lower limbs of CLI patients after regenerative therapy.

Objects and methods

Subject's characteristics

Three groups were studied: 10 healthy young subjects (age 22.1±1.6 years, weight 60.6±4.5 kg, height 171.3±3.4 cm, Table 1), 10 elderly subjects (68.0±4.3 years, weight 56.5±11.3 kg, height 158.4±7.9 cm, Hypertension 3, Diabetes 1, Dyslipidemia 3, Table 2), and 7 CLI patients after regenerative therapy (10 limbs of seven patients; five men and two women; 59.2±10.4 years, weight 59.1±16.0 kg, height 163.2±10.4 cm). The clinical details of the CLI patients are shown in Tables 3 and 4. Consecutive CLI patients who underwent regenerative

Table 3. Baseline characteristics of CLI patients.

	1	2	3	4	5	6	7
Age (years)	68	59	48	49	65	75	66
Male/female	F	M	F	M	M	M	M
Height (cm)	144.8	165.5	152.6	175.7	170	165.2	166.0
Weight (kg)	39.3	65.0	36.0	77.4	65.0	64.2	66.0
Body mass index (%)	18.7	23.9	15.5	25.1	22.5	25.0	24.0
Disease	ASO	Buerger	Systemic scleroderma	Buerger	ASO	ASO	ASO
Fontaine classification (right/left)	III(Lt)	III(Rt)	IV/IV	IV/IV	III/III	IV(Rt)	III(Rt)
ABI (right/left)	0.77(Lt)	0.65(Rt)	1.08/1.12	1.24/1.28	Unmeasurable	Unmeasurable	0.51(Rt)
TASC classification	Femoral popliteal lesions: Type D	Femoral popliteal lesions: Type D	Bilateral pedal artery occlusion	Bilateral femoral popliteal lesions: Type B	Bilateral femoral popliteal lesions: Type B	Femoral popliteal lesions: Type B	Femoral popliteal lesions: Type B
Previous vascular surgery	Lt FP bypass: occluded Lt P(AK) P(BK) bypass EVT (Lt ATA)	None	None	Bilateral regenerative cell therapy	EVT (Rt ATA, Lt CIA)	Unknown	EVT (Rt SFA, Rt ATA)

CLI: common iliac artery; ASO: arteriosclerosis obliterans; ABI: ankle-brachial pressure index; TASC: TransAtlantic Inter-Society Consensus; FP: femoro-popliteal; AK: above-knee; BK: below-knee; EVT: endovascular treatment; ATA: anterior tibial artery; SFA: superficial femoral artery; Lt: left; Rt: right.

therapy and participated in the cardiac rehabilitation programs since April–July 2012 were enrolled. (The patients in whom the electrodes could not be placed on the tibialis anterior (TA) muscle due to severe ulcer and pain were excluded.) Among the seven CLI patients, there were five limbs with atherosclerosis obliterans, three limbs with Buerger's disease, and two limbs with systemic scleroderma. In all CLI patients, severe main arterial ischemia below the knee was confirmed by angiography. The CLI patients had not only resting pain and ulcer for $\geqslant$3 months but also an average ankle-brachial pressure index (ABI) of 0.71 ± 0.49. Five limbs were classified as Fontaine stage III, and five limbs were classified as Fontaine stage IV.

Methods

At first, written informed consent was obtained from all participants after a detailed explanation about all procedures, the purpose of the study, and the possible risks and benefits of the participation. This study conformed to the Declaration of Helsinki, and the institutional ethical committee of Kyoto Prefectural University of Medicine and Doshisha University approved the purpose and all procedures of the study.

ES protocol

The ES therapy should be planned about 7 days after cell transplantation because tissue edema and acute inflammation were accompanied soon after the regenerative therapy. In the CLI patients, the ES therapy was performed on 8.8 ± 1.3 days after the regenerative cell therapy. The subject lay supine on a plinth during process. ES was applied by a portable electrical stimulator (TORIO300; ITO Physiotherapy & Rehabilitation, Tokyo) and with self-adhesive electrodes. One of the electrodes was placed on the motor point of the TA muscle, and the other electrode was placed on the distal point of the TA. After the subject rested in the supine position for 5 min, the ES was applied at 5-Hz frequency at 150-μs intervals for a 10-min duration. The intensity was set at 15–30 mA so that the subject experienced rhythmic and painless muscle contractions induced by the ES.[8] The generated pulse-forming wave was set as biphasic to avoid undesirable side effects (e.g. electrolysis).

