



Green coffee bean extract rich in chlorogenic acids prevents muscle capillary regression via inhibiting oxidative stress and enhancing angiogenesis.

邢, 吉昊

(Degree)

博士 (保健学)

(Date of Degree)

2025-03-25

(Date of Publication)

2026-03-01

(Resource Type)

doctoral thesis

(Report Number)

甲第9182号

(URL)

<https://hdl.handle.net/20.500.14094/0100496463>

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



博 士 論 文

Green coffee bean extract rich in chlorogenic acids
prevents muscle capillary regression via inhibiting
oxidative stress and enhancing angiogenesis.

(クロロゲン酸を豊富に含む生コーヒー豆抽出物は酸化ストレスの抑制と血管新生の促進を通じて、筋肉毛細血管の退行を防止する)

令和 7 年 1 月 20 日

神戸大学大学院保健学研究科保健学専攻

邢 吉昊

Biomedical Research (Tokyo)

Original Article

Green coffee bean extract rich in chlorogenic acids prevents muscle capillary regression via inhibiting oxidative stress and enhancing angiogenesis.

Jihao XING¹, Hao LIN¹, Ryosuke NAKANISHI¹, Kazuya IWAI², Noriaki MAESHIGE¹, Hiroyo KONDO¹,
and Hidemi FUJINO¹

¹Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences, Kobe, Japan

²R&D Department, UCC Ueshima Coffee Co., Ltd.

The e-mail addresses of all authors are as follows:

Jihao Xing: xingjihao1995@gmail.com; Hao Lin: linhao_china1995@yahoo.co.jp;

Ryosuke Nakanishi: rnakanishi@kobe-kui.ac.jp; Kazuya Iwai: [kazuya-](mailto:kazuya-iwai@ucc.co.jp)

iwai@ucc.co.jp; Noriaki Maeshige: nmaeshige@pearl.kobe-u.ac.jp; Hiroyo Kondo:

kondohiroyo@gmail.com; Hidemi Fujino: fujino@phoenix.kobe-u.ac.jp

Running title: CGA prevents capillary loss

Correspondence author

Hidemi Fujino, Ph.D.

Professor, Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma-Ku, Kobe 654-0142, Japan.

Tel: +81-78-796-4542, Fax: +81-78-796-4542 E-mail: fujino@phoenix.kobe-u.ac.jp

Abstract:

Reactive oxygen species (ROS) production in disused skeletal muscle induces capillary regression, which disrupts the balance of pro- and anti-angiogenic factors. We tested whether green coffee bean extract containing rich chlorogenic acid, which has antioxidant properties, can prevent capillary regression caused by muscle atrophy. The study included 24 female Sprague-Dawley rats, randomly assigned to four groups: control (CON), 2-week hindlimb unloading (HU), 2-week hindlimb unloading supplemented with coffee extract rich in chlorogenic acids (HU+50%CGA), and 2-week hindlimb unloading supplemented with trace amounts of chlorogenic acids (HU+5%CGA). Rats in the HU+50%CGA and HU+5%CGA groups received an oral dose of coffee extract at 850 mg/kg per day. The effects of chlorogenic acids in coffee extracts were investigated by comparing these groups. Unloading increased oxidative stress, disrupted mitochondrial oxidative activity, and upregulated TSP-1 expression, triggering endothelial cell apoptosis and leading to capillary regression. In contrast, the supplementation with coffee extract containing rich chlorogenic acids prevented ROS overproduction and improved metabolic activity, promoting angiogenesis by correcting the imbalance of pro- and anti-angiogenic factors, and inhibiting endothelial cell apoptosis. In conclusion, green coffee bean extract rich in chlorogenic acids inhibits ROS production, enhances mitochondrial metabolism, mitigates capillary regression by promoting angiogenesis and preventing apoptosis.

Key words: Muscle atrophy, Oxidative stress, Angiogenesis, Chlorogenic acid

Introduction

Capillaries are vital for transporting oxygen and nutrients to skeletal muscle tissues, and their functionality is directly related to exercise capacity (Olfert and Birot, 2011; Fujino et al., 2014). Muscle inactivity, such as during bed rest, immobilization, or spaceflight, contribute to both a decline in muscle size and strength as well as capillary regression in the muscle (Hendrickse et al., 2022). Long-term inactivity, exemplified by hindlimb unloading, contributes to increased oxidative stress, stemming from elevated levels of reactive oxygen species (ROS) (Wüst et al., 2009; Kanazashi et al., 2014). In addition, capillary networks are preserved through a delicate equilibrium of pro-angiogenic and anti-angiogenic factors, which control capillary growth or regression. Examples of such factors include vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) (Byrne et al., 2005; Chen et al., 2000). Moreover, Angiopoietin1 (Ang1) supports vascular maturation, while Angiopoietin2 (Ang2) breaks the connection between endothelial and perivascular cells, leading to cell death and vascular regression. The expression of these proteins is affected by apoptotic signaling (Fagiani and Christofori, 2013).

Chlorogenic acids (CGA) have garnered significant attention in recent research focused on the cardiovascular protection effect of coffee (Lukitasari et al., 2020). In general, Green coffee beans are currently regarded as the most important plant source of CGAs (Rojas-González et al., 2022; Farah and Donangelo, 2006; Raskar and Bhalekar, 2019). According to our previous study, green coffee extract proved to be a beneficial treatment in preventing capillary regression induced by muscle atrophy (Xing et al., 2008). However, the role of chlorogenic acids in green coffee bean extract

as a key component in mitigating capillary retraction remains uncertain, and further investigation is needed to determine whether its effectiveness is concentration-dependent.

Our study compared the effects of rich chlorogenic acids and trace amounts of chlorogenic acids intake from green coffee bean extract on the skeletal muscle capillary network by examining changes in angiogenic signaling and mitochondrial metabolism during hindlimb unloading. Our hypothesis was that an extract with higher levels of chlorogenic acids would be more effective in preventing capillary regression linked to muscle atrophy. If confirmed, this study could emphasize the role of chlorogenic acids in green coffee beans in strengthening the capillary network, suggesting it as a focused nutritional approach to counteract inactivity-related capillary regression.

