Title page

Original article

Zinc deficiency impairs ischemia-induced angiogenesis

Running Title: Zinc, angiogenesis, and chronic limb-threatening ischemia

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ARTICLE HIGHLIGHTS

Type of Research: Single-center retrospective cohort study and animal experiments.

Key Findings: Zinc deficiency impaired the rate of ischemia-induced revascularization through enhanced oxidative stress rates in mice experiments. Serum zinc levels were also positively associated with SPP in 51 patients with CLTI who referred for de novo revascularization for CLTI due to arteriosclerosis obliterans ($r = .538$, $P < .001$).

Take home Message: The intake of zinc could be useful for the prevention and/or treatment of ischemic limb disease. In addition, circulating zinc level could be a useful marker for the assessment of atherosclerosis-based vascular disease such as limb ischemia.

Table of Contents Summary

Zinc deficiency impaired the rate of ischemia-induced revascularization through enhanced oxidative stress rates in mice experiments. Serum zinc levels were also positively associated with SPP in 51 patients with CLTI who referred for de novo revascularization for CLTI due to arteriosclerosis obliterans.

Key word: zinc, ischemia, angiogenesis

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Abstract

Objectives: Zinc is an important essential trace metal that is involved in many physiologic functions and its deficiency can affect the development of multiple organs, including the vasculature. However, there is a lack of clarity on the impact of zinc deficiency in the regulation of angiogenesis. We aimed to investigate the impact of zinc deficiency on the revascularization process through animal experiments and examine the relationship between circulating zinc levels and tissue blood perfusion in patients with chronic limb-threatening ischemia (CLTI).

Methods: Zinc-deficient mice and control wild-type (WT) mice were subjected to unilateral hindlimb ischemic surgery. Next, we examined the relationship between serum zinc levels and skin perfusion pressure (SPP) as an index of tissue blood perfusion in patients with CLTI. A total of 51 patients with CLTI who referred for de novo revascularization for CLTI due to arteriosclerosis obliterans at our hospital from May 2012 to March 2016 were enrolled.

Results: The zinc-deficient mice exhibited impaired blood flow recovery rates and a decreased capillary density in the ischemic limb compared to the control WT mice. The zinc-deficient mice also showed increased reactive oxygen species production rates after hindlimb ischemia. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitors

ameliorated the zinc deficient-induced impairment of revascularization. Serum zinc levels were positively associated with SPP in the CLTI patients. Multivariate regression analysis also revealed that serum zinc levels were significantly correlated with SPP in patients with CLTI.

Conclusions: Zinc deficiency impaired the rate of ischemia-induced revascularization through enhanced oxidative stress rates, suggesting that nutritional approaches aimed at zinc intake may could be useful in CLTI prevention and treatment.

Clinical Relevance: This study aimed to investigate the impact of zinc deficiency on angiogenesis. We found that zinc deficiency impaired the rate of ischemia-induced revascularization through enhanced oxidative stress rates in animal model. In addition, serum zinc levels were positively associated with SPP in the CLTI patients. Thus, the intake of zinc could be useful for the prevention and/or treatment of ischemic limb disease. Circulating zinc level could be a useful marker for the assessment of atherosclerosis-based vascular disease such as limb ischemia. Possibly, nutritional improvement by zinc intake could lead to prevention and treatment ischemic vascular disease.

Introduction

Peripheral arterial disease (PAD) is increasing globally. In no less than half of PAD patients with chronic limb-threatening ischemia (CLTI), amputation of the affected limbs is required, which reduces patients' quality of life and life span [1]. PAD develops as a result of lifestyle changes, such as lack of physical activity, over-nutrition, and the dyshomeostasis of trace elements [2]. While the influence of selenium and copper on atherosclerosis and heart disease has attracted a high degree of attention [3, 4], the impact of trace metal deficiencies on this process is less well-defined. Of note, there is a lack of information of the effects of trace elements, including zinc, on angiogenesis. Moreover, no specific signaling pathways associated with angiogenesis have been identified.

In recent years, the importance of zinc nutrition has gained global recognition[5]. Zinc is an important essential trace metal required for many physiological functions, including growth and reproduction. Zinc deficiency can affect the development of multiple organs, including the brain, lungs, kidneys, heart, and vasculature[6]. Some major health impacts of zinc deficiency include reduced growth rates and immune function suppression[6, 7]. Thus, zinc deficiency is widely recognized as a leading risk factor for morbidity and mortality.

With regard to cardiovascular disease, arterial pressure is inversely correlated with

serum zinc concentrations[8]. Zinc deficiency that is induced by zinc metabolism disturbances is correlated with cardiovascular occurrences [6, 7]. A high zinc intake status provides protection against cardiovascular disease[9]. Recently, we investigated the relationship between zinc deficiency and clinical outcomes after bypass surgery for CLTI and found that zinc deficiency is associated with lower rates of limb salvage[10]. As described above, the development of cardiovascular diseases, including CLTI, is associated with blood zinc levels, and the reduced angiogenic capacity is majorly involved in the pathology of CLTI [11]. Therefore, we hypothesized that Zinc may regulate angiogenesis in CLTI. Accordingly, we aimed to investigate the impact of zinc deficiency on ischemia-induced revascularization processes employing a murine hindlimb ischemia model and also examine the relationship between circulating zinc levels and skin perfusion pressure (SPP) as an index of tissue blood perfusion in patients with CLTI.

Material and Methods

Materials

CD31 antibody was purchased from BD (Franklin Lakes, NJ). Dihydroethidium and apocynin were purchased from Wako Pure chemical (Osaka, Japan).

Zinc-deficient mouse model and mouse model of limb blood perfusion

Wild-type (WT) and diet-induced, zinc-deficient mice on a C57/BL6 background were used in this study. All study protocols were approved by the Institutional Animal Care and Use Committee at Nagoya University. Zinc deficiency was induced by the provision of *ad libitum* access to a zinc-deficient diet (0.01% zinc content, Oriental BioService, Inc.) to 3-week-old animals for 7 weeks[12]. The lean controls continued to be fed a normal chow diet until the time of the experiment. The number of the zinc-deficient mice and control mice was 8 in each group. At age 10 weeks, the serum zinc levels of the mice were measured and the mice were subjected to unilateral hind limb surgery under anesthesia with sodium pentobarbital (administered intraperitoneally at 50 mg/kg). In this model, the entire left femoral artery and vein were removed surgically, as described previously [13, 14]. All the mice were weighed at weekly intervals. The heart rate and systolic blood pressure in the conscious state were

determined using a tail-cuff pressure analysis system. In some experiments, the mice were treated with apocynin or a vehicle from age 3 weeks until sacrifice. Apocynin has the effect of inhibiting NADPH oxidase activity and suppressing the production of reactive oxygen species (ROS). Apocynin powder was added to hot (~60°C) sterile water. The water was allowed to cool to room temperature before being provided to the mice. The concentration of apocynin was 2 mg/ml in water. The conversion to mg/kg was estimated based on the average daily water intake of approximately 4 ml/mouse, resulting in a dose of approximately 300 mg/kg/day[15].

Laser Doppler blood flow analysis

A laser Doppler blood flow (LDBF) analyzer (Moor LDI, Moor Instruments) was used to measure the hindlimb blood flow immediately before surgery and on postoperative days 3, 7, 14, and 28. LDBF analysis was performed on the legs and feet. Blood flow was displayed as changes in the laser frequency using different color pixels. After scanning, stored images were analyzed for blood flow quantification. To avoid data variations owing to ambient light and temperature, hindlimb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF [13, 14].