Measurements and data analysis

The HHb and HbO_2 concentrations and the Hb_{tot} profiles in the TA were recorded using a customized continuous wave NIRS device (BOM-L1TR; OMEGAWAVE, INC., Tokyo). The system could continuously monitor the concentration changes in HbO_2, HHb, and Hb_{tot} at two different sites: skin and muscle as described below, which were calculated from the light attenuation changes using a modification of the Beer–Lambert law.[9] Pulsed light was emitted at 1-s interval from the emission probe with four different wavelengths (775, 810, 850, and 910 nm) and that was detected using a three-segment photodiode detection probe that received

Table 4. Medication in CLI patients.

1	2	3	4	5	6	7
Alprostadil alfadex	Alprostadil alfadex	Alprostadil alfadex	Alprostadil alfadex	Alprostadil alfadex	Sarpogrelate	Warfarin potassium
Clopidogrel	Cilostazol	Rosuvastatin	Ethyl icosapentate	Limaprost	Hydrochloride	Acetylsalicylic acid
Rosuvastatin	Acetylsalicylic acid	Beraprost	Pantethine	Clopidogrel	Cilostazol	Amlodipine besilate
Beraprost		Sodium ferrous citrate	Beraprost	Carbazochrome sodium	Pitavastatin	
Telmisartan		Elastase	Sarpogrelate	Sulfonate hydrate	Carvedilo	
Flecainide		Bosentan hydrate	Hydrochloride	Enalapril	Furosemide	
Sodium ferrous citrate		Etodolac	Morphine hydrochloride	Furosemide	Pregabalin	
		Pregabalin	Mirtazapine	Vildagliptin		
		Riboflavin	Methylcobalamin	Salazosulfapyridine		
		Tocopherol		Acetylsalicylic acid		
				Acetaminophen		
				Prednisolone		
				Methylcobalamin		
				Tocopherol		
				Precipitated calcium carbonate		
				Lanthanum carbonate		
				Alprazolam		
				Amezinium metilsulfate		
				d-Chlorpheniramine maleate		

NIRS signals at 1 Hz. The probes were housed in a black silicone holder. The distances between the emitter and receivers were 15 and 30 mm, respectively, and the penetration depth was approximately one-half of the distance between the emitter and the receivers, that is, 7.5 and 15 mm. The penetration depth at 7.5 mm was considered to reflect the states of the subcutaneous layer and the depth at 15 mm was considered to reflect the states of muscle layer. In this study, the 7.5-mm layer was defined as "skin" and the 15-mm layer was defined as "muscle." The TA muscle with the attached probe holder was then wrapped in a dark-colored, elastic bandage to further secure the probes and to eliminate ambient light that might contaminate the NIRS signal. This method has been validated in both healthy subjects and patients.[10]

The NIRS data reflected the relative concentration changes in the hemoglobin chromophores and therefore it did not reflect absolute tissue O_2 values. As $\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$ were measured as a change from the resting baseline values, the probe gain was set at 0 prior to testing with the subject at rest in the supine position. With the use of two detectors at the lower penetration depth of 7.5 mm, the "skin" $\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$ could be observed reflecting mostly cutaneous blood flow dynamics (i.e. $\Delta[HbO_2]_{skin}$, $\Delta[HHb]_{skin}$, and $\Delta[Hb_{tot}]_{skin}$). Otherwise, the data from the deep penetration depth of 15 mm, the "muscle" $\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$ could be observed. The $\Delta[HbO_2]_{muscle}$, $\Delta[HHb]_{muscle}$, and $\Delta[Hb_{tot}]_{muscle}$ were obtained by subtracting the $\Delta[HbO_2]_{skin}$, $\Delta[HHb]_{skin}$, and $\Delta[Hb_{tot}]_{skin}$ from the deeper ($\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$) according to the modified Beer–Lambert law. Simply, the tissue O_2 saturation (S_tO_2) was calculated as $[HbO_2]/[Hb_{tot}] \times 100\%$ in skin and muscle regions.