Materials and methods

1. Experimental groups

For the study, twenty female Sprague-Dawley rats (average weight: 320 ± 6 g) were used, and these rats were acquired from Japan SLC (Hamamatsu, Japan). Once the one-week acclimatization period was complete, the rats were divided into four groups: control (CON), hindlimb unloading (HU), hindlimb unloading combined with green coffee bean extract rich in chlorogenic acids (50%) (HU + 50% CGA), and hindlimb unloading combined with trace amounts of chlorogenic acids in green coffee bean (5%) (HU + 5% CGA). This research was conducted with the approval of the Institutional Animal Care and Use Committee and was performed in line with Kobe University

Animal Experimentation Regulations. All procedures adhered to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, as established by the National Research Council in 1996.

2. Hindlimb unloading

Following the procedure detailed by Morey et al. (Morey et al., 1979), hindlimb unloading was implemented with certain modifications. In summary, each rat was secured by its tail with adhesive tape and kite string, which kept its hindlimbs from touching the cage floor or walls. though the forelimbs remained in contact with the floor of the cage. Furthermore, the rats' hindlimbs remained unloaded for a period of two weeks. The animals were maintained in a dedicated room with environmental controls set to a temperature of $22 \pm 2^{\circ}\text{C}$ and a 12-hour light-dark schedule, and had ad libitum access to food and water.

3. Green coffee bean extract containing chlorogenic acids

The preparation of the green coffee extract followed a specific protocol. Initially, green coffee beans (Brazil Santos) were treated with supercritical fluid carbon dioxide for a set period to remove caffeine and other substances such as coffee oils (Patent No. P2023-29828A). After decaffeination, the decaffeinated green coffee bean extract was made by ethanol extraction, concentration, and spray drying. The extracts were then processed through column chromatography to divide them into two separate fractions: a 325-nm absorbent fraction with 50% chlorogenic acids, and a non-absorbent fraction with 5% chlorogenic acids. In this procedure, a minimal

amount of 0.005% (v/v) acetic acid was used to dissolve the coffee beans. The resulting solution was loaded onto a Toyopearl HW-40 column (dimensions: $2.0 \times 2.0 \times 3.14 \times 40.0$ cm) and eluted stepwise with 0.005% (v/v) acetic acid, followed by 50% (v/v) methanol, and then 99.5% (v/v) methanol, with the flow rate maintained 1.2 mL/min. The column effluent was continuously monitored for absorbance at 325 nm to detect chlorogenic acids. The fractions showing absorbance at 325 nm (chlorogenic acid-rich) and those without absorption were collected separately. Following the protocol outlined by Iwai et al. (2012) and UCC Ueshima Coffee Co. LTD., Japan, underwent ethanol extraction, concentration via evaporation, freeze-drying, and was stored for later use. Rats in the HU + 50% CGA and HU + 5% CGA groups received a daily oral dose of chlorogenic acids dissolved in distilled water (850 mg/kg/day). The rats in the other groups received oral doses of distilled water. Following a 4-day acclimatization period, all rats continued to receive either distilled water or chlorogenic acids during the HU period.

4. Muscle preparation

Twenty-four hours following the final oral administration, all rats were placed under deep anesthetized using 4% isoflurane inhalation and then euthanized with an intraperitoneal dose of sodium pentobarbital (100 mg/kg). The soleus muscles were removed, weighed, and swiftly frozen using a dry ice-isopentane bath, then stored at -80°C pending analysis.

5. Histological analysis

Transverse sections of the soleus muscle, each 10 μ m in thickness, were cut at -25°C on a cryostat (CM-1510S, Leica Microsystems, Mannheim, Germany) and placed on glass slides.

To visualize capillaries, the transverse sections were stained for alkaline phosphatase (AP). Sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 45 minutes at 37°C, followed by fixation with 4% paraformaldehyde. The capillary-to-fiber (C/F) ratio was calculated by counting capillaries and myofibers on each cryosection from AP-stained images under the microscope.

To evaluate skeletal muscle oxidative capacity, sections were stained for succinate dehydrogenase (SDH) as described by Kanazashi et al. (Kanazashi et al., 2019). The sections were incubated in a reaction buffer with nitroblue tetrazolium, 0.2 M sodium succinate, and 0.2 M phosphate buffer at 37°C for 45 minutes. SDH activity was assessed via densitometric analysis, and fiber cross-sectional area (FCSA) was measured across groups.

Additional sections underwent staining for myofibrillar adenosine triphosphatase (ATPase) after being preincubated at pH 4.2 to determine muscle fiber types. These sections were then used to assess the percentage of slow muscle fibers.

Dihydroethidium (DHE) was utilized as a fluorescent probe to evaluate intracellular ROS, producing oxyethidium and emitting fluorescence when it reacts with superoxide (O₂⁻). DHE is capable of permeating cells and binds with nucleic acids to emit a bright fluorescence, which can be detected qualitatively using fluorescent microscopy (model

BZ-X800; Keyence). Transverse sections were incubated with a solution of 5×10^{-6} mol/l DHE (Wako Pure Chemicals, Osaka, Japan) at 37 °C for 30 minutes in a darkened environment. Afterward, they were rinsed with phosphate-buffered saline (PBS) warmed to 37 °C and examined under a fluorescent microscope with a 545 nm excitation filter. The fluorescence area fraction was calculated to determine fluorescence intensity, based on grayscale analysis of five images for each muscle. NIH ImageJ software (Bethesda, MD, USA) facilitated these measurements.

6. Analysis of apoptosis on cross-sectional specimens.

The TUNEL staining method was employed to identify apoptotic endothelial cells within the soleus muscle. The 10 µm thick sections were fixed in 4% paraformaldehyde for 10 minutes, followed by blocking with 3% hydrogen peroxide in methanol and 3% bovine serum albumin in PBS, with incubation at 37°C for 30 minutes. They were then rinsed in PBS three times, with each rinse lasting for 10 minutes. Then sections were then immunohistochemically stained with a CD31 monoclonal antibody (1:200 dilution, sc-376764, Santa Cruz, USA) to identify endothelial cells. Afterward, the DyLight 594-coupled secondary antibody (1:500 dilution; Jackson, West Grove, PA, USA) was applied, followed by the TUNEL reagent (Takara, Tokyo, Japan) as per the manufacturer's instructions. The slides were ultimately mounted using DAPI-Fluoromount G (SouthernBiotech, Birmingham, AL, USA). Fluorescence microscopy (BZ-X800; Keyence, Osaka, Japan) was then employed to analyze the stained sections,

and yellow TUNEL-positive endothelial cells were counted in each soleus muscle section.

