〈Research Article〉

Effect of exercise on angiogenesis and oxidative stress of lower limbs ischemia in SMP30/GNL knockout mice

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Summary In this study, the authors examined the influence of different exercise periods on vascularization factors using model mice with lower limb ischemia. The subjects for the study were 98 mice with a deficiency in vitamin C synthesis. They received surgical procedure at 12 weeks of age and were randomly divided into 13 groups according to the presence of exercise and vitamin C intake and the difference in exercise periods. They were sacrificed at 13 weeks, 15 weeks, and 18 weeks of age (1 week, 3 weeks, and 6 weeks of exercise periods, respectively). The vascularization factors and the oxidative stress were measured in the plasma. The results revealed that mice without vitamin C intake and 1 week and 3 weeks of exercise had significantly higher potential antioxidative activity than that in mice with no exercise ($p < 0.01$). Mice without vitamin C intake and 3 weeks of exercise had significantly higher vascular endothelial growth factors (VEGF) than in mice of 1 week and 6 weeks of exercise ($p < 0.01$ for both), and mice with vitamin C intake and 1 week of exercise had significantly higher VEGF than those with 6 weeks of exercise ($p < 0.05$). Moreover, mice without vitamin C intake and 1 week, 3 weeks, and 6 weeks of exercise had significantly high levels of endostatin. In mice with exercise without vitamin C, the potential antioxidative activity was high because of the compensatory effects of vitamin C, and the gene expression for the vascularization-inhibiting factors exceeded that for the promoting factors; therefore, the equilibrium of the vascularization-promoting and vascularization-inhibiting factors has been determined using the V/E ratios. This study indicates that exercise has an influence on potential antioxidative activity and vascularization factors.

Key words: Exercise, Angiogenic factors, Oxidative stress, Lower limbs ischemia

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1. Introduction

In peripheral arterial disease (PAD), motor abilities decline because of abnormal skeletal muscles or a decline in blood flow due to arterial occlusion. Moreover, exercise is recommended for PAD patients, and their maximum walking distance is improved with exercise. The effects of exercise involve factors such as energy production by mitochondria, collateral circulation, vascular endothelium function, vascularization factors, and inflammatory reactions[1,2]. It has been reported that vascularization effects are inherent in a living body in response to ischemia, and vascular endothelial growth factors (VEGF) or fibroblast growth factors (FGF), which play a central role in vascular formation, are increased by exercise[3]. In a study with an ischemic lower limb model mouse, which had similar physiological clinical conditions to PAD, hypoxia inducible factors (HIF-1α) were activated by exercise, which then influenced the expression of VEGF[4]. However, one report supports the role of vascularization factors while another denies its role in the effects of exercise[4,5].

Capillary plexus in tissues needs to be controlled by a balance between the promoting factors and inhibiting factors for vascularization to occur. Therefore, the effects of exercise on PAD need to be studied by examining the balance between vascularization-promoting factors and vascularization-inhibiting factors. PAD is a disease with a poor prognosis, and risk reduction for arteriosclerosis is recommended. In particular, oxidative stress, which is important in the progression of arteriosclerosis, is a common mechanism that causes arteriosclerosis and artery lesions[6]. It has been suggested that oxidative stress may influence the DNA of cells or the protein on a cell membrane and be involved in atherosclerosis. It has also been reported that oxidative stress in PAD patients is reduced by intravascular treatment[6]. Moreover, oxidative stress increases with exercise and is affected by components of exercise, such as exercise periods, even though there are no definitive conclusions about this[7]. Therefore, it is necessary to examine the relationship between oxidative stress and vascularization factors to clarify the effects of exercise on PAD. By contrast, various substances such as vitamin C or reduced coenzyme Q10 (H_2CoQ_{10}: QH) are antioxidants, which defend a living body against oxidative stress. Commonly, vitamin C and QH are in food that is sensitive to oxidative stress as a radical capture type antioxidant[8]. Vitamin C is a strong water-soluble antioxidant, which effectively removes reactive oxygen species in cells and blood, improves antioxidative activity, and reduces oxidative stress[9]. Moreover, it has been reported that vitamin C acts on VEGF and HIF-1α and influences reperfusion of microcirculation[10-12]. Since wild-type mice were able to synthesize vitamin C, unlike humans, it was presumed that the antioxidative activity obtained by exercise could be evaluated using mice with a vitamin C synthesis deficiency. Therefore, in this study, the authors created an ischemic lower limb model using mice with a vitamin C synthesis deficiency and examined the influence of different exercise periods and vitamin C intake on vascularization factors and oxidative stress over time.

