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Combination of Cilostazol and L-Carnitine Improves Walking Performance in Peripheral Arterial Disease Model Rats

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Key Words
Peripheral arterial disease · Cilostazol · L-Carnitine · Combination therapy · Walking performance · Angiogenesis · Rats

Abstract
Cilostazol and L-carnitine have been used as a first-line drug and supplement, respectively, in patients with peripheral arterial disease with intermittent claudication. In this study, the effect of the combination of cilostazol and L-carnitine has been investigated in rats with unilateral hindlimb ischemia. For 28 days, cilostazol and L-carnitine were administrated separately or as a combination. The distance walked before gait disturbance developed was measured using a treadmill for 5 days a week. The capillary density of the ischemic hindlimb was evaluated by immunohistochemical staining at days 7, 14, 21, and 28. Angiogenic gene expression was measured by real-time RT-PCR at days 7 and 28. The greatest increase in the distance was observed in the combination therapy group when compared to the other groups. The capillary density in the adductor muscles of rats treated with cilostazol alone and combination therapy increased at day 28. Angiopoietin-2/Angiopoietin-1 expression ratios were higher, suggesting the promotion of angiogenesis, with cilostazol alone and combination therapy at day 7. This is the first study to show functional improvement of the hind limb following combination therapy with cilostazol and L-carnitine in experimental animals. This study also revealed that cilostazol promotes angiogenesis, and L-carnitine additively contributes to functional improvement via a non-angiogenic mechanism.

Introduction

Peripheral arterial disease (PAD), which is the arterial obstruction of extremities caused by atherosclerotic processes, is associated with significant morbidity and mortality [1]. Recently, it was reported that the mean annual incidence and prevalence of PAD in the United States were 2.35 and 10.69%, respectively [2]. Intermittent claudication (IC) is the most general symptom and induces aches, cramps, or fatigue, which occurs during exercise and is alleviated by rest. An insufficient blood flow to meet the metabolic demands of skeletal muscle for walking results in IC. Improvement of IC is one of the essential treatment strategies for patients with PAD.

Cilostazol is a first-line drug for patients with PAD exhibiting IC and has consistent evidence in many large-scale studies [3–5]. Cilostazol is a phosphodiesterase type 3 inhibitor, which leads to vasodilation and has an antiplatelet effect. However, the mechanism through which cilostazol improves IC remains to be determined. Researchers have focused on the possibility of an angiogenic effect of cilostazol, since it was reported that vascular endothelial growth factor (VEGF) levels increased in pa-

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tients following cilostazol administration [6]. Recently, several studies have demonstrated the angiogenic effect of cilostazol, such as promoting endothelial progenitor cell migration [7] and angiogenesis [8].

L-Carnitine is associated with beta-oxidation to a great extent and this helps convert fat into energy. Several studies have reported that L-carnitine administration increased the walking distance in patients with PAD with IC [9–11]. In addition, it was demonstrated that the combination of cilostazol and L-carnitine improves IC in patients with PAD through enhanced angiogenesis secondary to increased VEGF levels and increased energy production, respectively [12]. VEGF production is, however, enhanced under ischemic conditions [13]. The expression of angiogenic factors may therefore be downregulated when ischemic conditions are improved by L-carnitine, resulting in the attenuation of the effect of cilostazol. However, little is known about the interactive effect of cilostazol and L-carnitine.

In this study, we first evaluated the effect of cilostazol or L-carnitine alone, or their combination on the walking performance of PAD model rats. Then, alternations of capillary density and angiogenic factors, including VEGF, Angiopoietin-1 (Ang1), Ang2, Tie1, and Tie2, were investigated genetically in skeletal muscles of the PAD model rats.

Materials and Methods

Experimental Animal

Eight-week-old male Sprague-Dawley rats (SLC, Japan) were acclimated for 1 week prior to the start of the experiment. Rats were housed at 23 ± 2°C and 60 ± 10% humidity in a cage, lit daily from 7:00 a.m. to 7:00 p.m., in a controlled room and they received food and water ad libitum. The care and handling of the animals were in accordance with the 'Azabu University Animal Experiment Guidelines; April 2000'. All experiments complied with the guidelines of Ethics Committee of Azabu University, which reviewed and approved this study (approval number 130710-2).