7. Evaluation of Superoxide Dismutase (SOD) Activity

To assess SOD activity, a SOD Assay Kit -WST was employed, with procedures conducted according to the manufacturer's instructions (Dojindo Molecular Technologies Inc., Tokyo, Japan).

8. Western blot analysis

Frozen tissue samples (~10 mg each) were homogenized in EzRIPA lysis buffer (ATTO, Tokyo, Japan) containing 1% (v/v) phosphatase and protease inhibitor cocktails. To assess total protein concentration, the Bio-Rad protein determination kit (Hercules, CA, USA) was used following the manufacturer's protocol. The homogenates were dissolved in EzApply sample loading buffer (ATTO, Tokyo, Japan) and subsequently heated at 80°C for 10 minutes to ensure proper sample preparation.

A 5-20% SDS polyacrylamide gel (ATTO, Tokyo, Japan) was used to separate protein samples, with 30 µg loaded into each lane. Following transfer to PVDF membranes, the proteins were blocked for 1 hour in a solution of 3% bovine serum albumin in PBST (phosphate-buffered saline with Tween-20). The membranes were incubated at 4 °C overnight with primary antibodies, including mouse anti-Vascular Endothelial Growth Factor (VEGF) (diluted 1:1000 in PBST; MAA143Ra21; Cloud-

Clone USCN Life Science, Wuhan, China), mouse anti-Thrombospondin 1 (TSP-1) (diluted 1:1000 in PBST; #37879; Cell Signaling Technology Japan, Tokyo, Japan), rabbit anti- Angiopoietin 1 (ANG1) (1:1000 in PBST; 23302-1-AP; Proteintech, Chicago, IL, USA), goat anti- Angiopoietin 2 (ANG2) (diluted 1:200 in PBST; sc-7015; Santa Cruz), rabbit anti-Tie-2 (diluted 1:1000 in PBST; 19157-1-AP; Proteintech), rabbit anti-Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) (1:2000 in PBST; 16396-1-AP; Proteintech), rabbit anti-Heme oxygenase-1 (HO-1) (diluted 1:1000 in PBST; 10701-1-AP; Proteintech), rabbit anti-SOD2 (diluted 1:1000 in PBST; CSB-PA006154; Cusabio Technology LLC, Houston, USA) and rabbit anti-Caspase 3 (diluted 1:1000 in PBST; 19677-1-AP; Proteintech). Afterward, they were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (1:5000 in PBST) for 1 hour. Protein detection was carried out with EzWestLumi One (ATTO, Tokyo, Japan), and images were subsequently captured using the LuminoGraphI imaging system (ATTO, Tokyo, Japan).

9. Statistical analysis

All values are expressed as means $\pm$ standard error of the mean (SEM). A one-way ANOVA was used to analyze group differences, and Tukey's post hoc test was conducted to identify specific differences between groups. Statistical significance was considered at $P < 0.05$ for all tests.

Results

1. Body mass, soleus muscle and relative muscle weight

Compared to the CON group, the HU, HU+50%CGA, and HU+5%CGA groups showed significantly lower body mass, soleus muscle weight, and relative muscle weight. There were no significant differences among the HU, HU+50%CGA, and HU+5%CGA groups themselves (Table 1). These results suggest that unloading caused reductions in body mass and soleus muscle weight.

2. Capillary Quantity

Figure 1A-D shows representative images of stained capillaries in the soleus muscles. The C/F ratio in the HU, HU+50%CGA, and HU+5%CGA groups was significantly lower than in the CON group. Additionally, the HU+50%CGA group had a significantly higher C/F ratio than the HU and HU+5%CGA groups (Figure 1M). These results suggest that HU damaged the capillary network, whereas coffee extract rich in CGA helped prevent capillary regression in skeletal muscle.

3. Fiber cross-sectional areas and oxidative enzyme activity

In Figure 1E-H, representative images of SDH staining for the various groups are shown. SDH activity was significantly lower in the HU and HU+5%CGA groups compared to the CON and HU+50%CGA groups, the HU+50%CGA group exhibited significantly higher SDH activity than the HU and HU+5%CGA groups (Figure 1N). The soleus muscle fiber cross-sectional area (FCSA) was also significantly reduced in the HU, HU+50%CGA, and HU+5%CGA groups relative to the CON group, but FCSA

in the HU+50%CGA group was significantly larger than in the HU and HU+5%CGA groups (Figure 1O). These results suggest that coffee extract rich in CGA mitigated the HU-induced reductions in mitochondrial oxidase activity and FCSA.

4. Slow fiber composition

Figure 1I-L shows representative images of ATPase-stained sections for each group. Slow fiber composition was markedly decreased in both the HU and HU+5%CGA groups relative to the CON group. Additionally, the HU+50%CGA group exhibited a significantly higher slow fiber composition compared to the HU and HU+5%CGA groups (Figure 1P). These findings indicate that a transformation from slow to fast muscle fiber types occurred in the HU condition, while treatment with CGA-rich coffee extract prevented this transformation.

5. Generation of ROS

Nuclear DHE fluorescence was analyzed to determine intracellular ROS production. In Figure 2A-L, DHE-stained images of the soleus muscle are displayed for each group. The HU and HU+5%CGA groups exhibited significantly higher DHE fluorescence intensity compared to the CON and HU+50%CGA groups. Compared to the HU and HU+5%CGA groups, the HU+50%CGA group showed a significant decrease in fluorescence intensity (Figure 2M). These results indicate that treatment with coffee bean extract rich in CGA effectively reduces ROS overproduction.

6. Endothelial cell apoptosis evaluation

To identify apoptosis in endothelial cells, TUNEL and CD-31 staining was performed on transverse sections of the soleus muscle. Figure 3A-P provides representative images of endothelial cells that are TUNEL-positive. The frequency of TUNEL-positive cells was significantly higher in the HU and HU+5%CGA groups compared to the CON and HU+50%CGA groups. The HU+50%CGA group exhibited a significantly lower frequency than both the HU and HU+5%CGA groups, and the HU+5%CGA group also had a significantly lower frequency than the HU group (Figure 3Q).

7. SOD activity and SOD2, Nrf2, HO-1 and Caspase3 protein levels

As an antioxidant enzyme, SOD inhibits the excessive buildup of reactive oxygen species in tissues, helping to prevent oxidative damage (Zelko et al., 2002). Representative blot images are presented in Figure 4A. The HU+50%CGA and HU+5%CGA groups displayed significantly higher SOD2 activity compared to the CON group. Moreover, SOD2 activity in the HU+50%CGA group was significantly greater than in the HU and HU+5%CGA groups (Figure 4B). Regarding SOD2 protein expression levels (Figure 4C), the HU+50%CGA group was significantly higher than the CON group.