2. Materials and Methods

Animals

The animals for the study were 98 SMP30/GNL knockout mice (10-week-old males) that were sorted by the presence of exercise and vitamin C intake and then randomly divided into 13 groups by different exercise periods (1 week, 3 weeks, and 6 weeks) (Table 1). Groups A–G included mice without vitamin C, and Groups H–M included mice with vitamin C. Moreover, Groups B–M were those that underwent surgical procedure in the form of a right thigh artery ligation.

Protocol

All of the groups of mice received surgical procedure at 12 weeks age and were killed at 13, 15, and 18 weeks of age (1 week, 3 weeks, and 6 weeks of exercise periods, respectively). They were killed 5 hours after exercise in accordance with a precedent

Table1 The randomly divided into 13 groups in SMP30/GNL knockout mice

	surgical procedure	periods	exercise	vitamin C
Group A(sham)	none	1week	Without	Without
Group B	surgical procedure	1week	presence of exercise	Without
Group C	surgical procedure	1week	Without	Without
Group D	surgical procedure	3 weeks	presence of exercise	Without
Group E	surgical procedure	3 weeks	Without	Without
Group F	surgical procedure	6 weeks	presence of exercise	Without
Group G	surgical procedure	6 weeks	Without	Without
Group H	surgical procedure	1week	presence of exercise	vitamin C intake
Group I	surgical procedure	1week	Without	vitamin C intake
Group J	surgical procedure	3 weeks	presence of exercise	vitamin C intake
Group K	surgical procedure	3 weeks	Without	vitamin C intake
Group L	surgical procedure	6 weeks	presence of exercise	vitamin C intake
Group M	surgical procedure	6 weeks	Without	vitamin C intake

study[13], and the right gastrocnemius and whole blood was collected. The RNA of the collected gastrocnemius was stabilized with RNA later stabilization solution (Thermo Fisher Scientific, Japan) and kept at -20 °C until the analysis. Additionally, blood samples were collected (approximately 100 μL) and promptly centrifuged to perform measurements such as a d-ROM test, and plasma was collected and refrigerated. Exercise started 2 days after mice received surgical procedure. The frequency, time and intensity of the exercise was determined by the precedent study[13], and, therefore, moderate exercise was selected (frequency: 5 times/week, time: started with 15 min/day with a gradual increases of 3 min/day to reach a maximum of 60 min/day, intensity: 18–19m/min, gradient: 5%)[13]. Based on the precedent study[14], 100% of the vitamin C indicated that the vitamin C mice required per day (vitamin C content: 1.5 g/L) was administered with drinking water, and 0% of the vitamin C that vitamin C mice without vitamin C required per day was taken with drinking water. All mice were bred in an environment with a light and dark cycle, at a room temperature of 20 ± 1 °C and relative humidity of approximately 50%, 12 hours (7:00/19:00, light/dark cycle). They were freely fed chow (CL -2, Nippon Kurea) that did not contain vitamin C (intake started at 12 weeks of age), and their behaviors were not restricted. The subject mice for the present study were senescence-accelerating model mice, which were extremely similar to humans who could not synthesize vitamin C[15]. In the study where mice were fed with chow that did not contain vitamin C, typical scurvy symptoms such as fractures were seen along with weight loss, and mice died by 136 days[16]. The surgical procedure involved a transverse incision on the right groin, the right common femoral artery was exposed and exfoliated under general anesthesia by pentobarbital natrium, and two spots were ligated using a silk thread.

Real-time polymerase chain reaction (PCR)

The total RNA by the phenol chloroform extraction method based on the protocol of RNeasy fibrous tissue mini kit (QIAGEN, GER) was used for the mRNA analysis. The cDNA synthesis was performed based on a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Japan). For real-time PCR, the Taqman probe technique was applied using a PCR analysis system (Chromo 4 made of BIO-RAD, USA) for 40 cycles. The following target genes were used based on the precedent study: vascularization-promoting factors: 6 factors, vascularization-inhibiting factors: 2 factors, and GAPDH as endogenous standard genes[2,5] (Table 2). The vascularization-promoting factors that were