Capillary density analysis

The capillary density in the adductor muscle was analyzed for the obtainment of specific evidence on the vascularity at the microcirculation level. Tissue samples were obtained from the ischemic thigh adductor skeletal muscles on postoperative day 28. Frozen tissue slices (7 μm in thickness) were prepared and stained with CD31 and then treated with fluorescein isothiocyanate-conjugated secondary antibody for the detection of CD31. The signals were detected and analyzed by fluorescence microscopy. Fifteen random microscopic fields from three different sections in each tissue block were examined for the presence of capillary endothelial cells, and the number of capillary endothelial cells per field was determined as the capillary density [13].

In situ dihydroethidium staining

We evaluated the superoxide production rate by in situ dihydroethidium (DHE) staining. Samples were embedded in optimal cutting temperature compound and snap-frozen in liquid nitrogen. Tissue slices (7 μm in thickness) were prepared and incubated with DHE in phosphate-buffered saline (10 mmol/l) in a dark, humidified container at room temperature

for 30 min. DHE is oxidized through the reaction of superoxide with ethidium bromide, which binds to the DNA in the nuclei to emit red fluorescence. The signals were detected and analyzed by fluorescence microscopy. The excitation wavelength was 488 nm and emission fluorescence was detected using a 568 nm long-pass filter [16].

Biomarker analysis

The serum zinc levels in the mice blood samples were measured with enzymatic kits (Metallogenics, Chiba, Japan). This assay system measures both free and protein-bound zinc [17]. Human blood samples were obtained from the patients before the surgery. Complete blood counts were performed using a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan). Biochemical data, including those pertaining to zinc, were measured using a LABOSPECT 008 automatic analyzer (Hitachi Co., Tokyo, Japan).

The derivatives of reactive oxidative metabolites (d-ROMs) were measured using a Free Radical Analytic System, according to the manufacturer's instructions. The d-ROMs test is based on the concept that the amount of organic hydroperoxides in the blood is related to the amount of free radicals from which they are formed. In brief, when the sample is dissolved in an acidic buffer, the hydroperoxides react with the transition metal. The

concentrations of these persistent species can be determined at 505 nm using a spectrophotometer. The d-ROMs are expressed in Carratelli Units (Carr units), where 1 Carr unit corresponds to 0.8 mg/l of hydrogen peroxide [16].

Serum nitrotyrosine levels were measured by nitrotyrosine ELISA kit (StressMarq Biosciences Inc., Victoria BC, Canada) according to the manufacturer's instruction.

Measurement of mRNA

Total RNA was extracted from muscle tissues using a RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocols. Complementary DNA (cDNA) from 500 ng of total RNA was synthesized by reverse transcription using ReverTra Ace® qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) analysis was performed on a CFX-96 system using THUNDERBIRD® qPCR Mix (Toyobo Life Science) as a double-stranded DNA-specific dye according to the manufacturer's instruction (Bio-Rad; Hercules CA) [18]. Primers were designed as follows: 5'- CAGGCTGCTGTAACGATGAA-3' and 5'-AATGCTTTCTCCGCTCTGAA-3' for mouse VEGF; 5′-TTGGGTCAGCACTGGCTCTG-3′ and 5′-TGGCGGTGTGCAGTGCTATC-3′ for mouse $gp91^{phox}$; 5′-

GGCCATTGCCAGTGTGATCTA-3′ and 5′-TGCTTGATGGTGCCTCCAA-3′ for mouse $p22^{phox}$; 5′-GATGTTCCCCATTGAGGCCG-3' and 5′-GTTTCAGGTCATCAGGCCGC-3′ for mouse $p47^{phox}$; 5′-CTGGCTGAGGCCATCAGACT-3′ and 5′-AGGCCACTGCAGAGTGCTTG-3′ for mouse $p67^{phox}$; 5′- ATGGTGAAGGTCGGTGTG-3′, and 5′- ACCAGTGGATGCAGGGAT-3′ for GAPDH. The expression levels of the examined transcripts were compared to those of GAPDH and normalized to the mean value of the controls.

Clinical study

Fifty-one consecutive patients with CLTI who referred for de novo revascularization for CLTI due to arteriosclerosis obliterans at our hospital from May 2012 to March 2016 were retrospectively analyzed. Blood samples obtained from the patient before surgery were used to measure biochemical data, including those pertaining to zinc. This study was conducted in accordance with mandates of the Declaration of Helsinki. The Nagoya University School of Medicine Institutional Review Board approved the study (approval number:2019-0185), and all patients provided written informed consent for data collection.

CLTI was defined as tissue loss/gangrene and resting pain lasting >2 weeks, in

accordance with Inter-Society Consensus for the Management of Peripheral Arterial Disease guidelines [19]. A medical history was obtained to document past medical history, medications, and co-morbid disease. Body mass index (BMI) was calculated as the ratio of weight to height squared. Diabetes mellitus was defined according to World Health Organization criteria. Hypertension was defined as a systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg on repeated measurements or the reception of antihypertensive treatment. Dyslipidemia was defined as a total cholesterol level ≥220 mg/dL, triglyceride level ≥150 mg/dL, or the reception of lipid-lowering therapy. Coronary artery disease (CAD) was defined as a history of any revascularization of the coronary arteries. Cardiovascular disease was defined as a history of stroke, cerebral hemorrhage, and/or any revascularization of the carotid arteries.

In the present study, we measured skin perfusion pressure (SPP) as an index of tissue blood perfusion. SPP was measured with the patient in the supine position on a bed at room temperature, using a laser Doppler probe (SensiLase PAD3000 Doppler Waveform Analyzer, Kaneka Medix Corp., Osaka, Japan). SPP <40 mmHg with tissue loss/gangrene or SPP<30 mmHg with resting pain were defined as being indicative of CLTI [20]. SPP values were obtained by trained medical technologist from the patients before surgery.

Statistical analysis

All statistical analyses were performed using PASW Statistics 27 software (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation. Analysis of variance followed by Tukey's honestly significant difference tests and unpaired Student's t-tests were used for statistical analysis. For the identification of the covariates associated with SPP, Spearman's rank-order correlation was conducted. Variables with $P < .05$ in the univariate analysis were incorporated into the multivariable model. A multiple linear regression was calculated to predict SPP based on the variables. In all the analyses, $P<0.05$ was considered statistically significant.

Results

Zinc-deficient mice show reduction of ischemia-induced revascularization

To assess the impact of zinc deficiency on the revascularization process in response to ischemia, the WT mice treated with or without a zinc-deficient diet were subjected to unilateral hindlimb ischemia. All the mice survived after the surgical induction of unilateral hindlimb ischemia. All the mice also appeared healthy during the follow-up period. The body weight and blood pressure did not differ between the zinc-deficient mice and control mice. The skin conditions remained unchanged, whereas no behavioral problems were observed. The serum zinc concentration was 94.2 ± 3.8 μg/dL in the zinc-deficient mice and 123.0 ± 10.2 μg/dL in the control WT mice at age 10 weeks ($P < .05$) (Supplementary Figure 1).