We examined the protein expression of Nrf2 and HO-1 to investigate the antioxidant pathway mechanism. Both Nrf2 and HO-1 expression levels were significantly higher in the HU+50%CGA group than in the CON group (Figure 4D and E).

To determine apoptosis levels, Caspase 3 protein expression was analyzed. The HU group exhibited a significantly higher Caspase 3 level compared to the CON group. In contrast, the HU+50%CGA and HU+5%CGA groups showed significantly lower Caspase 3 levels than the HU group (Figure 4F).

8. Protein expression level of VEGF, TSP-1, ANG1, ANG2 and Tie2

Figure 5A presents representative images of protein blots. The VEGF protein expression, one of the pro-angiogenic factors examined, showed no statistically significant differences among the four groups (Figure 5B). The HU and HU+5%CGA groups showed a significant increase in TSP-1 expression, an anti-angiogenic factor, relative to the CON group. Additionally, the HU+50%CGA group exhibited significantly lower TSP-1 levels than the HU and HU+5%CGA groups (Figure 5C). The VEGF/TSP-1 ratio was significantly reduced in the HU, HU+50%CGA, and HU+5%CGA groups relative to the CON group; however, the ratio in the HU+50%CGA group was significantly higher than that observed in the HU and HU+5%CGA groups (Figure 5D). Regarding ANG1 protein expression, it was significantly reduced in the HU+50%CGA and HU+5%CGA groups compared to the CON group (Fig. 5E). Concomitantly, protein expression level of ANG2, was not statistically significant between the four groups (Fig. 5F). ANG2/ANG1 ratio was significantly increased at HU+50%CGA and HU+5%CGA groups than that at CON group (Figure 5G). There were no significant differences in Tie2 protein expression across the four groups (Fig. 5H).

Discussion

The key finding of this study is that green coffee bean extract containing rich chlorogenic acids prevents capillary degradation by reducing oxidative stress and preserving mitochondrial activity, thereby more effectively promoting capillary angiogenesis and inhibiting endothelial cell apoptosis. However, The CGA treatment had a limited effect on counteracting the decrease in muscle mass due to hindlimb unloading. We propose that green coffee bean extract, rich in chlorogenic acids, can effectively prevent capillary regression within the first 2 weeks of unloading by regulating the equilibrium between pro- and anti-angiogenic signals.

Previous studies have shown that two weeks of hindlimb unloading significantly increases oxidative stress and decreases muscle capillaries within the soleus muscle (Kanazashi et al., 2013). In the present study, we observed that HU led to an increase in oxidative stress, as indicated by the increased fluorescence intensity of DHE. Additionally, our study showed that 2weeks of HU decreased the capillary-to-fiber (C/F) ratio in soleus muscle, FCSA, and SDH activity, and consistent with previous studies, capillary regression resulting from prolonged inactivity is linked to reduced VEGF/TSP-1 signaling (Wagatsuma and Osawa, 2006). Two weeks of unloading did not impact VEGF expression at the protein level because VEGF protein expression changes significantly within the first week after hindlimb unloading, but these changes stabilize after two weeks (Tanaka et al., 2022). In contrast, it is well established that two weeks of hindlimb unloading enhances antiangiogenic signals, specifically through increased TSP-1 protein expression (Kanazashi et al., 2014). Therefore, two weeks of

unloading downregulated VEGF/TSP-1 signaling. In addition, in agreement with prior studies conducted using the hindlimb unloading model (Tanaka et al, 2019; Yoshihara et al, 2017), the current results reveal increased frequency in TUNEL-positive endothelial cells and increased caspase 3 protein expression within the unloaded soleus muscle. Moreover, excessive ROS production caused a reduction in SDH activity, a marker of mitochondrial oxidative potential that is associated with oxygen demand (Kondo et al., 1993; Kanazawa et al, 2014). The findings imply that oxidative stress induced by two weeks of hindlimb unloading disrupts mitochondrial oxidative activity, leading to a decrease in oxygen demand. This reduction in oxygen demand may upregulate TSP-1 expression, triggering endothelial cell apoptosis and consequently causing capillary regression.

In the current study, treatment with green coffee bean extract containing rich chlorogenic acids more effectively inhibited ROS overexpression induced by hindlimb unloading. This is supported by our observation of decreased ROS production, as indicated by reduced DHE staining, along with increased SOD2 activity and protein expression in the unloaded soleus muscle treated with rich chlorogenic acids compared to supplementation with trace amounts of chlorogenic acids in green coffee bean. Besides, we observed that the proteins Nrf2 and HO-1 were highly expressed in the rich chlorogenic acids group. These findings align with the results reported by Hada et al. and Yao et al., which showed that CGA prevents ROS overexpression through activation of the Nrf2-HO-1 pathway. This pathway is vital in defending against various oxidative stress-induced diseases, including cardiovascular conditions (Hada et al.,

2020; Yao et al., 2018). In addition, this study found that the green coffee extract rich chlorogenic acids also attenuated capillary regression by decreasing ROS production during the two-week hindlimb unloading process. This effect was attributed to changes in pro- and anti-angiogenic factors, including angiopoietins, VEGF, and TSP-1. Previous studies have demonstrated that the ANG1-Tie2 pathway promotes vessel stabilization, while ANG2 blocks the ANG1-Tie2 pathway, leading to vascular destabilization and creating conditions conducive to angiogenesis and promoting endothelial cell survival (Thurston and Daly, 2012; Kamiyama and Augustin, 2021). Furthermore, they suggest that angiogenesis is activated by the synergistic action of angiopoietins and VEGF (Ju et al., 2014). In line with previous research, our study demonstrated that a higher concentration of chlorogenic acids helped to prevent capillary regression in the unloaded soleus muscle via the downregulation of TSP-1 and ANG1 expression, thereby increasing the VEGF/TSP-1 and ANG2/ANG1 ratios.