Table 2 Primer sequences used for quantitative real-time PCR

Mouse Sequence(ABI TaqMan Gene expression assays)	
Gene name	TaqMan probe set ID
VEGF(VEGF-A)	Mm01281449_m1
FGF(FGF2)	Mm00433287_m1
HIF-1α	Mm00468875_m1
PGC-1α	Mm00447183_m1
IL-6	Mm00446190_m1
TSP-1	Mm00439498_m1
endostatin	Mm00487129_m1
Kdr(VEGFR2)	Mm00440099_m1
GAPDH	Mm99999915_g1

VEGF: Vascular endothelial growth factor, FGF: Fibroblast growth factors, HIF-1α: Hypoxia Inducible Factor, PGC-1α: PPAR gamma coactivator 1-alpha, IL-6: Interleukin-6, TSP-1: Thrombospondin-1, Kdr: Vascular Endothelial Growth Factor Receptor, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

analyzed were VEGF (VEGF-A), FGF, HIF-1α, PGC-1α (PPAR gamma coactivator 1-alpha), Interleukin-6 (IL-6), Kdr (vascular endothelial growth factor receptor), which are related to the VEGF signal, and Thrombospondin-1 (TSP-1) and endostatin, which suppress vascularization in conjunction with exercise. The relative levels of the obtained Ct (cycle threshold) values were calculated using the comparison Ct method (the ΔΔCt method) and compared with ΔΔCt of Group A as a proportion value (1.0 times). The Ct was the intersection between the amplification curve and threshold line[17]. In addition, ratios of endostatin as the inhibiting factor that competes with VEGF as a vascularization-promoting factor were calculated (VEGF/endostatin: V/E ratio) to examine the balance between promoting factors and inhibiting factors. No reference value for the V/E ratio has been reported[18].

Oxidative stress protection system

The degrees of oxidative stress (reactive oxygen metabolites test: d-ROM test) and antioxidation activity (biological antioxidant potential: BAP test) were measured with a free radical autoanalyzer (FREE made of H&D), and the potential antioxidative activity of the plasma (BAP test level / d-ROM test level: BAP / d-ROM ratio) was also calculated.

In the d-ROM test, the free radical levels in the living body, particularly the hydroperoxide concentrations, were measured using an optical measurement method (color reaction) (Unit: U.CARR, 1 U.CARR = hydrogen peroxide 0.08 mg/dL)[19]. The levels obtained in the d-ROM test indicate degrees of oxidative stress (oxidase reaction). The BAP test measures the reduction effects of the plasma antioxidant (unit: μmol/L). The plasma was mixed with reagent-containing ferric ions, and the quantity of the reduction to ferrous ions was measured using an optical measurement method. The levels that were obtained in the BAP test are the quantity of ferrous ions that are reduced by plasma and indicate the levels of antioxidative activity[19]. Furthermore, the BAP / d-ROM ratios were calculated from the levels of the BAP test and the d-ROM test. The ratios indicate the potential antioxidative activity, which is the balance of the degrees of oxidative stress and the antioxidation activity. As for plasma vitamin C concentration, the reduction type (ascorbic acid) and oxidation type (dehydroascorbic acid) were measured by HPLC, and the total vitamin C (ascorbic acid + dehydroascorbic acid) concentration was calculated.

Surface skin temperature and weight

The surface skin temperatures were measured

before the surgical procedure (Pre), after the surgical procedure (Post) and at the time mice were sacrificed with thermography (E60 made by an FLIR company), and the ratio between the right foot skin temperature and the left foot skin temperature was calculated. Further, for acute ischemic findings, normal results (no findings), discoloration and toe necrosis were confirmed macroscopically.

Statistical analysis

SPSS (Ver 21.0 for Windows) was used for statistical processing, and significant differences were determined using ANOVA test for the comparison between groups and Tukey's test.

Animal ethics

Prior to its initiation, the study was approved by the study promotion committee for animal experiments in the authors' organization (approval No. 26-2, Saitama Prefectural University).

3. Results

Change in surface skin temperature

A comparison of the surface skin temperatures during the Post and Pre periods of measurement showed no change in Group A, whereas a significant decrease was seen in Groups B-M, for which surgical procedure was performed ($p < 0.05$ for both). However, discoloration and toe necrosis were not macroscopically observed (Table 3). Further, a change in surface skin temperature was seen until the time mice were killed. In the groups for which surgical procedure was performed, the surface skin temperature of the ischemic lower limb continued decreasing until the time mice were killed.