LDBF analysis was performed before surgery and on postoperative days 3, 7, 14, 21, and 28 for the evaluation of the blood flow recovery rate after ligation of the femoral artery of the control and zinc-deficient mice. Representative images of blood flow, as measured by LDBF, are shown in Figure 1A. In the control mice, the hindlimb blood flow perfusion rate fell precipitously after surgery, remained impaired for 3 days, increased to 25-35% of that in the nonischemic limb by day 7, and ultimately returned to 45-55% of that in the nonischemic limb by day 28 (Figure 1B). In contrast to the blood flow in the control mice,

that in the ischemic hindlimb was markedly reduced in the zinc-deficient mice compared to the level in the nonischemic hindlimb on postoperative days 7, 14, 21, and 28 ($P<.05$).

To investigate the extent of revascularization at the microcirculatory level, the capillary density was measured in ischemic tissues by staining with anti-CD31 antibody (Ab). Representative photomicrographs of histological sections stained with anti-CD31 Ab are shown in Figure 1C. Quantitative analysis revealed that the capillary density was significantly reduced in the zinc-deficient mice compared to that in the control mice on postoperative day 28 (Figure 1D and E). Furthermore, we assessed the capillary density by alkaline phosphatase staining. As shown by the result of CD31 immunostaining, the counts of alkaline phosphatase-positive cells were lower in the zinc-deficient mice than in the control mice (Supplementary Figure 2).

In addition, we measured the expression levels of VEGF mRNA as one of the angiogenic factors in the ischemic muscle in the zinc-deficient and control mice on day 28, following hindlimb surgery by real-time PCR. The zinc-deficient mice showed lower VEGF mRNA levels compared to that of the control mice (Figure 1F).

Increased oxidative damage in zinc-deficient mice subjected to hindlimb ischemia

To investigate the production of reactive oxygen species (ROS) in the ischemic muscle, we performed DHE staining analysis of the ischemic muscle of the mice by fluorescence microscopy. Ischemic injury increased the ROS production rate in the adductor muscle to a greater extent in the zinc-deficient mice than in the control mice (Figure 2A).

We next measured the serum levels of d-ROM and nitrotyrosine, an index of oxidative stress, 4 weeks after the hind limb ischemia surgery. The serum d-ROM and nitrotyrosine levels were significantly greater in the zinc-deficient mice than in the control mice (Figure 2B and C).

Furthermore, the mRNA levels of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase components in the ischemic muscle in the zinc-deficient and control mice on day 28 following hindlimb surgery were measured by real-time PCR. The zinc-deficient mice showed higher expression levels of NADPH oxidase components, including Nox2, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ compared to the control mice (Figure 2D). Thus, zinc deficiency impaired the rate of ischemia-induced angiogenesis, which is accompanied by excessive ROS production.

NADPH oxidase inhibitors restore ischemia-induced angiogenesis in zinc-deficient mice

To examine whether excessive ROS production is involved in reducing the angiogenic capacity caused by zinc deficiency, mice were treated with both a zinc-deficient diet and the NADPH oxidase inhibitor apocynin in drinking water from 3 weeks of age (Figure 3A). Then, the mice were subjected to unilateral hindlimb surgery at the age of 10 weeks. Treatment with apocynin partly restored the reduced angiogenic capacity caused by zinc deficiency on day 14 after hindlimb surgery (Figure 3B). Also, apocynin treatment partly restored the reduced capillary density caused by zinc deficiency (Figure 3C).

Association between zinc levels and SPP in patients with CLTI

Finally, we examined the relationship between serum zinc levels and SPP as an index of tissue blood perfusion in patients with CLTI. The clinical characteristics of the patients are shown in Table I and II. A total of 51 patients with CLTI who underwent de novo revascularization were enrolled in this study. The patients were aged 71.4 ± 8.1 years and 66.7% of them were male. Patients' mean BMI was 21.0 ± 4.4 kg/m^2. The average serum zinc and copper levels were 62.3 ± 16.9 μ/dL and 129.3 ± 28.3 μ/dL, respectively. The mean levels of alkaline phosphatase, albumin, hemoglobin, and C-reactive protein were 292.8 ± 97.4 IU/L, 3.4 ± 0.6 g/dL, 11.0 ± 2.0 g/dL, and 2.8 ± 3.5 mg/dL, respectively. The proportions

of smokers and those undergoing hemodialysis were 78.4% and 37.2%, respectively. Type 2 diabetes mellitus in 33 patients (57%), hypertension in 39 patients (78%), dyslipidemia in 27 patients (35%), CAD in 29 patients (41%), and cerebrovascular disease in 10 patients (24%) were observed as concomitant diseases. According to the Rutherford classification, 38 (40.9%) patients had category 4 PAD, 51 (54.8%) showed category 5 disease, and 51 (54.8%) category 6 disease. The wound, ischemia, foot infection stages were as follows: stage 1 (n=4), stage 2 (n=6), stage 3 (n=10), and stage 4 (n=28). Mean SPP was 18.7 ± 8.9 and average ankle brachial index was 0.45 ± 0.30. No patient with CLTI underwent primary major amputation.

We performed univariate linear regression analysis to examine the relationship between SPP and the clinical parameters of the patients with CLTI. Serum zinc levels were positively associated with SPP (r= .538, *P*< .001) (Table III and Figure 4). Albumin levels and the prevalence of diabetes, but not copper levels, were also associated with SPP (Table III). The oral administration of statins, antiplatelet agents, or antihypertensives was not associated with SPP. Multiple linear regression analysis revealed that serum zinc levels were correlated positively with SPP (*P*= .001) (Table III). Thus, serum zinc level was an independent predictor of SPP as an index of tissue blood perfusion in patients with CLTI.

Discussion

The major findings of the present study are as follows: (1) Zinc deficiency contributed to impaired revascularization rates in response to tissue ischemia; (2) Zinc deficiency led to an increase in the production of ROS; (3) an NADPH oxidase inhibitor ameliorated the zinc deficient-induced impairment of revascularization; and (4) Serum zinc levels were positively associated with SPP in patients with CLTI. Thus, our data suggest that zinc can function as a regulator of vascular response to ischemia.

Therapeutic strategies to promote collateral vessel formation and angiogenesis in patients with vascular insufficiency such as CLTI are important for ischemic tissue salvage [21]. Zinc supplementation could be potentially useful in the treatment of such diseases. In addition, mesenchymal stromal cells (MSCs) are known to have the ability to differentiate into a variety of cell lineages, secrete several cytokines, and exert an angiogenic effect by implantation [22-24]. MSC implantation has been performed for CLTI in several institutions with favorable results [22, 24]. A recent study demonstrated that zinc supplementation caused a marked attenuation in the rate of cell apoptosis, enhanced cell viability, and increased the expression of angiogenic cytokines, resulting in the enhancement of the angiogenic effect associated with MSC implantation [25]. These findings suggest that MSC implantation in

combination with zinc supplementation may be highly effective for therapeutic angiogenesis in CLTI patients.

Zinc plays an important role in the activation of antioxidant enzymes, elimination of oxygen-derived free radicals, and generation of free radical scavengers in various organs including the blood vessels [26]. The prophylactic administration of zinc resulted in decreasing rates of oxidative stress and increasing glutathione peroxidase and superoxide dismutase enzyme (SOD) expression levels in a cerebral hypoxia-ischemia rat model [27]. The severity of pressure ulcers (PUs) was exacerbated by zinc deficiency in a cutaneous ischemia-reperfusion injury (I/R) mice model and zinc deficiency increased the degree of cutaneous I/R injury-induced vascular damage, oxidative stress, and apoptosis [12]. The oral supplementation of zinc also improved the severity of zinc deficiency-associated PUs [12]. Clinically, zinc supplementation increases the enzymatic activity of SOD in the blood in patients with type 2 diabetes mellitus, suggesting that it may be useful in patients with PUs [28]. Consistent with these observations, we demonstrated that zinc deficiency impaired the rate of ischemia-induced angiogenesis and increased the ROS levels in ischemic muscles and the blood stream. Furthermore, an NADPH oxidase inhibitor ameliorated the zinc deficiency-

induced impairment of revascularization. Thus, increased ROS production rates may contribute to the impaired angiogenic actions of zinc deficiency under ischemic conditions.