After the 10-min ES therapy, the residual effects of the ES derived from central and peripheral vascular responses were continuously observed for 10 min. The subjects' systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded using a beat-by-beat computer-based automated tonometer technique (JENTOW; NihonKorin, Tokyo) of the upper right arm rested at heart level alongside the body. The central hemodynamic values of stroke volume (SV) and cardiac output (CO) were continuously calculated by a pulse counter technique.[11] Total peripheral resistance (TPR) and mean arterial pressure (MAP) were automatically calculated with the formula TPR = MAP/CO and MAP = DBP + 1/3 (SBP − DBP).

The beat-by-beat heart rate (HR) was continuously monitored by electrocardiography (ECG) using transistor–transistor logic signal intervals synchronized with the R-wave of the ECG by CM5 leads. All data were adopted using a built-in A/D converter (A&D Instruments, Tokyo) with an interval resolution of 5 ms (i.e. the sampling interval

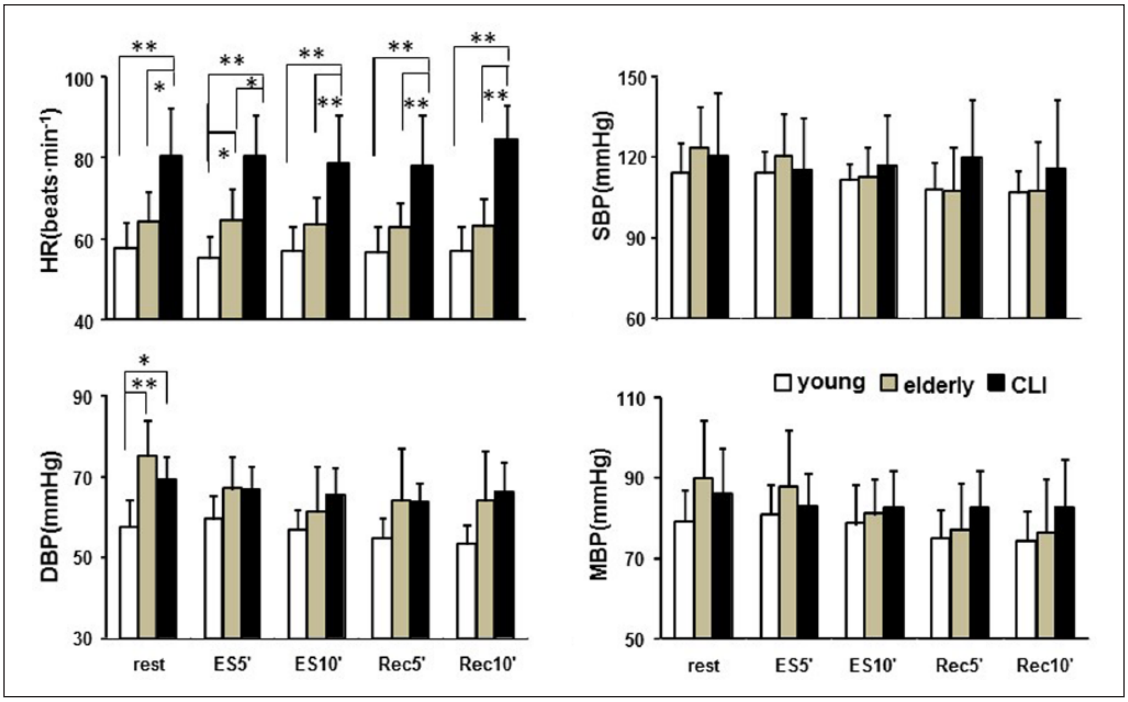

Figure 1. Mean data of HR, SBP, DBP, and MBP at each period among young, elderly, and CLI groups. A significant increase in HR was shown in CLI group while blood pressure responses were not different among three groups.
*$p < 0.05$, **$p < 0.01$.