PAD Model Rats and Groups

We generated the PAD model as reported by Orito et al. [14]. In short, rats were anesthetized, and the left iliac artery was ligated with sutures via laparotomy. In our previous study, we showed that cilostazol increased the gait disturbance developed (D_{GD}) significantly at dose of 30 mg/kg in rats with unilateral hindlimb ischemia [14]. It has been reported that an oral dose of 300 mg/kg L-carnitine improves age-associated decline in mitochondrial respiratory chain activity of rat heart muscles [15] and antioxidant status via reduction in tissue lipid peroxidation in atherosclerotic rats [16]. One-week post ligation, rats were administered daily doses of vehicle, 30 mg/kg cilostazol, 300 mg/kg L-carnitine, or cilostazol and L-carnitine orally twice a day for 28 days.

Table 1. Primer for quantitative real-time RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
VEGF	tggagcgttcactgtgagcc	tctgtcgacggtgacgatgg
Ang1	cagtcagaggcagtacatgc	gcataagggcgcatttgcac
Ang2	ggcctactgtgacatggaca	tacagagagtgtgcctcgct
Tie1	ctcgagagcatggaacagcct	gtgcctccaaggctcactatc
Tie2	ggagcatgtgaacacagaggc	ctcaattgccatccagcgcac
GAPDH	cagagctgaacgggaagctc	cattgagagcaatgccagcc

Walking Performance Evaluation

Throughout experiments, a rodent treadmill was maintained at a 15% incline and a running speed of 15 m/min. Rats that had been running normally on a treadmill developed a gait disturbance when a distal portion of the iliac artery was occluded. We found that the distance that the rats run until exhibiting D_{GD} was a good parameter to evaluate ischemic severity of the hindlimb [14]. The rats were divided into 4 groups according to the D_{GD} before administration, so that the average D_{GD} in each group was equal. All rats exercised on the treadmill for 5 min/day for 5 days a week, and D_{GD} was measured at each exercised day. D_{GD} was also evaluated for comparison at days 7, 14, 21, and 28.

Histological Analyses

Following histological and quantitative real-time RT-PCR analyses were performed in rats separately from walking performance evaluation. At days 7, 14, 21, and 28 after drug administration, adductor muscles were collected from the left limbs; two thirds of the muscles collected were snap-frozen in isopentane chilled by liquid nitrogen and stored at –80°C until use in the histological assay, while the remaining muscles were put into RNA stabilization reagent (Qiagen, Hilden, Germany) overnight and stored at –80°C until use in real-time RT-PCR analysis. Tissues were cut into 6-μm sections and mounted on silane-coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan). Sections were immunostained using anti-mouse CD31 antibodies (Santa Cruz Biotechnology, Calif., USA) to visualize vascular endothelial cells. Capillary density was defined by the number of vascular endothelial cells/muscle fibers counted.

Quantitative Real-Time RT-PCR Analysis

The method we adopted was basically similar to the one reported by Mori et al. [17]. The total RNA of ischemic adductor muscle was prepared using an SV Total RNA Isolation System (Promega, Wisc., USA) according to the manufacturer's instructions and then reverse transcribed to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). The measurement of gene expression by quantitative analysis was carried out using a LightCycler system (Roche). Primers were synthesized by Invitrogen (table 1). Quantitative real-time RT-PCR analysis of *VEGF*, *Ang1*, *Ang2*, *Tie1*, *Tie2*, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene expression was carried out using a LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche Applied Science) with primer sets described in table 1. PCR amplification of the housekeeping gene *GAPDH* was carried out for each sample as a control for sample loading and