Comparison of oxidative stress protection systems against oxidative stress

The plasma vitamin C concentration in Groups A–G was <11.4 μmol/L and >11.4 μmol/L in Groups H–M (Tables 4 and 5). Groups A–G did not present typical scorbutic symptoms such as fractures accompanied with weight loss. The plasma concentrations of vitamin C were not influenced by exercise, and 1 week, 3 weeks, and 6 weeks mice exhibited vitamin C deficiency in mice without vitamin C[20]. The BAP/d-ROM ratios were significantly higher in Groups B

Table 3 Change in the surface skin temperature

	Surface skin temperature ,ratios			n
	Pre	Post**	killed**	
A	99.4 ± 1.2	100.3 ± 1.6	100.6 ± 1.0	8
B†	99.4 ± 1.0	96.3 ± 1.4	97.5 ± 1.7	6
C†	99.3 ± 0.9	96.1 ± 1.8	96.1 ± 2.8	9
D†	99.5 ± 1.0	97.5 ± 0.7	97.5 ± 0.6	6
E†	99.5 ± 0.8	95.8 ± 2.1	95.4 ± 2.4	8
F†	100.0 ± 1.0	96.6 ± 1.7	95.7 ± 1.6	9
G†	100.4 ± 0.8	93.9 ± 2.7	96.4 ± 2.2	8
H†	100.4 ± 1.3	97.4 ± 1.1	96.7 ± 1.1	7
I†	100.0 ± 1.1	96.3 ± 2.3	96.0 ± 2.6	7
J†	99.7 ± 1.3	97.2 ± 1.1	96.4 ± 1.3	7
K†	99.3 ± 1.1	94.2 ± 2.9	95.3 ± 2.6	7
L†	100.1 ± 1.0	95.5 ± 2.5	96.3 ± 1.9	8
M†	99.8 ± 1.8	95.0 ± 1.6	96.0 ± 3.3	8

A comparison of the surface skin temperatures. Values are mean±SD. p Value by ANOVA test for the comparison between groups and Tukey's test for the Pre vs Post or slaughter.†p＜0.05, **p＜0.01

Table 4 Change in the plasma vitamin C values and oxidative stress regulation system (without vitamin C)

	Total vitamin C** ,μ mol/L	d-ROMs test** ,U.CARR	BAP test** ,μmol/L	BAP/d-ROMs** ,ratio
A	2.6 ± 0.5[e]	109.6 ± 11.8	2290.5 ± 238.5	21.2 ± 3.7[c]
B	3.0 ± 0.5[e]	96.0 ± 11.2[a]	2679.8 ± 307.2[b]	28.0 ± 2.0[aa, bb, ee]
C	6.1 ± 3.3[d]	120.8 ± 20.5	2187.5 ± 288.4	18.5 ± 3.8[dd]
D	2.9 ± 0.4	96.0 ± 8.3[a]	2526.5 ± 246.7	26.3 ± 1.8[aa]
E	2.7 ± 0.1	124.0 ± 16.8	2114.1 ± 202.1	17.3 ± 2.5[c, dd]
F	2.7 ± 0.4[e]	105.8 ± 11.9	2458.6 ± 217.9	23.6 ± 4.6
G	3.2 ± 0.5	124.8 ± 20.4	2206.8 ± 291.2	18.0 ± 3.4[c, d]

Levels are mean±SD. p Value by ANOVA test for the comparison between groups and Tukey's test for the Total vitamin C levels vs d-ROMs test vs BAP test or d-ROM/BAP ratio.

**p < 0.01, a:p < 0.05 comparison G, aa:p < 0.01 comparison G, b:p < 0.05 comparison E, bb:p < 0.01 comparison E, c:p < 0.05 comparison B, d:p < 0.05 comparison D, dd:p < 0.01 comparison D, e:p < 0.05 comparison C, ee:p < 0.01 comparison C.

Table 5 Change in the plasma vitamin C values and oxidative stress regulation system (vitamin C intake)

	Total vitamin C** ,μmol/L	d-ROMs test** ,U.CARR	BAP test ,μmol/L	BAP/d-ROMs* ,ratio
A	2.6 ± 0.5	109.6 ± 11.8	2290.5 ± 238.5	21.2 ± 3.7
H	19.4 ± 7.1	100.0 ± 6.7[ff]	2554.2 ± 149.2	25.7 ± 2.8[ff]
I	44.4 ± 2.9[gg, h]	97.8 ± 2.1[ff]	2301.9 ± 258.8	23.6 ± 2.9
J	32.6 ± 6.4[g]	101.3 ± 9.8[ff]	2376.8 ± 246.8	23.5 ± 1.8
K	46.5 ± 11.5[gg, h]	107.8 ± 10.8	2355.2 ± 323.5	22.2 ± 4.7
L	37.3 ± 14.3[gg]	111.2 ± 22.6	2443.8 ± 306.1	22.9 ± 5.8
M	41.5 ± 8.6[gg]	130.6 ± 6.4	2235.6 ± 133.5	17.1 ± 0.8

Levels are mean±SD. p Value by ANOVA test for the comparison between groups and Tukey's test for the Total vitamin C values vs d-ROMs test vs BAP test or d-ROM/BAP ratio.