at 200 Hz). A customized program was used to identify a stable and noise-independent fiducial point on all R-waves for each recording. The data of the cardiovascular indexes and NIRS were calculated at the periods of rest, ES at 5 min, and ES at 10 min (ES5′, ES10′), and recovery at 5 min and 10 min (Rec5′ Rec10′), averaging the values for 30 s at each period. Most of the seven CLI patients had an ulcer and/or arterial obstruction in the upper arms and thus their blood pressure could not be measured using the automated tonometer device.

Statistical analysis

Regarding the sample size determination in the "Methods" section, even though we selected that the sample size (n = 7) for the CLI patients was smaller than other groups (each n = 10), the subjected legs using ES for CLI patients were 10 limbs. Thus, the sample size among three groups was same as 10 samples, total of 30 samples. First, we are planning a study of a continuous response variable from independent control (healthy elderly) and experimental (CLI) subjects. In a previous study,[12] HbO_2 response using NIRS within each subject group was normally distributed with standard deviation (SD) of 0.27. If the true difference in the experimental and control means is 0.34, we will need to study 11 experimental subjects and 11 control subjects to be able to reject the null hypothesis so that the population means of the experimental and control groups are equal with probability (power) 0.8. The type I error probability associated with this test of this null hypothesis is 0.05. This argument justifies the number of participants and statistical power used in this study.

All values are presented as means ± SD. The effect of ES in each group was also analyzed by a one-way analysis of variance (ANOVA), and a post hoc Tukey–Kramer's test was used to compare the data obtained at baseline, ES, and the recovery periods of rest. Comparisons of all cardiovascular and NIRS data among the three groups in each interval were analyzed by one-way ANOVA. A post hoc Scheffe's F-test was used to compare the young, elderly, and CLI groups. For the comparison between the young and elderly groups, the unpaired t-test was used. All analyses were performed using SPSS software (Abacus Concepts, USA) with significance in all cases set at the 5% level.

Results

Central circulation during ES and recovery

There were no adverse events in any of the subjects throughout the experiments. The mean HR value was the greatest in the CLI group and showed significant differences compared to the young and elderly groups throughout the entire process ($p < 0.05$, $p < 0.01$, Figure 1). However, the HR remained constant in each group. In addition, the SBP values did not show any significant changes in each group (Figure 1). The mean blood pressure (MBP) in the CLI group tended to decline, from 86.4 ± 11.2 to 82.8 ± 12.1 mmHg. The DBP in the elderly subjects was significantly high compared to the