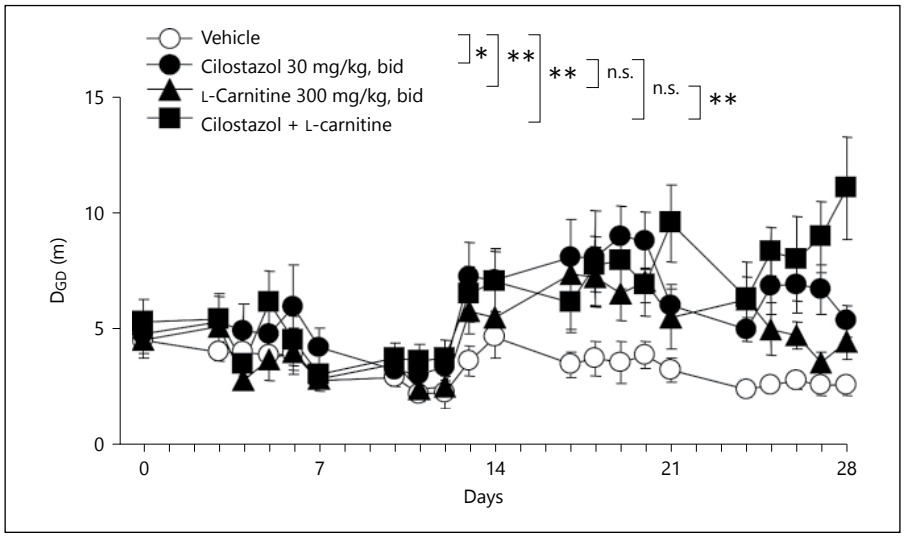


Fig. 1. Effects of cilostazol, L-carnitine, or their combination on the distance walked before D_{GD} in rats with unilateral hindlimb ischemia. D_{GD} was measured as walking performance. All rats were exercised on the treadmill for 5 min/day for 5 days a week. D_{GD} significantly increased in groups administered cilostazol, L-carnitine, and their combination compared with vehicle. * $p < 0.05$, ** $p < 0.01$ 2-way ANOVA with repeated measures, mean ± SD (n = 9). n.s. = Not significant.

to allow normalization among samples. To determine the absolute copy number of the target transcripts, the amplified fragments of *GAPDH* or target gene amplified by PCR using the primer sets were constructed using a pGEM cloning vector (Promega), and the concentrations of these purified plasmids were measured. The absorbance at 260 nm and the copy numbers were calculated from the concentrations of the samples. A standard curve was constructed by plotting the threshold cycle versus the known copy number for each plasmid template in the dilutions. The copy numbers for all unknown samples were determined according to the standard curve using LightCycler software 3.5.3 (Roche Applied Science). To correct the differences in both RNA quality and quantity between samples, each target gene was first normalized by dividing the copy number of target with the copy number of *GAPDH*. The initial value corrected for the amount of *GAPDH* was indicated as 100% to evaluate the sequential alteration of the mRNA expression level.

Statistical Analysis

All data were expressed as the mean ± SD. The difference in walking performance among groups was determined by 2-way analysis of variance (ANOVA) with repeated measures. Differences in capillary density were determined by the unpaired t test. Differences in distance walked at each day and the expression of mRNA among groups were determined by 1-way ANOVA followed by multiple comparisons using the Bonferroni's test.

Results

Effect of Repeated Oral Administration of Cilostazol and/or L-Carnitine on Walking Performance in PAD Model Rats

We investigated the effects of cilostazol and/or L-carnitine administration on the walking performance in rats with hindlimb ischemia (fig. 1). Administration of cilostazol, L-carnitine, and their combination significantly increased the D_{GD} compared to the control group. Combined administration of cilostazol and L-carnitine resulted in a greater increase in D_{GD} than that observed with cilostazol alone and a significant increase compared to L-carnitine administration alone.

We also evaluated the D_{GD} at days 0, 7, 14, 21, and 28; an improvement in walking performance was observed with cilostazol and/or L-carnitine (fig. 2). There was no significant difference in the D_{GD} among the groups at days 0, 7, and 14. Combined administration of cilostazol and L-carnitine significantly increased the D_{GD} at day 21 compared with the control group, and at day 28 compared with the other groups.

