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Original Article

Therapeutic effect of *Cnidium officinale* Makino extract on ovariectomized hind-limb ischemic mice

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ABSTRACT

Background: Cnidium officinale Makino (COM) has been used traditionally to treat female menstrual disorders, such as amenorrhea, hypomenorrhea and oligomenorrhea, by improving blood circulation. The present study aimed to investigate the alleviating effect of COM extracts on surgical injuryinduced ischemia in the hind-limb of mice.

Methods: In this study, female C57BL/6 mice were ovariectomized, and the vessels of the hind-limb were excised after ligation by surgical silk (6–0). The mice were orally administered with COM (150 or 300 mg/kg/day) for 3 weeks, and the blood flow rate of hind-limbs was evaluated by using a laser Doppler system after hind-limb ischemic surgery in an *in vivo* study. Additionally, the migration and tube formation of human umbilical vein endothelial cells (HUVECs) were evaluated in an *in vitro* study.

Results: The blood flow rate was synchronized to the nonischemic lesion of the hind-limb, and its elevation compared to the vehicle was observed at 14 and 21 days after hind-limb ischemic surgery in COM-treated groups. The number of capillaries increase in a dose-dependent manner in the COM-treated groups (150 and 300 mg/kg). In HUVECs, the activities of cell migration were significantly increased by 50 and 75 μg/mL for the COM-treated groups. In addition, the number of tubule branches and junctions was also increased by doses of COM (50 and/or 75 μg/mL).

Conclusion: The results of our study suggested that the COM extract may have therapeutic application for the treatment of hind-limb ischemic damage, which is due to the improvement of the peripheral angiogenetic system.

1. Introduction

Cnidium officinale Makino (COM), which is called "Chungung" in Korea, is a medicinal plant that has been used in oriental medicine to decrease inflammation and improve blood circulation. Ancient documents, which describe principles and practices of oriental medicine, state that COM is a main ingredient of 202 prescriptions that relieve pain, inflammation and menstrual disorders. Previously, COM was used to reduce inflammation and the relaxation of blood congestion. In addition, COM has been reported to have anti-cancer effects on oral cancer, hepatic cancer and colorectal cancer.[1–3]

Compared to the Western population, Asian women experience fewer symptoms related to menopause; this difference may be explained by differences in Asian cultures, i.e., folk medicine, dietary habits and traditional alternative medicine.[4] Previous studies reported that various herbal remedies attenuated symptoms of menopausal dysfunction, such as weight gain, dyslipidemia and the development of mood disorders, etc.[4,5] In menopausal women, the disturbance of menstrual cycles reduces the serum levels of sex-steroid hormones; therefore, hormone replacement therapy (HRT) with a kind of estradiol is the most effective remedy for the lacking estradiol of menopausal women.[6] However, inadequate HRT is accompanied by several adverse effects, such as uterine bleeding and endometrial hyperplasia. An additional uncertain risk includes breast cancer.[6,7] Phytoestrogens, an alternative to HRT, are natural herbal compounds that are structurally similar to estrogen and its metabolites, and they are a prevalent alternative to synthetic estrogen and/or progesterone therapy.[6,7] The consumption of phytoestrogens can improve menopausal symptoms, i.e., hot flashes and night sweats; however, the consumption of phytoestrogen is also a limit to the treatment of menopausal symptoms.[6–8]

Menopause is associated with an increase in lipid profiles, including low-density cholesterol and triglycerides, and a decrease in high-density cholesterol levels. This may lead to problems with

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circulation, including sluggish blood flow and the occurrence of varicose and spider veins.[5] In addition, peripheral vascular disease commonly results in changes to serum lipid levels, and it is associated with an increased incidence with age during the menopausal period.[5,9] Several studies have shown that changes in low-density lipoprotein cholesterol and changes in the serum level of estrogen are closely related.[5] In addition, decreased estrogen production in the ovaries leads to genitourinary atrophy, osteoporosis, and vascular and circulatory disturbances in various organs during menopause.[5,8] The role of estrogen in mediating lipid profiles may indirectly and/or directly affect vascular dysfunction, and it may be recovered from the ischemic damage resulting in atherosclerosis and embolism in the limbs.[8,9] Therefore, we selected a hind-limb ischemic mouse model because it is associated with peripheral blood circulation disturbance due to thrombosis and atherosclerosis in postmenopausal women.[10] And the use of this model is very effective in understanding the effects of angiogenic reactivity on *in vivo* vascular remodeling.[11] In the ischemic lesion, neovascularization depends on pathological conditions, such as immunity and the inflammatory response, and it is also associated with cellular and molecular recognition.[12] The process of neovascularization, including vasculogenesis, angiogenesis and arteriogenesis, contributed to the repair and remodeling of damaged tissues during the ischemia-related disease.[12]

In this study, we investigated whether the COM extract regulates the expression of angiogenetic factors and alters blood flow rate and capillary density in an established menopausal model of peripheral vascular disease. The ischemic/nonischemic blood perfusion ratio was evaluated using a noninvasive laser doppler perfusion imaging system, and the number of capillaries were visualized and counted using immunohistochemistry with CD31 antibodies against endothelial cells in the ischemic limb muscle.