A previous report showed that low serum zinc levels are associated with an increased prevalence of CAD. Significantly lower serum levels of zinc were observed in CAD patients than in those with a normal angiogram among patients who had undergone coronary catheterization [29]. In addition, the serum zinc/urine zinc ratio was inversely associated with CAD [30]. In the present study, serum zinc levels were positively associated with the SPP in patients with CLTI. In addition, we previously showed that zinc deficiency is associated with lower rates of limb salvage in CLTI patients who had undergone bypass surgery [10]. These data suggest that zinc levels may reflect the severity of CLTI. Taken together, circulating zinc levels may be not only a functionally important factor for the modulation of angiogenesis under pathological conditions but also a useful marker for the assessment of atherosclerosis-based vascular disease such as limb ischemia.

In our preliminary data, serum zinc levels were not significantly correlated with albumin levels or the Controlling Nutritional Status (CONUT) Score, which is one of the nutritional indices CONUT scores, in patients with CLTI. In the present study, a multivariate analysis including albumin levels revealed that SPP was strongly correlated with serum zinc

levels alone. Although malnutrition is a factor which reduces zinc levels[5], our results suggest that various factors associated with the pathology of CLTI may affect blood zinc levels more than the nutritional status.

In various guidelines that have been reported for zinc deficiency, zinc deficiency is defined as a serum zinc level of less than 60 μg/dL, and marginal zinc deficiency is defined as a serum zinc level of 60–80 μg/dL [31 32]. In the present study, the mean zinc level in patients with CLTI tended to be as low as 62.3 ± 16.9 μg/dL. It may be preferable for patients with CLTI to actively consume foods with a high zinc content, such as oysters and pork liver. Due to the ease of measurement, zinc levels should be regularly measured in patients with CLTI. If serum zinc levels are extremely low, it may be beneficial to consider treatment with zinc adjuvants.

This study has several limitations. First, in the present study, we measured serum zinc levels at only one time point and did not evaluate the subsequent changes over time. Second, we did not examine whether zinc supplementation could enhance angiogenic capacity or could improve the prognosis of patients with CLTI. We believe that future studies are needed to evaluate the effects of long-term administration of zinc. Third, from an ethical perspective, we could not evaluate angiogenesis by performing immunostaining with muscle

tissue samples collected from patients with CLTI. Thus, in the present study, we were unable to directly evaluate the association between serum zinc levels and angiogenic capacity in humans. Finally, due to the small sample size, we were unable to examine how the different therapeutic strategies used in the patients with CLTI affected the prognosis in the present study.

In conclusion, zinc deficiency impaired the rate of revascularization in response to tissue ischemia, which is accompanied by oxidative damage. Nutritional approaches aimed at the intake of zinc may be useful for the prevention and/or treatment of ischemic limb disease. In addition, the preventive effects and usefulness of zinc need to be further investigated in large-scale studies in the future.

Conflict of interest statement

None.

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References

1. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. Circ Res. 2015;116(9):1509-26.

2. Delaney CL, Smale MK, Miller MD. Nutritional Considerations for Peripheral Arterial Disease: A Narrative Review. Nutrients. 2019;11(6).

3. Strain JJ. Trace elements and cardiovascular disease. Bibl Nutr Dieta. 1998(54):127-40.

4. Alissa EM, Bahijri SM, Ferns GA. The controversy surrounding selenium and cardiovascular disease: a review of the evidence. Med Sci Monit. 2003;9(1):RA9-18.

5. Roohani N, Hurrell R, Kelishadi R, Schulin R. Zinc and its importance for human health: An integrative review. J Res Med Sci. 2013;18(2):144-57.

6. Tomat AL, Costa Mde L, Arranz CT. Zinc restriction during different periods of life: influence in renal and cardiovascular diseases. Nutrition. 2011;27(4):392-8.

7. Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. Maternal and child undernutrition: global and regional exposures and health consequences. Lancet. 2008;371(9608):243-60.

8. Tubek S. Zinc content in lymphocytes and the activity of zinc ion efflux from

lymphocytes in primary arterial hypertension. Biol Trace Elem Res. 2005;107(2):89-99.

9. Kok FJ, Van Duijn CM, Hofman A, Van der Voet GB, De Wolff FA, Paays CH, et al. Serum copper and zinc and the risk of death from cancer and cardiovascular disease. Am J Epidemiol. 1988;128(2):352-9.

10. Koyama A, Kodama A, Tsuruoka T, Fujii T, Sugimoto M, Banno H, et al. Zinc Deficiency and Clinical Outcome After Infrainguinal Bypass Grafting for Critical Limb Ischemia. Circ Rep. 2020;3:167-73.

11. Duscha BD, Robbins JL, Jones WS, Kraus WE, Lye RJ, Sanders JM, et al. Angiogenesis in skeletal muscle precede improvements in peak oxygen uptake in peripheral artery disease patients. Arterioscler Thromb Vasc Biol. 2011;31(11):2742-8.

12. Nakamura H, Sekiguchi A, Ogawa Y, Kawamura T, Akai R, Iwawaki T, et al. Zinc deficiency exacerbates pressure ulcers by increasing oxidative stress and ATP in the skin. J Dermatol Sci. 2019;95(2):62-9.

13. Hori A, Shibata R, Morisaki K, Murohara T, Komori K. Cilostazol stimulates revascularisation in response to ischaemia via an eNOS-dependent mechanism. Eur J Vasc Endovasc Surg. 2012;43(1):62-5.

14. Yamaguchi S, Shibata R, Ohashi K, Enomoto T, Ogawa H, Otaka N, et al. C1q/TNF-

Related Protein 9 Promotes Revascularization in Response to Ischemia via an eNOS-Dependent Manner. Front Pharmacol. 2020;11:1313.

15. Harraz MM, Marden JJ, Zhou W, Zhang Y, Williams A, Sharov VS, et al. SOD1 mutations disrupt redox-sensitive Rac regulation of NADPH oxidase in a familial ALS model. J Clin Invest. 2008;118(2):659-70.

16. Kito T, Shibata R, Kondo M, Yamamoto T, Suzuki H, Ishii M, et al. Nifedipine ameliorates ischemia-induced revascularization in diet-induced obese mice. Am J Hypertens. 2012;25(4):401-6.

17. Kamei S, Fujikawa H, Nohara H, Ueno-Shuto K, Maruta K, Nakashima R, et al. Zinc Deficiency via a Splice Switch in Zinc Importer ZIP2/SLC39A2 Causes Cystic Fibrosis-Associated MUC5AC Hypersecretion in Airway Epithelial Cells. EBioMedicine. 2018;27:304-16.

18. Kambara T, Shibata R, Ohashi K, Matsuo K, Hiramatsu-Ito M, Enomoto T, et al. C1q/Tumor Necrosis Factor-Related Protein 9 Protects against Acute Myocardial Injury through an Adiponectin Receptor I-AMPK-Dependent Mechanism. Mol Cell Biol. 2015;35(12):2173-85.

19. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, et al.

Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg. 2007;33 Suppl 1:S1-75.

20. Yamada T, Ohta T, Ishibashi H, Sugimoto I, Iwata H, Takahashi M, et al. Clinical reliability and utility of skin perfusion pressure measurement in ischemic limbs--comparison with other noninvasive diagnostic methods. J Vasc Surg. 2008;47(2):318-23.

21. Grochot-Przeczek A, Dulak J, Jozkowicz A. Therapeutic angiogenesis for revascularization in peripheral artery disease. Gene. 2013;525(2):220-8.

22. Hou L, Kim JJ, Woo YJ, Huang NF. Stem cell-based therapies to promote angiogenesis in ischemic cardiovascular disease. Am J Physiol Heart Circ Physiol. 2016;310(4):H455-65.

23. Kondo K, Shintani S, Shibata R, Murakami H, Murakami R, Imaizumi M, et al. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. Arterioscler Thromb Vasc Biol. 2009;29(1):61-6.

24. Katagiri T, Kondo K, Shibata R, Hayashida R, Shintani S, Yamaguchi S, et al. Therapeutic angiogenesis using autologous adipose-derived regenerative cells in patients with critical limb ischaemia in Japan: a clinical pilot study. Sci Rep. 2020;10(1):16045.

25. Zhang D, Li Y, Zhu T, Zhang F, Yang Z, Miao D. Zinc supplementation results in

improved therapeutic potential of bone marrow-derived mesenchymal stromal cells in a mouse ischemic limb model. Cytotherapy. 2011;13(2):156-64.

26. Maret W. The redox biology of redox-inert zinc ions. Free Radic Biol Med. 2019;134:311-26.

27. Tomas-Sanchez C, Blanco-Alvarez VM, Martinez-Fong D, Gonzalez-Barrios JA, Gonzalez-Vazquez A, Aguilar-Peralta AK, et al. Prophylactic Zinc and Therapeutic Selenium Administration Increases the Antioxidant Enzyme Activity in the Rat Temporoparietal Cortex and Improves Memory after a Transient Hypoxia-Ischemia. Oxid Med Cell Longev. 2018;2018:9416432.

28. Nazem MR, Asadi M, Jabbari N, Allameh A. Effects of zinc supplementation on superoxide dismutase activity and gene expression, and metabolic parameters in overweight type 2 diabetes patients: A randomized, double-blind, controlled trial. Clin Biochem. 2019;69:15-20.

29. Kazemi-Bajestani SM, Ghayour-Mobarhan M, Ebrahimi M, Moohebati M, Esmaeili HA, Parizadeh MR, et al. Serum copper and zinc concentrations are lower in Iranian patients with angiographically defined coronary artery disease than in subjects with a normal angiogram. J Trace Elem Med Biol. 2007;21(1):22-8.

30. Giannoglou GD, Konstantinou DM, Kovatsi L, Chatzizisis YS, Mikhailidis DP. Association of reduced zinc status with angiographically severe coronary atherosclerosis: a pilot study. Angiology. 2010;61(5):449-55.

31. Kodama H, Tanaka M, Naito Y, Katayama K, Moriyama M. Japan's Practical Guidelines for Zinc Deficiency with a Particular Focus on Taste Disorders, Inflammatory Bowel Disease, and Liver Cirrhosis. Int J Mol Sci. 2020;21(8).

32. Singh P, Nee J. Zinc Deficiency. BMJ Best Practice. 2018.

Clinical Relevance: This study aimed to investigate the impact of zinc deficiency on angiogenesis. We found that zinc deficiency impaired the rate of ischemia-induced revascularization through enhanced oxidative stress rates in animal model. In addition, serum zinc levels were positively associated with SPP in the CLTI patients. Thus, the intake of zinc could be useful for the prevention and/or treatment of ischemic limb disease. Circulating zinc level could be a useful marker for the assessment of atherosclerosis-based vascular disease such as limb ischemia. Possibly, nutritional improvement by zinc intake could lead to prevention and treatment of ischemic vascular disease.