**p < 0.01, *p < 0.05, ff:p < 0.01 comparison M, gg:p < 0.01 comparison A, h:p < 0.05 comparison H.

and D than in Groups C and E ($p < 0.01$ for both), and no difference was seen for those with vitamin C (Tables 3 and 4). The d-ROM and BAP test levels for mice without vitamin C were lower and higher in Groups B and D than that in Groups C and E, respectively. The potential antioxidative activity was higher in mice with exercise of 1week and 3 weeks for mice without vitamin C than in mice without exercise, whereas there was no difference in the mice with vitamin C.

Comparison of vascularization factors

In the mice without vitamin C, gene expression of VEGF was significantly higher in Group D (3.5 times) than in Groups B (0.5 times) and F (0.7 times) ($p < 0.01$ for both), whereas no significant difference was seen in HIF-1α and FGF (Fig. 1). Further, the endostatin level was high in Groups B (4.3 times), D (6.1 times) and F (3.5 times). In the mice with vitamin C, the gene expression of FGF was significantly lower in Group L (0.2 times) than in Groups H (1.4 times) and J (0.8 times) ($p < 0.01$ for both). Likewise, gene expression of HIF-1α was significantly lower in Group L (0.2 times) than in Groups H (1.1 times) and J (0.5 times) ($p < 0.01$, $p < 0.05$). Further, gene expression of VEGF was significantly higher in Group H (1.3 times) than in Group L (0.5 times) ($p < 0.05$) (Fig. 2). The VEGF for the mice without vitamin C was significantly higher in the mice with 3 weeks of exercise than in the mice with

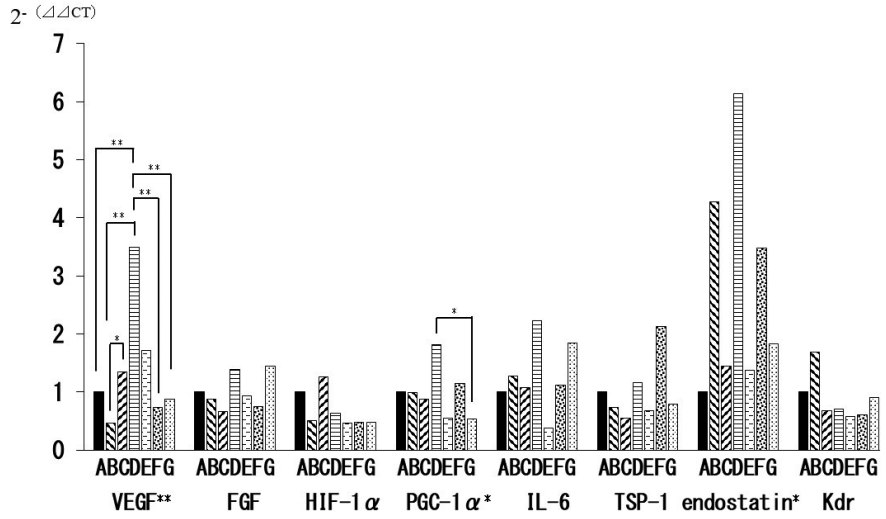

Fig. 1 Effects of lower limbs ischemia on mRNA expression leveis of angiogenic factors enzymes. mRNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. p Value by ANOVA test for the comparison between groups and Tukey's test. VEGF: Vascular endothelial growth factor, FGF: Fibroblast growth factors, HIF-1α: Hypoxia Inducible Factor, PGC-1α: PPAR gamma coactivator 1-alpha, IL-6: Interleukin-6, TSP-1: Thrombospondin-1, Kdr: Vascular Endothelial Growth Factor Receptor, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. **indicate significant differences at levels of p <0.01, *significant differences at levels of p <0.05.