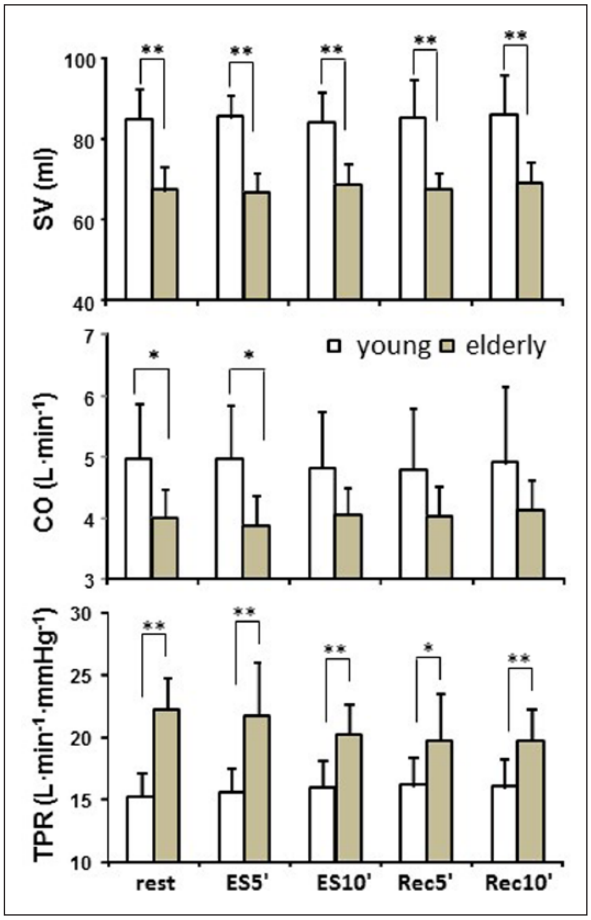

Figure 2. Mean data of TPR, SV, and Qc at each period among young and elderly groups. Significant decrease in SV and increase in TPR were shown in elderly group compared with young group. *$p < 0.05$, **$p < 0.01$.

young ($p < 0.01$) and CLI ($p < 0.05$) groups at only the rest condition.

In the comparison of the young and elderly groups, a significantly declined SV was observed at each period ($p < 0.01$, Figure 2), and an interaction between greater HR and lower SV was observed. The resultant CO was significantly lower in the elderly group than in the young group ($p < 0.01$ at rest and ES5′), but the CO values remained constant throughout the experiment in each groups (Figure 2). There was a significant difference in TPR values between the young and elderly groups, and the TPR in the elderly group gradually decreased during the recovery period following ES.

Alterations of blood flow of skin and muscle measured by NIRS

In the subcutaneous layer, the $\Delta[HbO_2]_{skin}$ and the $\Delta[Hb_{tot}]_{skin}$ tended to increase during the recovery period in the CLI patients (Figure 3), but no significant difference was observed among the three groups. The $\Delta[HbO_2]_{muscle}$, $\Delta[HHb]_{muscle}$, and $\Delta[Hb_{tot}]_{muscle}$ values in TA were gradually increased after ES5′ and remained at significantly greater levels during the recovery period in the CLI patients.

In contrast, in the elderly group, both the skin and muscle $\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$ showed less changes during experiment, indicating that the skin and muscle blood flow were not influenced by the ES. In the young group, the skin blood flow tended to increase at early ES5′, but it was not significantly different from the baseline. The muscle blood flow did not also significantly change throughout the experiment as the same pattern in the elderly group.

As shown in Figure 4, regarding the S_tO_2 at the cutaneous portion ($S_tO_{2\ skin}$), there was a common trend in which the $S_tO_{2\ skin}$ in all three groups remained relatively constant varying with narrow-ranging throughout the experiment. Although the $S_tO_{2\ skin}$ in the CLI group was not significantly different from those in the young and elderly groups, it was relatively lower at approximately 56%–57%. Relatively lower S_tO_2 at muscle portion ($S_tO_{2\ muscle}$) was also observed compared to the $S_tO_{2\ skin}$ in each group, but the values of $S_tO_{2\ muscle}$ remained constant at approximately 53% in the CLI patients throughout the experiment.

Discussion

The main result of this study was that low-frequency ES on the TA in CLI patients after regenerative therapy markedly increased muscle blood flow (i.e. the alterations in $\Delta[HbO_2]_{muscle}$ and $\Delta[Hb_{tot}]_{muscle}$) assessed by NIRS, without influences on the systemic circulation (i.e. HR and MBP). The effect was not apparent in the healthy young and elderly subjects. The reason for the differences among three groups was considered as the markedly decreased blood flow in the skin or muscle due to the arterial branch occlusion in the CLI patients, in contrast to the normal blood flow in the other two groups.

There were several reports regarding the improvement of local circulation by TENS, but the results brought about by the effects of TENS were equivocal because of the variation in methodology, electrode placement, position of blood flow probe, treatment time, and so on.[6] Cramp et al.[6] reported that low-frequency TENS (4 Hz) significantly increased the local cutaneous blood flow in the healthy subjects, and regarding to the intensity of TENS, the effect of TENS on cutaneous blood flow depended on whether muscle activity was induced in the healthy subjects. They concluded that low-frequency TENS applied above the motor threshold significantly increased local cutaneous blood flow, but our results showed no cutaneous blood flow increase in healthy subjects. This might be because the intensity or duration of ES on our protocol, which was effective for the CLI patients, was insufficient for them.