Meanwhile, this study found that the group with rich chlorogenic acids exhibited elevated SDH activity, which is a key rate-limiting enzyme in the TCA cycle and serves as a marker for mitochondrial respiratory capacity linked to oxygen demand (Hepple, 2000). This result is in agreement with Charifi et al., who found that enhanced mitochondrial metabolic activity increases oxygen delivery to the soleus muscles, thus encouraging angiogenesis in skeletal muscles (Charifi et al., 2004). In addition, rich chlorogenic acids opposed the unloading-induced transition of muscle fibers from oxidative to glycolytic types. This is important because angio-adaptation is strongly linked to the intensity of the oxidative metabolic profile and the distribution of muscle

377 fiber types. These results align with the findings demonstrated that ingesting
378 antioxidant supplements such as chlorogenic acids and astaxanthin during a 2-week
379 period of hindlimb unloading lowers ROS production and enhances SDH activity, thus
380 preventing capillary regression (Xing et al., 2021; Kanazashi et al., 2014). In addition,
381 Fan et al. have shown that chlorogenic acids supplementation stimulates angiogenesis
382 and mitigates apoptosis in cases of cerebral ischemia-reperfusion injury (Fan et al.,
383 2022). Consistent with this prior study, our results from TUNEL staining and Caspase-
384 3 protein expression indicated that ingestion of green coffee bean extract containing
385 chlorogenic acids inhibited endothelial cell apoptosis, particularly with the extract
386 containing a rich concentration of chlorogenic acids. Although trace amounts of
387 chlorogenic acid in green coffee bean extract may inhibit endothelial cell apoptosis, its
388 lower concentration may not be sufficient to reduce oxidative stress and promote
389 capillary neogenesis by modulating the VEGF/TSP-1 and ANG2/ANG1 ratios.
390 Moreover, in the presence of trace amounts of chlorogenic acid, caspase-3 activity is
391 reduced, but DNA fragmentation persists. This may be in line with Wang et al. and
392 Yamagata et al., who proposed that chlorogenic acid may activate alternative caspases
393 or non-caspase pathways, resulting in DNA cleavage without fully triggering the classic
394 apoptotic pathway (Wang et al., 2016; Yamagata et al., 2018). Higher concentrations
395 of chlorogenic acids in green coffee extracts could more effectively prevent ROS
396 overproduction by enhancing antioxidant capacity through the activation of Nrf2/HO-
397 1 under disuse conditions. Additionally, the reduction in ROS may help maintain

oxygen demand by preserving mitochondrial activity, thereby mitigating capillary regression through the inhibition of TSP1 expression and endothelial cell apoptosis.

In conclusion, green coffee bean extract containing rich chlorogenic acids effectively prevented the increase in ROS production in the soleus muscle and maintained mitochondrial enzyme activity (such as SDH), thereby promoting angiogenesis by elevating the VEGF/TSP-1 and ANG2/ANG1 protein expression ratios and inhibited endothelial cell apoptosis. According to these results, the intake of a diet enriched with green coffee bean extract containing rich chlorogenic acids prevents increased oxidative stress and enhances mitochondrial metabolism to improve oxygen demand. These results, in turn, indicate to prevention of capillary regression under unloading condition by reversing the dysregulation of pro- and anti-angiogenic factors and inhibiting endothelial cell apoptosis.

Acknowledgments

The study received financial support from Grants-in-Aid for Scientific Research (20KK0227 and 21K19742) awarded by the Japanese Ministry of Education, Culture, Sports, Science, and Technology, along with JST SPRING, Grant Number JPMJSP2110. Chlorogenic acid was provided by UCC Ueshima Coffee Co. LTD. (Japan) for use in this research.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

Byrne, A. M., Bouchier-Hayes, D. J., & Harmey, J. H. (2005). Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *Journal of cellular and molecular medicine*, 9(4), 777–794.

Charifi, N., Kadi, F., Féasson, L., Costes, F., Geyssant, A., et al. (2004). Enhancement of microvessel tortuosity in the vastus lateralis muscle of old men in response to endurance training. *Journal of Physiology*, 554(2), 559–569.

Chen, H., Herndon, M. E., & Lawler, J. (2000). The cell biology of thrombospondin-1. *Matrix biology: journal of the International Society for Matrix Biology*, 19(7), 597–614.

Fagiani, E., & Christofori, G. (2013). Angiopoietins in angiogenesis. *Cancer letters*, 328(1), 18–26.

Fan, Y., Li, Y., Yang, Y., Lin, K., Lin, Q., et al. (2022). Chlorogenic acid promotes angiogenesis and attenuates apoptosis following cerebral ischaemia-reperfusion injury by regulating the PI3K-Akt signalling. *Pharmaceutical biology*, 60(1), 1646–1655.

Farah, A., & Donangelo, C. M. (2006). Compostos fenólicos em café. *Brazilian Journal of Plant Physiology*, 18, 23-36.

Fujino, H., Kondo, H., Nagatomo, F. & Ishihara, A. (2014). Capillary growth and regression in skeletal muscle. *J Phys Fitness Sports Med* 3(5), 483–491.

Hada, Y., Uchida, H. A., Otaka, N., Onishi, Y., Okamoto, S., et al. (2020). The Protective Effect of Chlorogenic Acid on Vascular Senescence via the Nrf2/HO-1 Pathway. *International journal of molecular sciences*, 21(12), 4527.

Hendrickse, P. W., Wüst, R. C. I., Ganse, B., Giakoumaki, I., Rittweger, J., et al. (2022). Capillary rarefaction during bed rest is proportionally less than fibre atrophy and loss of oxidative capacity. *Journal of cachexia, sarcopenia and muscle*, 13(6), 2712–2723.

Hepple R. T. (2000). Skeletal muscle: microcirculatory adaptation to metabolic demand. *Medicine and science in sports and exercise*, 32(1), 117–123.

Iwai, K., Narita, Y., Fukunaga, T., Nakagiri, O., Kamiya, T., et al. (2012). Study on the postprandial glucose responses to a chlorogenic acid-rich extract of decaffeinated green coffee beans in rats and healthy human subjects. *Food Science and Technology Research*, 18(6), 849-860.

Ju, R., Wen, Y., Gou, R., Wang, Y., & Xu, Q. (2014). The experimental therapy on brain ischemia by improvement of local angiogenesis with tissue engineering in the mouse. *Cell transplantation*, 23(1_suppl), 83-95.

Kamiyama, M., & Augustin, H. G. (2021). Alternatively spliced form of angiopoietin-2 as a new vascular rheostat. *Cancer research*, 81(1), 35-37.