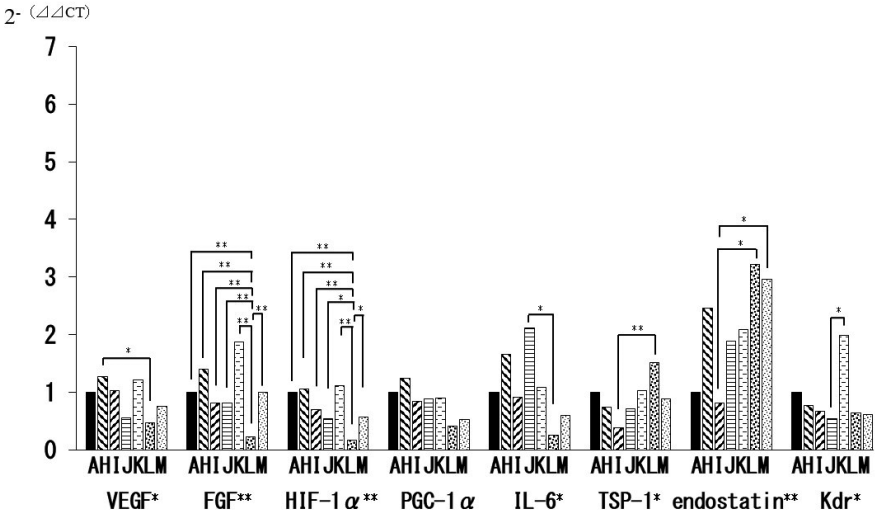

Fig. 2 Effects of lower limbs ischemia on mRNA expression leveis of angiogenic factors enzymes. mRNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. p Value by ANOVA test for the comparison between groups and Tukey's test. Reference Fig.1. **significant differences at levels of p <0.01, *significant differences at levels of p <0.05.

1week and 6 weeks of exercise, whereas the VEGF for the mice with vitamin C was significantly higher in the mice with 1week of exercise than in mice with 6 weeks of exercise. Further, FGF and HIF-1α for mice with vitamin C was significantly lower in mice with 6 weeks of exercise than in mice with 1week and 3 weeks of exercise. Endostatin levels for mice without vitamin C were higher in mice with 1 week, 3 weeks, and 6 weeks of exercise than in mice without exercise.

The V/E ratios were significantly higher in Group D (0.6 fold) than that in Groups B (0.1 fold) and F (0.2 fold) ($p < 0.01$ and $p < 0.05$, respectively), and Group H (0.5 fold) than that in Groups L (0.1 fold) (Fig. 3). In both with and without vitamin C, all were within the range that did not over 1.0 fold; however, groups with over 1.0 fold (Groups E and I) were observed in the group without exercise. Further, the V/E ratios were higher in mice with exercise than that in mice without exercise. The V/E ratios in mice with exercise had a trend that was similar to that of the VEGF, and they did not exceed 1.0 fold, whereas there were the groups with over 1.0 fold in mice without exercise.

4. Discussion

Since the change in surface skin temperature of the ischemic lower limbs significantly decreased in the groups that received surgical procedure, we found it necessary to create a mild ischemic lower limb model without discoloration and toe necrosis by ligation of the femoral artery. Moreover, since the surface skin temperature significantly decreased until mice were killed, a reduction in the surface skin temperature by ligation continued regardless of the presence of exercise and vitamin C and exercise periods. Since it has been reported[21] that the most common exercise period for PAD is 3-6 months, it is possible that the surface skin temperature did not improve by controlling exercise and vitamin C for the period of 6 weeks of exercise in the study. It is necessary to create models with mild ischemia and no necrosis and with severe ischemia and toe