Effect on Capillary Density in Ischemic Adductor Muscles of Rats Administered Cilostazol and/or L-Carnitine

We next investigated the capillary density of adductor muscles in PAD model rats administered cilostazol and/or L-carnitine at days 0, 7, 14, 21, and 28. There was no significant difference in capillary density among the groups at days 0, 7, 14, and 21 (data not shown). At day 28, the capillary densities of the cilostazol group and groups that were administered a combination of cilostazol and L-carnitine significantly increased compared to those of the control group (fig. 3a, b).

mRNA Expression of Angiogenic-Related Genes in Ischemic Adductor Muscles of Rats Administered Cilostazol and/or L-Carnitine

We demonstrated that the administration of cilostazol alone and the combination of cilostazol and L-carnitine

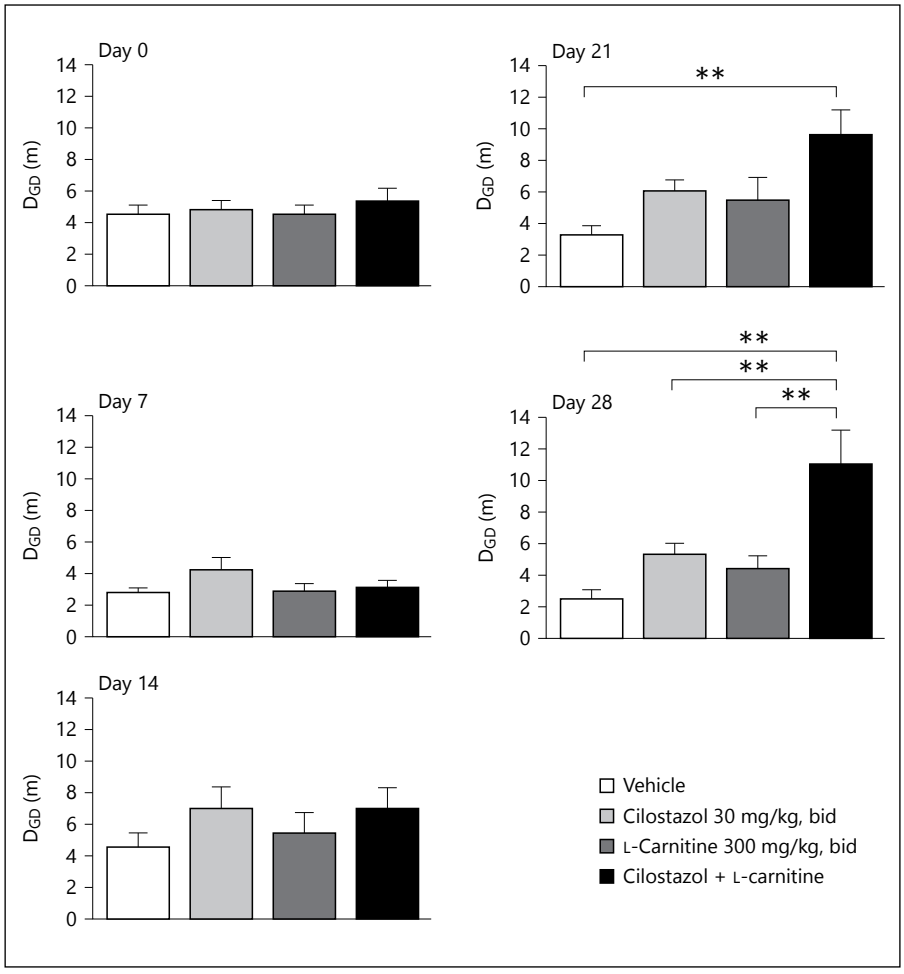


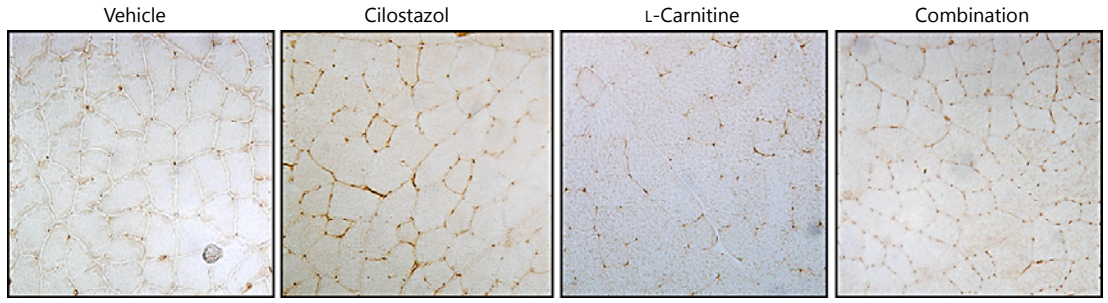
Fig. 2. Effects of cilostazol, L-carnitine, or their combination on the distance walked before D_{GD} at days 0, 7, 14, 21, and 28 in rats with unilateral hindlimb ischemia. No significant difference was observed in D_{GD} among all groups at days 0, 7, and 14. D_{GD} was significantly higher in the combination group at day 21 compared with that of the control group and at day 28 compared with all other groups. ** $p < 0.01$ vs. vehicle Bonferroni's multiple comparison test, mean ± SD (n = 9).