Kanazashi, M., Okumura, Y., Al-Nassan, S., Murakami, S., Kondo, H., et al. (2013). Protective effects of astaxanthin on capillary regression in atrophied soleus muscle of rats. *Acta physiologica (Oxford, England)*, 207(2), 405–415.

- 461 Kanazashi, M., Tanaka, M., Murakami, S., Kondo, H., Nagatomo, F., et al. (2014).
462 Amelioration of capillary regression and atrophy of the soleus muscle in hindlimb-
463 unloaded rats by astaxanthin supplementation and intermittent loading. *Experimental*
464 *physiology*, 99(8), 1065–1077.
- 465 Kanazashi, M., Tanaka, M., Nakanishi, R., Maeshige, N., & Fujino, H. (2019).
466 Effects of astaxanthin supplementation and electrical stimulation on muscle atrophy
467 and decreased oxidative capacity in soleus muscle during hindlimb unloading in rats.
468 *The journal of physiological sciences: JPS*, 69(5), 757–767.
- 469 Kanazawa, Y., Maekawa, K., Okumura, Y., Fujita, N., & Fujino, H. (2014).
470 Preventive effect of nucleoprotein on hindlimb unloading-induced capillary regression
471 in rat soleus muscle. *Biotechnic & histochemistry: official publication of the Biological*
472 *Stain Commission*, 89(3), 220–227.
- 473 Kondo, H., Nakagaki, I., Sasaki, S., Hori, S. & Itokawa, Y. (1993). Mechanism of
474 oxidative stress in skeletal muscle atrophied by immobilization. *American Journal of*
475 *Physiology-Endocrinology and Metabolism*, 265(6), E839-E844.
- 476 Lukitasari, M., Saifur Rohman, M., Nugroho, D. A., Widodo, N., & Nugrahini, N.
477 I. P. (2020). Cardiovascular protection effect of chlorogenic acid: focus on the
478 molecular mechanism. *F1000Research*, 9, 1462.
- 479 Morey, E. R., Sabelman, E. E., Turner, R. T., & Baylink, D. J. (1979). A new rat
480 model simulating some aspects of space flight. *The Physiologist*, 22(6), S23–S24.

Olfert, I. M., & Birot, O. (2011). Importance of anti-angiogenic factors in the regulation of skeletal muscle angiogenesis. *Microcirculation* (New York, N.Y.: 1994), 18(4), 316–330.

Raskar, V., & Bhalekar, M. R. (2019). Formulation of coffee bean extract (Chlorogenic Acid) solid lipid nanoparticles for lymphatic uptake on oral administration. *Journal of Drug Delivery and Therapeutics*, 9(4), 477-484.

Rojas-González, A., Figueroa-Hernández, C. Y., González-Rios, O., Suárez-Quiroz, M. L., González-Amaro, R. M., et al. (2022). Coffee Chlorogenic Acids Incorporation for Bioactivity Enhancement of Foods: A Review. *Molecules* (Basel, Switzerland), 27(11), 3400.

Tanaka, M., Kanazashi, M., Kondo, H., & Fujino, H. (2022). Time course of capillary regression and an expression balance between vascular endothelial growth factor-A and thrombospondin-1 in the soleus muscle of hindlimb unloaded rats. *Muscle & nerve*, 65(3), 350–360.

Tanaka, M., Kanazashi, M., Maeshige, N., Kondo, H., Ishihara, A., et al. (2019). Protective effects of Brazilian propolis supplementation on capillary regression in the soleus muscle of hindlimb-unloaded rats. *The journal of physiological sciences: JPS*, 69(2), 223–233.

Thurston, G., & Daly, C. (2012). The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. *Cold Spring Harbor perspectives in medicine*, 2(9), a006550.

Wagatsuma, A., & Osawa, T. (2006). Time course of changes in angiogenesis-related factors in denervated muscle. *Acta Physiologica*, 187(4), 503-509.

Wang, J., Li, J., Liu, J., Xu, M., Tong, X., et al. (2016). Chlorogenic acid prevents isoproterenol-induced DNA damage in vascular smooth muscle cells. *Molecular medicine reports*, 14(5), 4063–4068.

Wüst, R. C., Gibbings, S. L., & Degens, H. (2009). Fiber capillary supply related to fiber size and oxidative capacity in human and rat skeletal muscle. *Advances in experimental medicine and biology*, 645, 75–80.

Xing, J., Pan, H., Lin, H., Nakanishi, R., Hirabayashi, T., et al. (2021). Protective effects of chlorogenic acid on capillary regression caused by disuse muscle atrophy. *Biomedical research (Tokyo, Japan)*, 42(6), 257–264.

Yamagata, K., Izawa, Y., Onodera, D., & Tagami, M. (2018). Chlorogenic acid regulates apoptosis and stem cell marker-related gene expression in A549 human lung cancer cells. *Molecular and cellular biochemistry*, 441(1-2), 9–19.

Yao, H., Sun, J., Wei, J., Zhang, X., Chen, B., et al. (2020). Kaempferol Protects Blood Vessels From Damage Induced by Oxidative Stress and Inflammation in Association With the Nrf2/HO-1 Signaling Pathway. *Frontiers in pharmacology*, 11, 1118.

Yoshihara, T., Yamamoto, Y., Shibaguchi, T., Miyaji, N., et al. (2017). Dietary astaxanthin supplementation attenuates disuse-induced muscle atrophy and myonuclear apoptosis in the rat soleus muscle. *The journal of physiological sciences: JPS*, 67(1), 181–190.

524 Zelko, I. N., Mariani, T. J., & Folz, R. J. (2002). Superoxide dismutase multigene
525 family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD
526 (SOD3) gene structures, evolution, and expression. *Free radical biology & medicine*,
527 33(3), 337–349.
528

Table 1. Body weight, soleus weight and relative soleus muscle weight

	Body weight (g)	Soleus muscle weight (mg)	Relative soleus muscle weight (mg/100 g)
CON	367.8 ± 17.1	175.8 ± 8.3	47.9 ± 1.3
HU	297.5 ± 18.3*	107.2 ± 8.3*	36.9 ± 4.8*
HU+50%CGA	302.4 ± 4.7*	98.1 ± 3.7*	32.4 ± 1.1*
HU+5%CGA	313.4 ± 3.7*	106.2 ± 4.1*	33.9 ± 1.4*

Values are means ± SEM (n = 6). Abbreviations: CON, control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50% chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The symbols * indicates significant differences from the CON group, respectively, at $P < 0.05$.