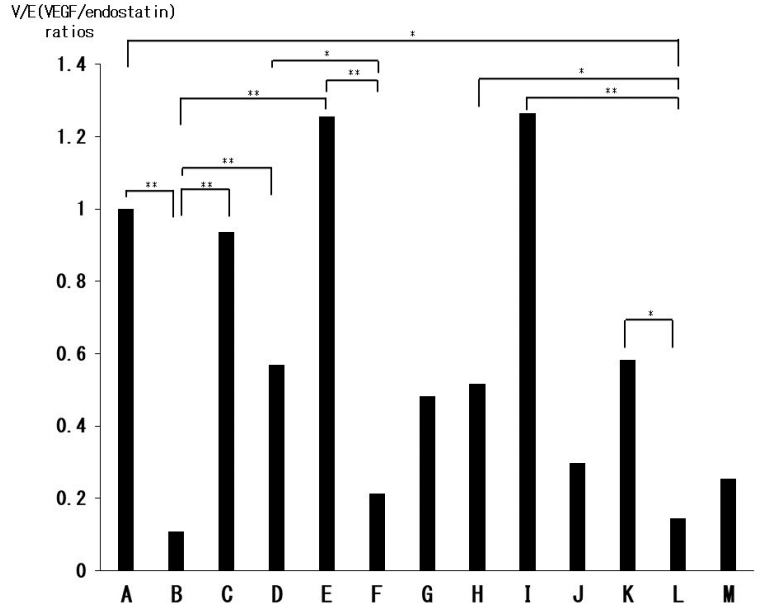

Fig. 3 Effects of lower limbs ischemia on mRNA expression leveis of V/E (VEGF/endostatin) ratios. mRNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. p Value by ANOVA test for the comparison between groups and Tukey's test. **indicate significant differences at levels of $p < 0.01$, *significant differences at levels of $p < 0.05$.

necrosis for future studies.

Oxidative stress is caused by an excess of active oxygen products in living bodies or the deterioration of the antioxidative system. It has been reported that the onset and aggravation of diseases such as PAD are involved in organ damage caused by oxidative stress[20]. In exercised mice, oxidative stress is commonly caused by an increase in oxygen intake, but oxidative stress is not increased with moderate exercise[7,21]. The balance between active oxygen products and antioxidative systems is well harmonized in moderate exercise, and this harmony breaks down with heavy exercise, which causes oxidative stress. Further, vitamin C decreases in muscle tissues and increases in blood with heavy exercise[22]. Therefore, it has been reported that vitamins are redistributed by the effective elimination of oxidative stress[9]. In this study, the concentration of vitamin C in plasma was not influenced by exercise with vitamin C; therefore, the redistribution of vitamins was not caused by moderate exercise, which we presume does not increase oxidative stress.

It has been reported that the antioxidative system generally and sufficiently functions sufficiently in a living body, and, furthermore, the expression and activity of antioxidant enzymes are increased by performing moderate exercise for a long term, which increases defense against oxidative stress[7,22]. The oxidative stress caused by exercise influences the immunity and the antioxidative system, acting on signal transduction systems involved in the activation of macrophages and the expression of cytokines[24]. Further, antioxidants such as vitamin C, which remove oxidative stress caused by exercise, are extremely important for biophylaxis. In this study, the BAP test in mice with 1 week and 3 weeks of exercise without vitamin C had high levels (BAP test), and the potential antioxidative activity had significantly high levels. In other words, although vitamin C, which removes oxidative stress caused by exercise, was deficient in the group without vitamin C, the potential antioxidative activity was high. This is probably because mice without vitamin C experienced the compensatory effects of vitamin C (i.e., some substance other than vitamin C) by exercise, and the expression and activity of the antioxidant enzyme became prominent. Contrarily, vitamin C, which removes oxidation caused by exercise, existed in mice with vitamin C, and no difference was seen in the potential antioxidative activity. This suggests that mice with vitamin C experienced the compensatory effects of vitamin C by exercise. Vitamin C might have been related to the oxidative stress that was caused by exercise. In the future, it is necessary to examine the balance between vitamin C and antioxidant enzymes.

In general, various factors involved in vascularization have been clarified, and the effects of the promoting factors need to overwhelm the inhibiting factors. It is inferred that HIF-1α might have been involved with the improvement of claudication in PAD patients by exercise, and the involvement of vascularization through the increase of VEGF has been suggested[1,4]. However, it has been reported that sustained exercise promotes the decomposition of HIF-1α, which suppresses the gene expression of the VEGF[5]. In this study, mice without vitamin C, 3 weeks of exercise resulted in significantly higher values of VEGF gene expression than mice with 1 week and 6 weeks of exercise, whereas no significant difference was seen in HIF-1α. The involvement of HIF-1α or IL-6 was not recognized for mice with 3 weeks of exercise, and the expression of vascularization-promoting factors through VEGF signals was observed. It can be inferred that the expression of VEGF by the ischemic lower limb was supported by transcription factors with which HIF-1α or IL-6 are not involved. Generally, there are five genes including placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF (VEGF-A) in the VEGF superfamily, and each of them has characteristic effects. Further, VEGF is a basic factor for vascularization that has many subtypes[25]. Therefore, since vascularization by VEGF signals works cooperatively with various vascularization induction mechanisms depending on the conditions, it is necessary to study it with the VEGF superfamily and subtypes. Further, the precedent study that used ischemic lower limb model rats reported that the

VEGF protein concentration of the thigh adductor reached its peak value at 1 day after surgical procedure, and no significant difference was seen 7 days after surgical procedure in comparison with the sham[26]. Although 3 weeks of exercise group had significantly high values in gene expression of VEGF, the VEGF protein concentration was not measured, and temporal analyses were not performed after surgical procedure. In the future, it is necessary to investigate the relationships between VEGF gene expression and protein concentration and perform temporal analyses after surgical procedure.