increased capillary density. Thus, we quantified the mRNA copy numbers of angiogenic-related genes, *VEGF*, *Ang1*, *Ang2*, *Tie1*, and *Tie2*, in ischemic adductor muscles of rats administered cilostazol and/or L-carnitine. The expression of *VEGF* in all groups was unchanged at days 7 and 28 (fig. 4a). *Ang2/Ang1* ratios in the cilostazol alone and combination groups were significantly higher than that in the control group at day 7, and the ratio in cilostazol group was higher even at day 28 (fig. 4b). The *Ang2/Ang1* ratio in the L-carnitine group and the expression of *Tie1* (data not shown) and *Tie2* in all groups were unchanged at days 7 and 28 (fig. 4c).

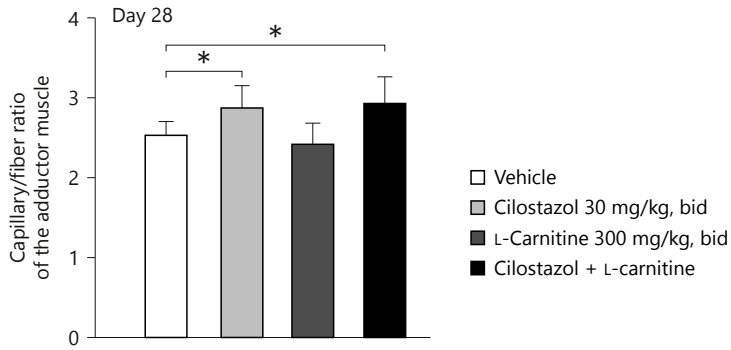
Discussion

We demonstrated that cilostazol and L-carnitine improved walking performance, and the improvement was greater with a combination of these drugs in rats with unilateral hindlimb ischemia. The effect of cilostazol on ischemic hindlimb has been examined using mouse and rat models of ischemic hindlimbs [8, 18]. These studies, however, focused on evaluating the superficial blood flow of the hindlimb under anesthesia, which may not precisely predict the function of the hindlimb. This is the first study to demonstrate the functional improvement of the hindlimb following administration of cilostazol, L-carnitine, and their combination in experimental animals. Goldenberg et al. [12] demonstrated that L-carnitine plus cilostazol used for the treatment of IC in patients with PAD improved walking performance compared to using cilostazol alone. This finding is consistent with our results, suggesting that the ischemic model and evaluation system used in this study is useful to examine the mechanisms of improvement of ischemic hindlimbs following the administration of cilostazol, L-carnitine, or their combination.