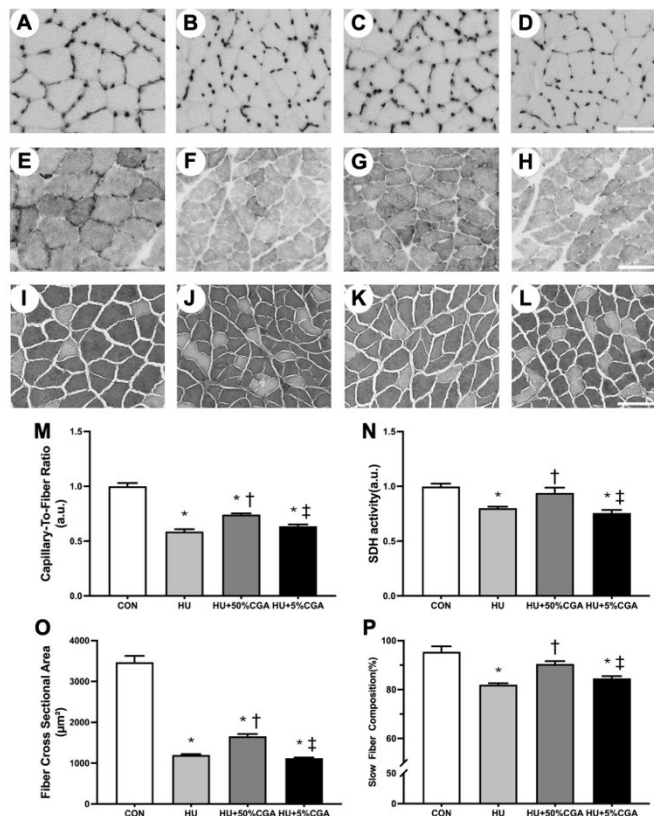


Fig 1. Transverse sections stained for alkaline phosphatase (A–D), succinate dehydrogenase (SDH) (E–H), adenosine triphosphatase (ATPase) (I–L, PH: 4.2, dark: type I; light: type II), C/F ratio (M), SDH activity (N) and Fiber cross sectional area (O) and Slow fiber composition (P) of the soleus muscles. CON (A), HU (B), HU+50%CGA (C), HU + 5%CGA (D). Capillaries were visualized as black dots. Bar = 100 μm. Values are means ± SEM (n = 6). Abbreviations: CON, control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50% chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, HU and HU+50%CGA groups, respectively, at $P < 0.05$.

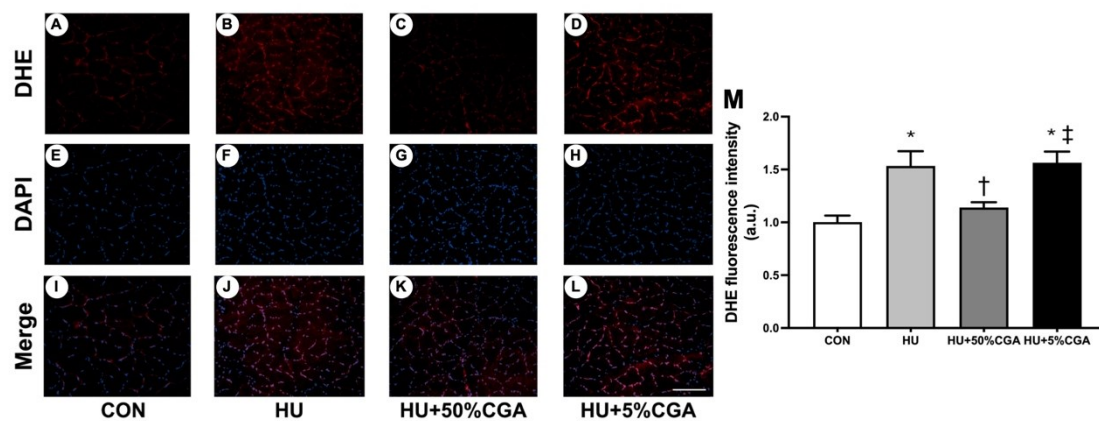


Fig 2. Cross-sectional images of dihydroethidium (DHE) staining (A-D) and DAPI staining (E-H) and merged images (I-L) of the soleus muscle in each group. The graph for DHE fluorescence intensity depicts the ratio of DHE and corresponding DAPI fluorescence intensity(M). Bar = 100 μ m. Values are means $\pm$ SEM (n = 6). Abbreviations: CON, control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50% chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, HU and HU+50%CGA groups, respectively, at $P < 0.05$.