Since antioxidants such as vitamin C generally catch reactive oxygen species caused by exercise, they are extremely important in biophylaxis. Vitamin C intake suppresses the increase of inflammatory cytokines in exercise, though opinions differ depending on the amount of vitamin C intake and period[27]. Further, it has been reported that since vitamin C is a coenzyme of iron-containing dioxygenase enzymes that promote the decomposition of HIF-1α, it regulates HIF-1α values[28]. In this study, mice with vitamin C, 1 week of exercise resulted in significantly higher VEGF gene expression than that in mice with 6 weeks of exercise. By contrast, for FGF and HIF-1α, 6 week of exercise resulted in significantly lower values than those in mice with 1 week and 3 weeks of exercise. Mice with 6 weeks of exercise promoted the decomposition of HIF-1α that is promoted in mice with vitamin C. The suppression of the expression of vascularization factors through the VEGF signal was supposed. The function of FGF is to adjust the expression levels of the vascularization factors such as VEGF to induce vascularization. Further, when an artery is occluded, the blood flow to the existing arterioles that connect collaterally and proximally to the obstruction site and its distal region is increased by the pressure gradient. It has been reported that FGF, which is a growth factor, acts on vascular endothelial cells in the vascular lumen, causing the blood vessels to be rebuilt[29]. In this study, since the gene expression values of FGF were low in the with 6 weeks of exercise for vitamin C intake mice, the growth factors for vascular endothelial cells and the expression of vascularization factors such as VEGF were presumably suppressed.

It has been reported that exercise increases endostatin, suppresses the expression of vascularization factors, and acts on the growth of endothelial cells[30,31]. Complicated control systems are involved in the development of a capillary plexus by exercise, whereas inhibiting factors such as endostatin typically. In this study, mice of with 1 week, 3 weeks, and 6 weeks of exercise exhibited high values of endostatin expression for mice with vitamin C. Further, mice with 1 week and 3 weeks of exercise, the potential antioxidative activity had high values that due to the compensatory effects of vitamin C. This seemed to suggest a relationship between the potential antioxidative activity and gene expressions of endostatin for mice without vitamin C. It is necessary to study the relationships between vitamin C and endostatin and examine their molecular mechanism.

The V/E ratios indicates the balance between the promoting factors and the inhibiting factors of vascularization. However, when the ratio exceed 1.0 fold, the promoting factors become less than that of the inhibiting factors. Since inhibiting factors such as endostatin become suppress the promoting factors, the V/E ratios might not exceed 1.0 fold. The V/E ratios are used for studies on the effects of therapy[32] and are an index that indicates the equilibrium of the promoting factors and the inhibiting factors. In this study, the V/E ratios did not exceed 1.0 fold in mice with exercise for both mice with and without vitamin C, whereas they exceeded 1.0 fold in some groups in mice without exercise. In particular, the group without vitamin C exhibited the expression of vascularization-promoting factors through VEGF signals in mice with exercise and endostatin gene expression by compensatory effects of vitamin C without the involvement of HIF-1α or IL-6. It was possible that the expression of the inhibiting factors exceeded those of the promoting factors in the exercise. The equilibrium between the inhibiting factors and promoting factors has been determined through the V/E ratios. Further, since the gene expression values of endostatin for mice

without vitamin C were low in mice without exercise, we presume that the expression of promoting factors exceeded that of inhibiting factors. In other words, disequilibrium of the inhibiting factors and promoting factors has been determined through the V/E ratios.

5. Conclusion

In mice of with exercise without vitamin C, the potential antioxidative activity was high because of the compensatory effects of vitamin C, and the gene expression for the vascularization-inhibiting factors exceeded that for the promoting factors; therefore, the equilibrium of the vascularization-promoting and vascularization-inhibiting factors has been determined using the V/E ratios. These results revealed that exercise affects the potential antioxidative activity and vascularization factors.

Conflicts of Interest

The authors have no conflicts of interest.

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