L-Carnitine alone did not increase the capillary density or mRNA expression of the genes involved in angio-



a



b

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Fig. 3. Effects of cilostazol, L-carnitine, or their combination on the capillary/fiber ratio in the ischemic adductor muscle. **a** Endothelial cells were immunostained by anti-CD31 antibody (brown). **b** Capillary/fiber ratio was significantly higher in cilostazol and the combination group compared with that of the control group at day 28. No significant difference was observed in the capillary/fiber ratio at days 7, 14, and 21. Muscular fibers and endothelia were counted by 2 observers. * $p < 0.05$ vs. vehicle unpaired t test, mean ± SD (n = 6–7).

genesis. Carnitine enhances long-chain fatty acid transfer from the cytosol to the mitochondrion. It is reported that carnitine improves microcirculation in the ischemic critical limb in patients [19]. Carnitine also improves mitochondrial respiratory chain activity in rats [15] and phosphocreatine synthesis after exercise in patients with PAD [20]. All these results cumulatively indicate that metabolic changes in the ischemic hindlimb may contribute to the improvement in walking performance by L-carnitine.

Cilostazol and the combination of cilostazol and L-carnitine increased the capillary densities of skeletal muscles. Ang1 has been identified as the major ligand for the angiogenic tyrosine kinase receptor, Tie2, and has been assigned the responsibility for recruiting and sustaining periendothelial cells. Ang2 is considered a natural Ang1/Tie2 inhibitor. Inhibition of Ang1 by Ang2 drives angiogenesis by loosening contacts between endothelial and periendothelial cells. The *Ang2/Ang1* ratio indicates the condition of vascular remodeling: a high *Ang2/Ang1* ratio, as was seen in this study, indicates the facilitation of vascular remodeling [21, 22]. Cilostazol may promote angiogenesis via destabilization of capillary conditions.

There was no significant difference in capillary density among the groups before day 28 although the cilostazol and combination therapy showed significant improvement in walking performance as compared to the capillary density in the control group. Cilostazol has several beneficial effects on microcirculation other than angiogenesis. It causes vasodilation, inhibits platelet aggregation, reduces plasma triglyceride levels, and preserves endothelial cells [23]. In addition, cilostazol has been shown to preserve the mitochondrial function against oxidative stress in in vitro experiment [24]. These effects may contribute to improving the walking performance before angiogenesis.

Although increases in capillary densities were observed, *VEGF* levels remained consistent in all groups.