Potential angiogenesis after autologous bone marrow cell therapy in CLI patients

The injection of BM-MNCs significantly improved CLI patients' pain-free walking time, rest pain, and tissue oxygen

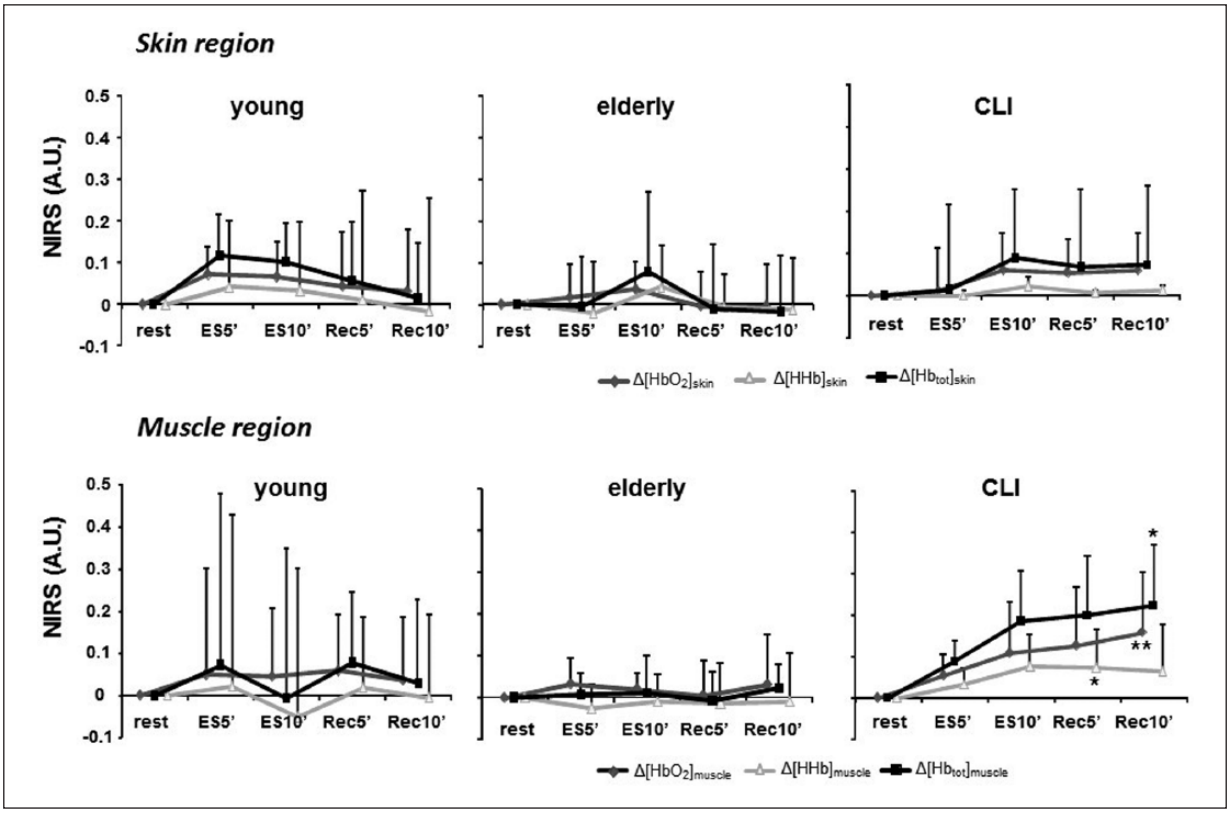

Figure 3. The change in $\Delta[HbO_2]$, $\Delta[HHb]$, and $\Delta[Hb_{tot}]$ at skin and muscle regions at each period among young, elderly, and CLI groups. The $\Delta[HbO_2]_{muscle}$, $\Delta[HHb]_{muscle}$, and $\Delta[Hb_{tot}]_{muscle}$ values in TA were gradually increased after ES5′ and remained at significantly greater levels during the recovery period in the CLI patients.
*$p < 0.05$, **$p < 0.01$ versus baseline.

pressure 6 months after treatment.[13] Apparently, the incorporation of endothelial progenitor cells (EPCs) in newly formed vessels as well as angiogenesis/arteriogenesis by angiogenic factors released from injected cells contribute to the increase in blood flow in the limbs of CLI patients.