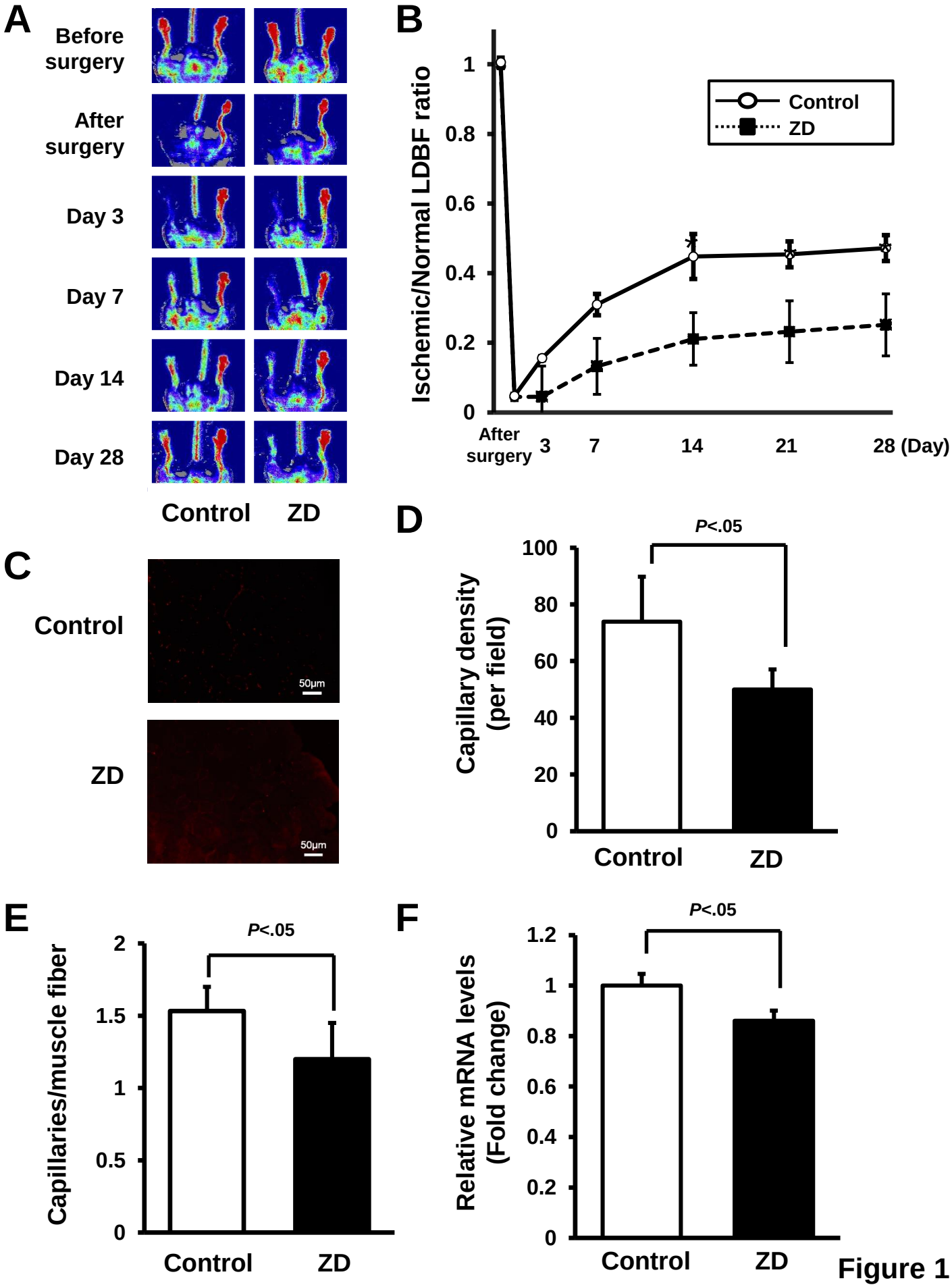

A
Before surgery
After surgery
Day 3
Day 7
Day 14
Day 28
Control
ZD
B
Ischemic/Normal LDBF ratio
1
0.8
0.6
0.4
0.2
0
Control
ZD
After surgery
3
7
14
21
28 (Day)
C
Control
ZD
50μm
50μm
D
Capillary density (per field)
P<.05
100
80
60
40
20
0
Control
ZD
E
Capillaries/muscle fiber
P<.05
2
1.5
1
0.5
0
Control
ZD
F
Relative mRNA levels (Fold change)
P<.05
1.2
1
0.8
0.6
0.4
0.2
0
Control
ZD

Figure 1

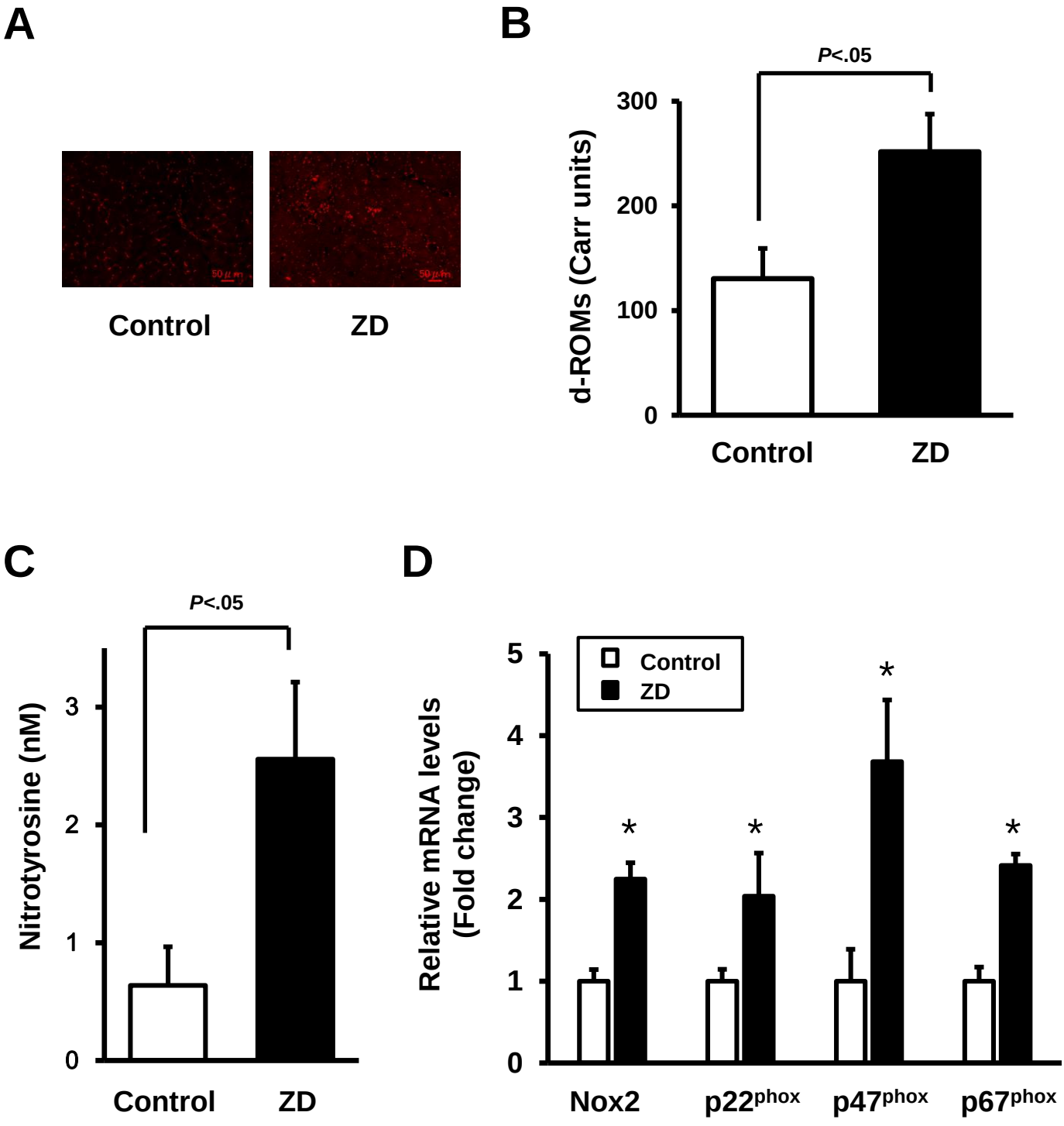

A
Control
ZD
B
P<.05
d-ROMs (Carr units)
300
200
100
0
Control
ZD
C
P<.05
Nitrotyrosine (nM)
3
2
1
0
Control
ZD
D
Relative mRNA levels
(Fold change)
Control
ZD
5
4
3
2
1
0
*
Nox2
p22phox
p47phox
p67phox