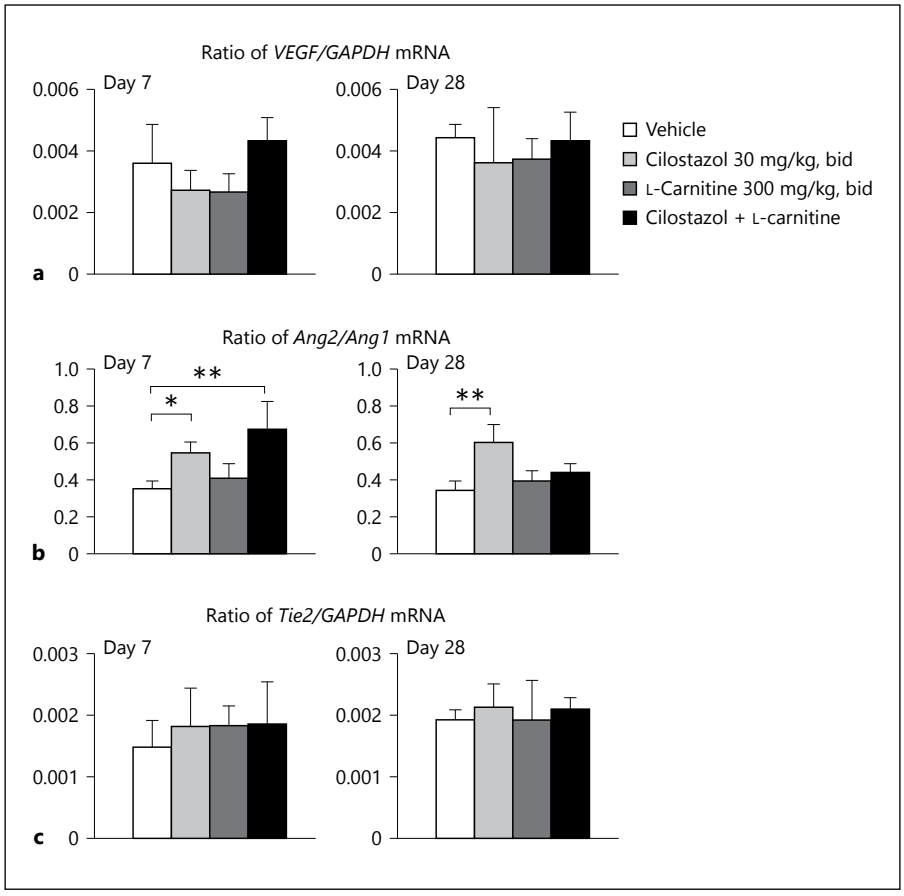

Fig. 4. Effects of cilostazol, L-carnitine, or their combination on mRNA expression of angiogenesis-related genes in the ischemic adductor muscles. **a** No significant difference was observed in the *VEGF* gene expression among all groups. **b** *Ang2/Ang1* ratio in adductor muscles. The *Ang2/Ang1* gene expression ratio was significantly higher in cilostazol and the combination group compared with the group administered vehicle at day 7. At day 28, *Ang2/Ang1* ratio was higher only in the cilostazol group compared with that of the control group. **c** No significant difference was observed in the *Tie2* gene expression among all groups. * $p < 0.05$, ** $p < 0.01$ 1-way ANOVA followed by Bonferroni's multiple comparison test, mean ± SD (n = 3–5).

This result is however not consistent with that of the previous report by Biscetti et al. [23]. There are several differences between our study and the one conducted by Biscetti et al. [23]; differences have been observed in the following areas: (i) animal species (mouse vs. rat), (ii) ischemic severity (severe; femoral and saphenous artery dissection vs. mild; iliac artery ligation), (iii) doses of cilostazol (0.3–30 mg/kg, i.p. vs. 30 mg/kg, p.o.), (iv) timing of treatment initiation (30 min before ligation vs. 1 week after ligation), and (v) administration period (single dose vs. 4 weeks). Further, the expression level of VEGF was evaluated 3 days after the administration of a single cilostazol dose and ligation in the study by Biscetti et al. [23]; in contrast, we evaluated the VEGF expression after repetitive administration of cilostazol for 14 and 35 days after ligation. These differences may have caused the disparity in results. Angiogenesis is induced not only by VEGF but also by the platelet-derived growth factor [25] and transforming growth factor beta [26]. Cilostazol inhibits the activity of phosphodiesterase type 3 [27], leading to the upregulation of the hepatocyte growth factor [28] and possible induction of angiogenesis [29]. Although further studies are necessary, these growth factors might contribute to the angiogenesis induced by cilostazol.

As described earlier, *Ang2/Ang1* ratios were high in the cilostazol and combination groups at day 7. However, at day 28, it was no longer high in the combination group, although it remained high in the cilostazol group. It is reported that the *Ang2* gene of endothelial cells is upregulated in hypoxia [30]. In combination, L-carnitine may ameliorate ischemic severity by enhancing energy production. Resultant lower hypoxic condition may inhibit increases in the *Ang2/Ang1* ratio.

In conclusion, we demonstrated that cilostazol and L-carnitine increased walking performance in rats with hindlimb ischemia. Greater improvement was observed with combination therapy than with either of these drugs separately. Angiogenesis induced by cilostazol under high Ang2/Ang1 ratio conditions and enhancement of energy production by L-carnitine may additively contribute to the improvement of walking performance.

Acknowledgments

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Conflicts of interest

The authors have no conflict of interest directly relevant to the content of this article.

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