The timing of ES measurement in CLI patients was 8.8 ± 1.3 days after regenerative cell therapy, and it might be the appropriate period in which to promote vascular angiogenesis. Tateishi-Yuyama et al.[13] reported that a laser Doppler image analysis performed 7–14 days after implantation revealed a striking improvement of blood perfusion in BM-MNC-injected limbs compared with the contralateral saline-injected limbs. Therefore, the timing that we performed ES therapy in the CLI patients might be supportive for the promotion of angiogenesis.

ES in CLI patients

TENS was successfully employed for the treatment of Raynaud's phenomenon, diabetic neuropathy, and chronic skin ulceration due to circulatory disorder of the feet[14] and leprous ulcers.[15] Anderson et al.[16] reported that repeated muscle ES for 4 weeks alleviated intermittent claudication, but there were little clinical experiences of ES therapy for CLI.

Several mechanisms of ES had been inferred such as muscle pumping, sympathetic inhibition, and vasodilator substances. First, effect of muscle pumping brought about ES applied above the motor threshold. Several previous papers[17–20] reported that stimulated muscle contraction was accompanied with increase in arterial flow and venous return in healthy subjects or animal models. Second, sympathetic inhibition resulted in the sympathetic reflex by afferent type III and IV fibers were demonstrated in the animal models.[17,18] Third, Kaada and colleagues[14,21,22] established that the release of vasodilator substances such as cholinergic, histaminergic, and dopaminergic and of vasoactive intestinal polypeptides, prostaglandins, and plasma kinins dilated skin vessels during ES. These mechanisms might also play a role in our study. Furthermore, studies are necessary for the determination of appropriate setting and protocol of ES therapy. The following parameters for setting include targets of the future studies: current amplitude, frequency, current type, pulse duration, position of electrodes, and duration of therapy.

Kaada and colleagues[21,22] reported the skin vasodilation with ES which applied 45 min/day and continued for 1 or 2 weeks for the patients with peripheral ischemia. In a previous investigation, the intensity of transcutaneous nerve stimulation (TNS) applied over the median nerve above the

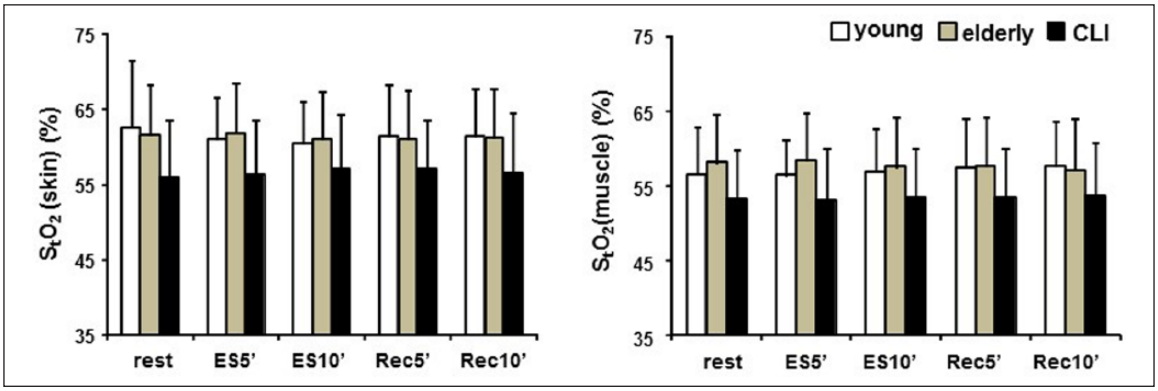

Figure 4. Tissue O_2 saturation (StO_2) in skin and muscle regions at each period among young, elderly, and CLI groups. Both $S_tO_{2\ skin}$ and $S_tO_{2\ muscle}$ of each group were not altered throughout the process.

motor threshold increased the cutaneous blood flow at the forearm, by contract the intensity of TNS below the motor threshold had no significant effect on fingertip or forearm cutaneous blood flows.[6] This finding suggests that an intensity above the motor threshold would be required for TNS to produce an increase in cutaneous blood flow.