Figure 2

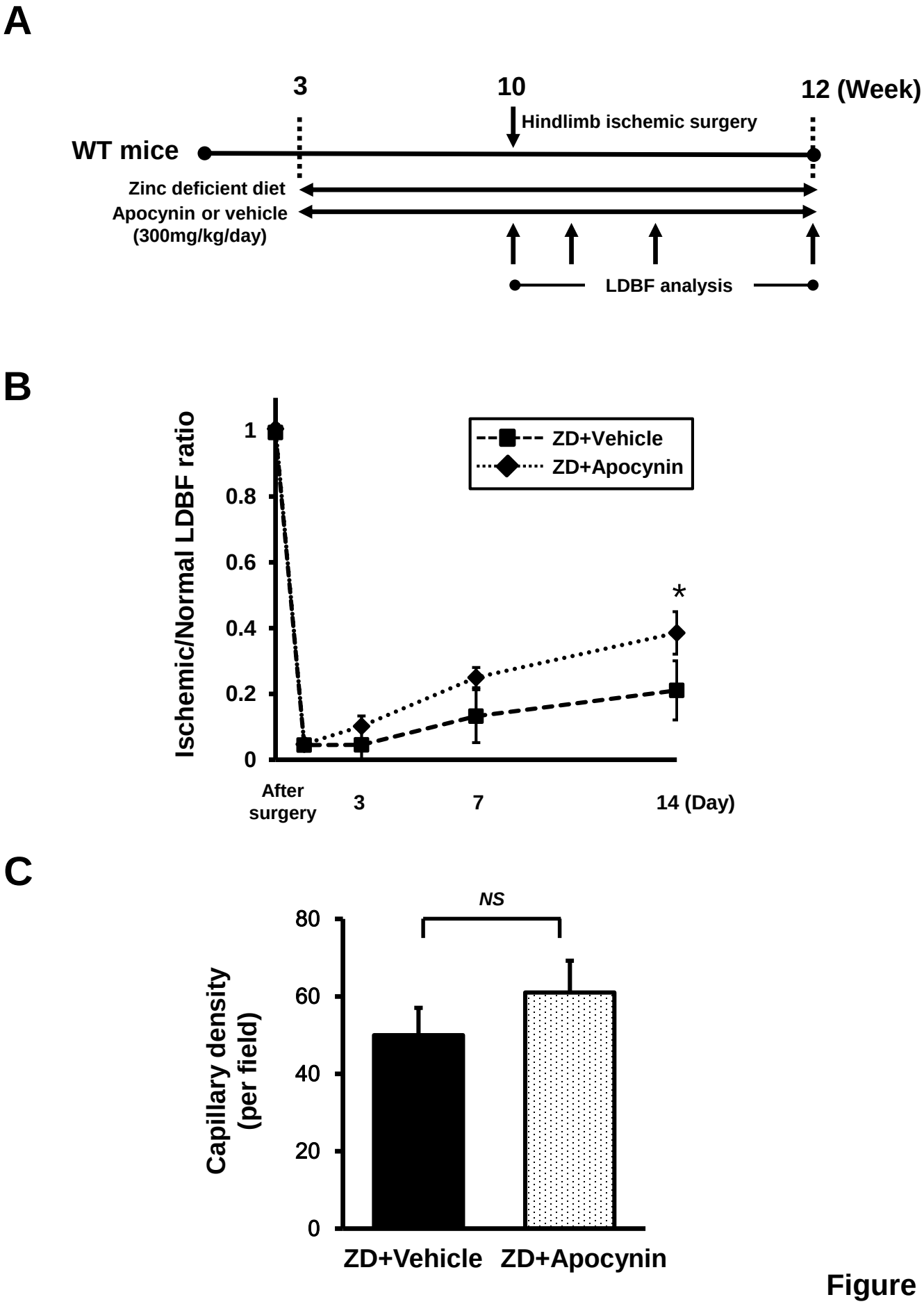

A
3
10
12 (Week)
Hindlimb ischemic surgery
WT mice
Zinc deficient diet
Apocynin or vehicle
(300mg/kg/day)
LDBF analysis
B
Ischemic/Normal LDBF ratio
1
0.8
0.6
0.4
0.2
0
ZD+Vehicle
ZD+Apocynin
*
After surgery
3
7
14 (Day)
C
NS
Capillary density (per field)
80
60
40
20
0
ZD+Vehicle
ZD+Apocynin

Figure 3

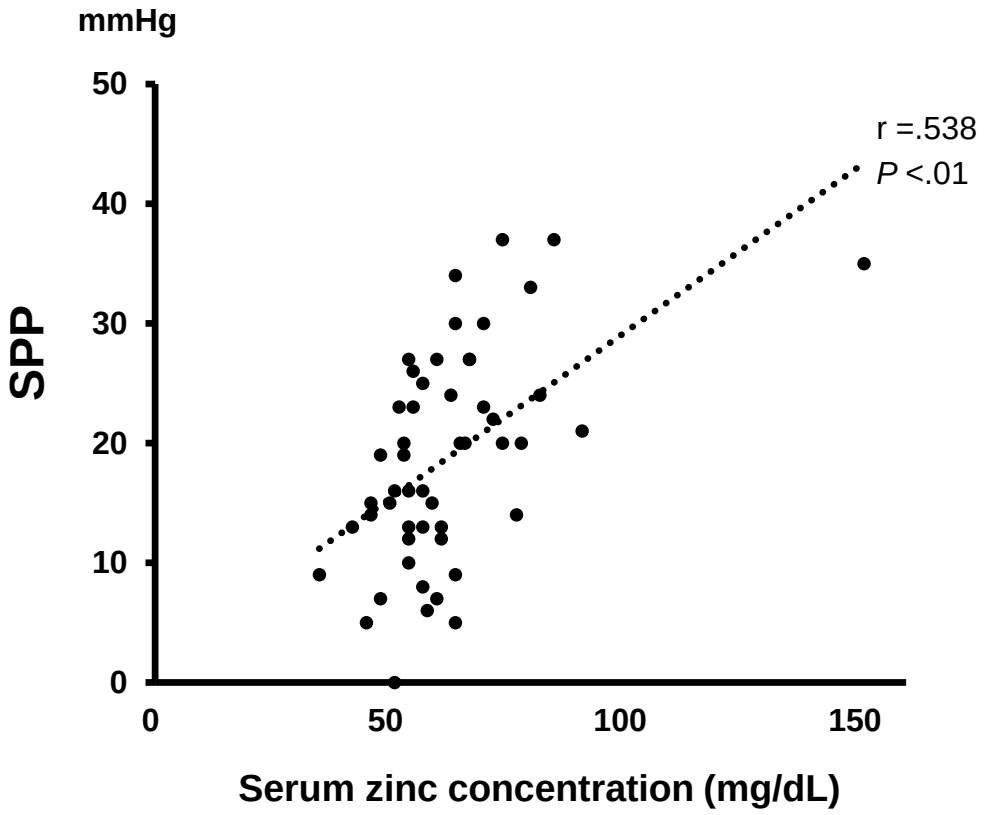
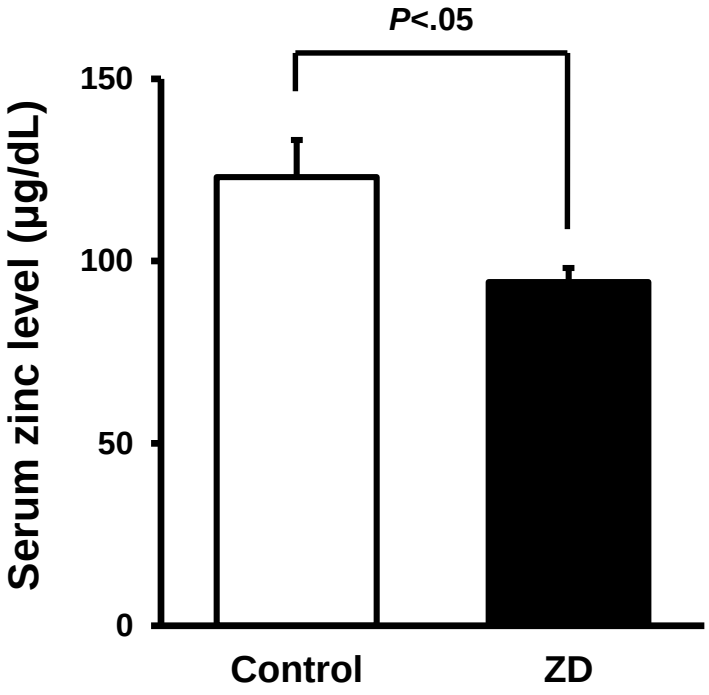
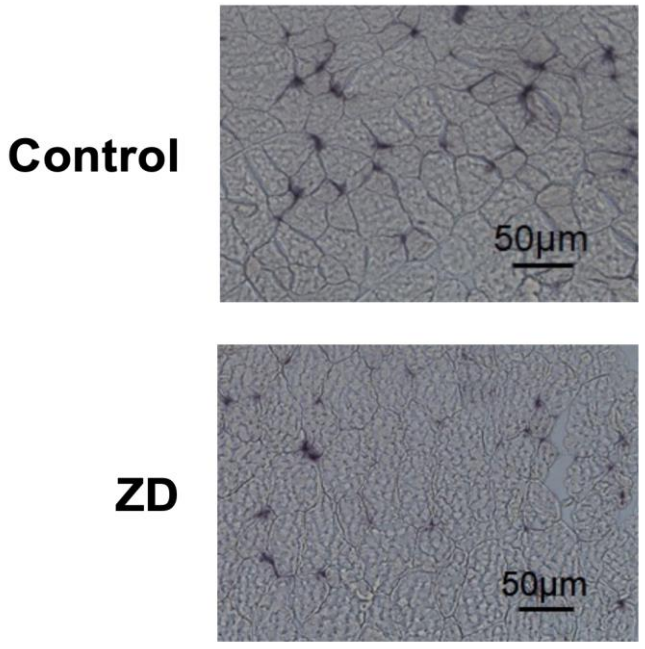

mmHg
50
40
30
20
10
0
SPP
r =.538
P <.01
0
50
100
150
Serum zinc concentration (mg/dL)