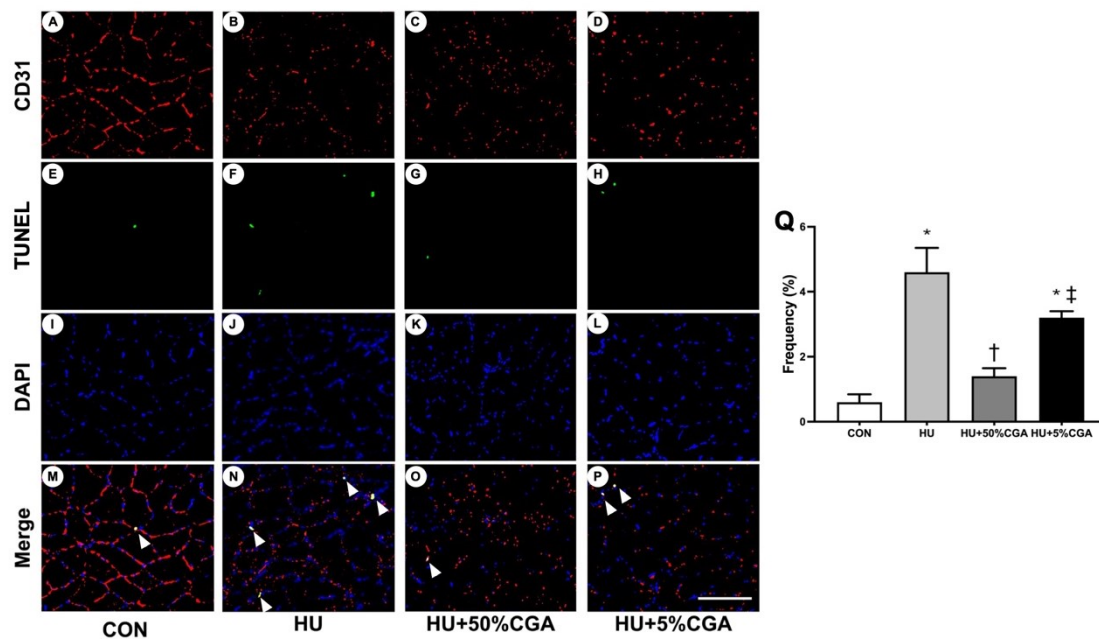


Fig 3. Confocal laser scanning microscopic images of the soleus muscle stained with cluster of differentiation 31 (CD31; A-D), terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; E-H), DAPI-Fluoromount G (DAPI; I-L) and merged images (M-P). Arrowheads indicate TUNEL-positive endothelial cells. Q mean number of the TUNEL- positive endothelial cells of the soleus muscle in each group. Bar = 100 μ m. Values are means $\pm$ SEM (n = 6). Abbreviations: CON, control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50% chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, HU and HU+50%CGA groups, respectively, at $P < 0.05$.

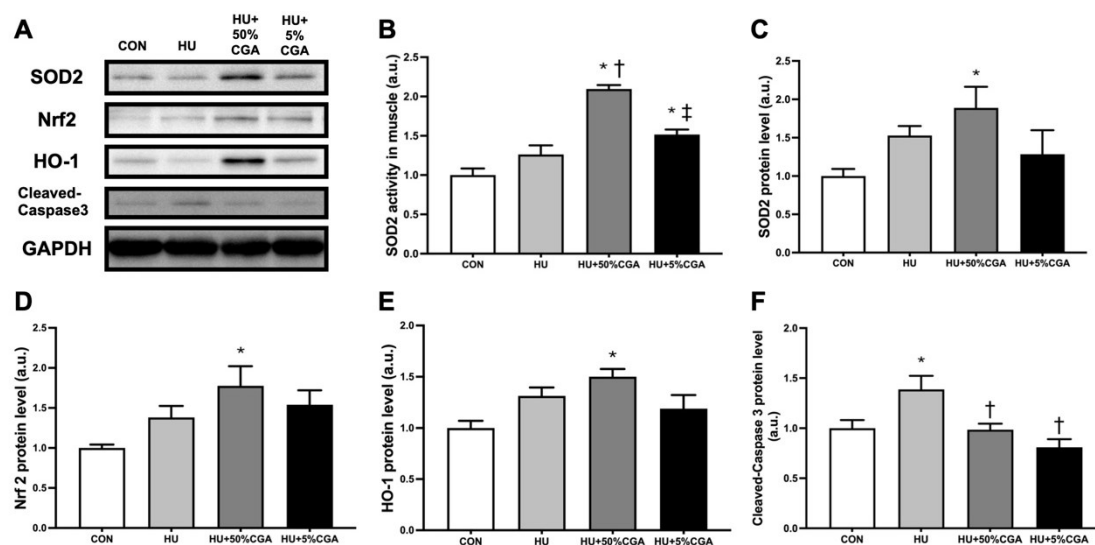
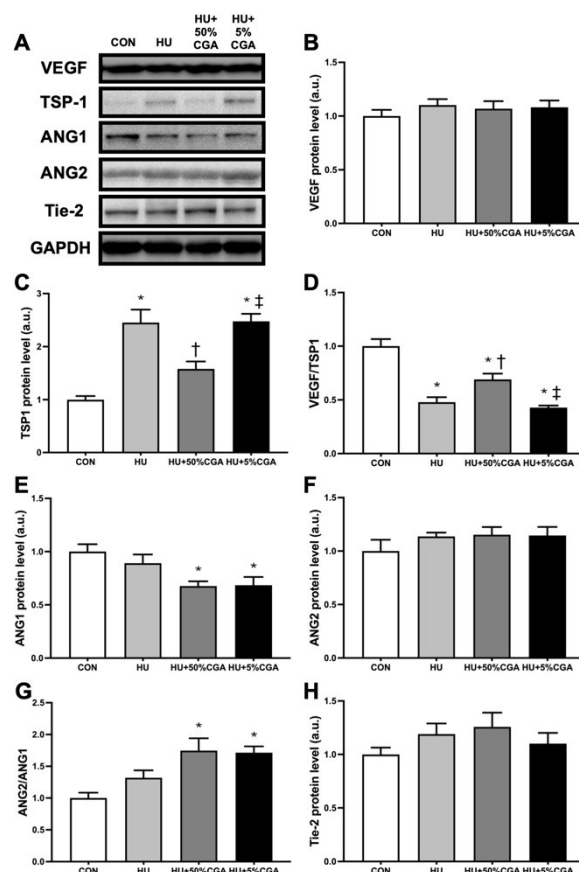


Fig 4. Effects of 50% and 5% chlorogenic acid on activity of SOD2(B) and protein levels of SOD2, Nrf2, HO-1 and Caspase 3 in the soleus muscles. Band images are representative western blots (A), and histograms show the quantification of band densities. Mean levels of SOD2(C), Nrf2(D), HO-1(E) and Caspase 3(F) protein expression in the soleus muscles of each group. Values are means $\pm$ SEM (n = 6). Abbreviations: CON, control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50% chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The symbols *, † and ‡ indicate significant differences from the CON, HU and HU+50%CGA groups, respectively, at $P < 0.05$.



579

580 **Fig 5.** Effects of 50% and 5% chlorogenic acid on VEGF, TSP-1, ANG1, ANG2 and
 581 Tie-2 protein expressions. Band images are representative western blots (A), and
 582 histograms show the quantification of band densities. Mean levels of VEGF (B), TSP-
 583 1 (C), VEGF/TSP-1 ratio (D), ANG1 (E), ANG2 (F), ANG2/ANG1 ratio (G) and Tie-
 584 2(H) in the soleus muscles of each group. Values were calculated as the fold changes
 585 relative to the control group. Values are means $\pm$ SEM (n = 6). Abbreviations: CON,
 586 control; HU, hindlimb unloaded; HU+50%CGA, hindlimb unloaded plus 50%
 587 chlorogenic acid; HU+5%CGA, hindlimb unloaded plus 5% chlorogenic acid. The
 588 symbols *, † and ‡ indicate significant differences from the CON, HU and
 589 HU+50%CGA groups, respectively, at $P < 0.05$.