Skin blood flow with ES

Skin blood flow in CLI patients is very important because the decrease in blood flow is one of the aggravating factors of skin ulcer. Blood flow in the microcirculation is composed of the skin nutrition capillaries and thermoregulatory arteriovenous (AV) shunts, and the former proportional contribution is 25% in the glabrous skin.[8] AV shunts in glabrous skin cause a more sustained vasoconstrictor tone, which may increase the risk for the ulcer of the PAD patients.[8] Microcirculatory dysfunction in patients with PAD had been already shown by Matsen et al.[23] Midttun et al.[24] showed the microcirculatory dysfunction in the claudicants due to the impaired AV shunt function. Bongard and Fragrell[25] evaluated the microcirculation of patients with PAD, and nutrition capillary blood cell velocity was similar to that of control, but total blood flow was increased in patients with PAD. They concluded that the microcirculation was impaired in patients with PAD, with increased but maldistributed blood flow. Although we did not directly evaluate the skin blood flow of the toe or the foot sole in this study, sympathetic inhibition brought about by ES might result in the increase in the skin blood flow in the toe of CLI patients.

Skin and muscle tissue O_2 saturation

We also observed that the S_tO_2 which was calculated as $[HbO_2]/[Hb_{tot}] \times 100\%$ in skin and muscle regions of TA remained constant even during ES and recovery. The mean value of $S_tO_{2\ skin}$ at 56%–57% (Figure 4) is higher than that of $S_tO_{2muscle}$ at 53% in the CLI patients in our study. Lower $StO_{2\ skin}$ at the toe or the foot sole might reflect the risk of the pressure ulcer. Mawson et al.[26] reported that spinal cord injury patients have significantly lower transcutaneous oxygen tension ($P_{tc}O_2$) levels at the sacrum compared to able-bodied controls, especially when lying supine. It suggested that lower $P_{tc}O_2$ may be a useful indicator of susceptibility to pressure ulcer. They also reported that after 30 min of ES, $P_{tc}O_2$ increased from 49 to 66 mmHg ($p < 0.001$) at the sacral region via the restoration of sympathetic tone. Debreceni et al.[27] observed that long-term 20-min 1-Hz ES on the lower limbs improved both the saturation of O_2 (from 73.5% to 95.5% at the toes) and the pain-free walking distance (87.5–421.25 m, $p < 0.01$) in patients with lower-limb ischemic wounds. Peters et al.[28] also demonstrated that 5-min external subsensory ES resulted in transient increase in $P_{tc}O_2$ subsequently to increasing local perfusion in diabetic subjects. In our study, we did not directly measure the $StO_{2\ skin}$ in the toe or the foot sole, but the improvement of that might be obtained after ES therapy in the CLI patients.

Study limitation

We only evaluated the acute effects of ES and its long-term effect was unclear. The skin blood flow was not measured in the predilection sites of ulcer such as toe or foot sole but measured in TA. The optimal protocol of ES for the CLI patients after regenerative therapy should also be examined. Future clinical examinations about an effect of lower-frequency ES therapy on the ulcer healing or the improvement of claudication are needed.

Conclusion

After lower-frequency ES therapy administered to CLI patients after they underwent regenerative cell therapy, we observed significant increase in $\Delta[HbO_2]_{muscle}$ and $\Delta[Hb_{tot}]_{muscle}$ in implanted muscle. In addition, the $S_tO_{2\ muscle}$ remained constant during the ES and then maintained at 56%–57% even during recovery. Therefore, ES therapy may be an effective treatment for improving muscle blood flow which does not cause significant muscle oxygen desaturation. These findings seemed to provide that ES therapy can

be clinically useful to increase the peripheral blood flow and might promote angiogenesis in the CLI patients who have undergone regenerative therapy.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

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Informed consent

Written informed consent was obtained from all subjects before the study.

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