Figure 4



Supplementary Figure 1



Supplementary Figure 2

Table I Patient characteristics

Characteristic	(n = 51)
Age (years)	71.4 ± 8.1
Male/Female	34/17
Body mass index (kg/m^2)	21.0 ± 4.4
Serum zinc concentration (μg/dL)	62.3 ± 16.9
Serum copper concentration (μg/dL)	129.3 ± 28.3
Alkaline phosphatase (IU/L)	292.8 ± 97.4
Albumin (g/dL)	3.4 ± 0.6
Hemoglobin (g/dL)	11.0 ± 2.0
C-reactive protein (mg/dL)	2.8 ± 3.5
Hypertension	39
Diabetes mellitus	33
Hemodialysis	19
Dyslipidemia	27
Smoker	40
Coronary artery disease	29
Cerebrovascular disease	10
Ankle Brachial index	0.45 ± 0.30
Skin perfusion pressure	18.7 ± 8.9
Drug	
α-blocker	4
β-blocker	17
Ca-blocker	31
Angiotensin II receptor blocker	18
Angiotensin-converting-enzyme inhibitor	3
Diuretic	6
Cilostazol	25
Clopidogrel	19
Aspirin	36
Warfarin	9
Direct oral anticoagulant	1
Statin	21
Insulin	6
Prednisolone	4

Categorical data are expressed as numbers. Continuous data are expressed as the means ± SD

Table II Patient classification and treatments

Characteristic	(n = 51)
Rutherford classification	
4	5(10)
5	38(74)
6	8(16)
WIfI clinical stage	
1	2(4)
2	8(16)
3	17(33)
4	24(47)
GLASS stage	
1	2(4)
2	2(4)
3	47(92)
Treatments	
Bypass surgery	34(67)
Endovascular surgery	2(4)
Bypass & Endovascular surgery	15(29)

Categorical data are expressed as numbers (%).

GLASS = Global Limb Anatomic Staging System

Table III Correlation with skin perfusion pressure

Variable	Univariate		Multivariate	
	ρ	*P*-value	β	*P*-value
Serum zinc level	.538	**.000**	0.445	**.001**
Albumin	.302	**.031**	0.147	.265
Diabetes mellitus	.288	**.041**	0.240	.058
Hemoglobin	.255	.071		
Age	- .195	.171		
Body mass index	.194	.173		
Antiplatelets	.193	.174		
C-reactive protein	- .191	.179		
Hemodialysis	.178	.212		
Statin	.163	.254		
Cerebrovascular disease	.158	.268		
Antihypertensives	.147	.302		
Hypertension	.082	.568		
Female	.061	.671		
Smoker	- .047	.743		
Alkaline phosphatase	.042	.772		
Coronary artery disease	.024	.865		
Dyslipidemia	- .017	.904		
Serum copper level	.008	.956		

Figure Legends

Figure 1. Zinc-deficient mice showing reductions in the rates of perfusion recovery and capillary vessel formation in ischemic limbs. A. Representative LDBF images in the ischemic limb of zinc-deficient mice or control WT mice. A low perfusion signal (dark blue) was observed in the ischemic hindlimb of zinc-deficient mice, whereas a high perfusion signal (red) was detected in the control WT mice at postoperative days 14, 21, and 28. **B.** Quantitative analysis of the ischemic to nonischemic LDBF ratio in the zinc-deficient mice or control WT mice before surgery and at different time points after surgery. Results are presented as the mean ± standard deviation (n=8 in each group). *P< .05 vs Zinc-deficient mice. **C.** Fluorescence staining of ischemic tissues with anti-CD31 monoclonal antibody (red) on postoperative day 28. **D and E.** Quantitative analysis of capillary density in zinc-deficient mice or the control WT mice on postoperative day 28. Capillary density was expressed as the number of capillaries per high power field (×400) (left) and capillaries per muscle fiber (right). **F.** mRNA levels of VEGF in the ischemic muscle in the zinc-deficient mice or control WT mice on postoperative day 28. Levels of mRNA were measured using the real-time polymerase chain reaction method (n=8 in each group). All results are normalized to GAPDH. *P< .01 vs control. Results are presented as mean ± standard deviation (n=8 in each group). LDBF, laser Doppler blood flow; WT,

wild type; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 2. Zinc deficiency increases the rate of oxidative damage in ischemic muscles. **A.** Production of reactive oxygen species (ROS) was evaluated by immunostaining with dihydroethidium on postoperative day 28 (×400 each field; red). **B and C.** Serum levels of the derivatives of reactive oxygen metabolites (d-ROMs) **(B)** and nitrotyrosine **(C)** in the zinc-deficient mice or the control WT mice after hindlimb surgery (n=8 in each group). **D.** mRNA levels of Nox2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ in the ischemic muscle in the zinc-deficient mice or control WT mice on postoperative day 28. Levels of mRNA were measured using the real-time polymerase chain reaction method (n=8 in each group). All results are normalized to GAPDH. *P< .01 vs control. WT, wild type; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Figure 3. NADPH oxidase inhibitor, apocynin, restores ischemia-induced angiogenesis in zinc-deficient mice. A. Schematic illustration of the experimental protocol. Zinc-deficient and control mice were treated with both a zinc-deficient diet and an NADPH oxidase inhibitor, apocynin (300 mg/kg/day), in drinking water from 3 weeks of age. Then, the mice were subjected to unilateral hindlimb surgery at the age of 10

weeks. **B.** Quantitative analysis of the ischemic to nonischemic LDBF ratio in the zinc-deficient mice treated with apocynin at different time points after surgery. **C.** the quantitative analysis of capillary density of the ischemic muscle in the zinc- deficient mice treated with apocynin at 14 days after surgery. Results are shown as the mean ± standard deviation (n=8 in each group). *P< .05. NADPH, nicotinamide adenine dinucleotide phosphate; LDBF, laser Doppler blood flow, NS, nonsignificant.

Figure 4. Association of serum zinc level with skin perfusion pressure (SPP) as an index of tissue blood perfusion in patients with chronic limb-threatening ischemia (CLTI). A total of 51 patients with CLTI who referred for de novo revascularization were enrolled in this study. The results are presented as the mean ± standard error.

Table Legends

Table I The clinical characteristics of the patients are shown. The patients with CLTI who underwent de novo revascularization were enrolled in this study (N=51).

Table II The classification and treatments of the patients are shown. The patients with CLTI who underwent de novo revascularization were enrolled in this study (N=51).

Table III Univariate linear regression analysis to examine the relationship between SPP and the clinical parameters of the patients with CLTI (N=51).