2. Methods

2.1. Preparation of COM extract

Dried COM was purchased from a commercial supplier at Backjaedang (Daejeon, South Korea). The dried COM was ground, and COM root (100 g) was extracted three times for 24 hours with 70% ethanol at room temperature. The layer was then filtered with filter paper and concentrated using a vacuum rotary evaporator. The dried COM root ethanol extracts was obtained 12.62 g. The plants were deposited in the Herbal Medicine Research Division of Korea Institute of Oriental Medicine (KIOM) in Daejeon, Korea; the voucher specimen (KIOMM 160012).

2.2. Experimental animals and treatments

Female 6-week-old C57BL/6 mice were obtained from Daehan Biolink (Eumseong, South Korea), and mice ($n = 28$) were divided into five-groups ($n = 7$ for each a group). The mice were allowed to adapt to laboratory conditions (temperature: 20 ± 2, relative humidity: $45 \pm 5\%$, light/dark cycle: 12 h) for 1-week. The mice were anesthetized with an intraperitoneal (i.p.) injection of a Zoletil–Rompun–saline mixture (2:1:2) and were ovariectomized (OVX). For hind-limb ischemia (HLI), the mice were also anesthetized, the vessels (artery and vein) of the hind-limb were excised, and its surgery was modified according to the criteria.[11] 17β-Estradiol (E2) and the COM extract were prepared in 0.2% CMC (carboxymethyl cellulose). The mice were treated daily for 3-weeks by p.o. injection with E2 and the COM extract (150 and 300 mg/kg/day) and then euthanized 12 hours after the final injection. The normal and positive controls of this study have already been described in previous studies.[13] All animal experiments were approved by the Ethics Committee of Korea Institute of Oriental Medicine (approved No. 17-028).

2.3. Laser Doppler blood perfusion analysis

The ischemic/nonischemic (ISCH/nISCH) blood flow rate in the hind-limb was evaluated using a laser doppler blood perfusion imager (PeriScan-PIM3; Perimed AB, Järfälla, Sweden) in accordance with the manufacturer's recommendations. An increased flow rate is indicated by yellow to red, and a decreased flow rate is indicated by deep blue to black. The ISCH/nISCH blood flow rates were calculated at −7, 0, 7, 14 and 21 days after HLI on colorimetric assay using PIMSoft (v1.5; Perimed AB).

2.4. Immunohistochemical stain

The limb muscle tissues were fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin before they were sectioned (5 μm thick), deparaffinized and hydrated in descending grades of ethyl ethanol. The endogenous peroxidase and alkaline phosphatase activities were blocked with BLOXALL (Vector Lab. Inc., CA, USA) for 30 minutes. The nonspecific reactions were blocked by incubating the sections in CAS-BLOCK (Thermo-Fisher Scientific, PA, USA) for 1 hour at room temperature. The sections were subsequently incubated at room temperature for 4 hours with the antibody against CD-31 (rabbit polyclonal, 1:250; Abcam). After washing with TBS-T (Tris-buffered saline including 0.5% Tween-20), the sections were incubated with a Dako REAL Envision/HRP (Rabbit/Mouse; Dako, CA, USA) for 30 minutes at room temperature. Dako REAL DAB+ Chromogen (Dako) was used, and the sections were counterstained with Mayer's hematoxylin (Sigma–Aldrich, Saint Louis, MO, USA) before mounting in the mounting medium (Thermo-Fisher Scientific).

2.5. Cell culture and experimental conditions

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Cat No. CC-2519; Allendale, NJ, USA) and cultured in endothelial cell basal medium (EBM, Cat. No. CC-3121; Lonza) supplemented with endothelial cell growth medium (EGM BulletKit, Cat. No. CC-3124, Lonza) at 37°C in an atmosphere of 5% CO_2 in humidified air.[14] Before treating the drug in all experiments, cells were cultured in complete phenol-red free endothelial basal medium (EBM, Cat. No. CC-3129; Lonza) supplemented with 0.2% charcoal-stripped FBS.[15,16] Confluent cultures of HUVECs were consecutively passaged and used between passages 3 and 6.

2.6. Cell viability assay

HUVECs were plated at a density of 2×10^3 cells/well in 96-well plates in complete medium and allowed to adhere overnight. The culture medium was then aspirated and phenol-red free (0.2% charcoal-stripped FBS) medium containing COM extract (0, 10, 25, 50, 75, 100 μg/mL) was added to each well. After 24 hours later, cell viability was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Cat. No. M5655; Sigma–Aldrich).[3] After removal of the insert, 100 μL of MTT solution was added per well, and HUVECs were cultured for 4 hours in the dark. After removing the medium, the generated formazan crystal was dissolved in 500 μL of dimethylsulfoxide (DMSO) and the value of optical density was measured at 570 nm with a microplate reader (Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA). We calculated percentage viability using the formula optical density of treated sample/optical density of untreated control × 100.

2.7. Migration assay

The migration of HUVECs was analyzed using trans-well plates with 8 μm pore size. (Cat. No. 3422; Corning, Inc., Acton, MA, USA).[17] HUVECs were seeded in the upper chamber with or without 50 ng/mL VEGF (Cat. No. 293-VE; R&D Systems, Inc., Minneapolis, MN, USA) and 10, 50, 75, 100 μg/mL COM in the serum-free medium. The lower chamber was used as a chemoattractant by adding 500 μL of medium containing 2% charcoal-stripped FBS. After 6 hours incubation at 37°C under 5% CO_2, the cells on the top of the polycarbonate membrane were wiped off using cotton swabs and the migrating cells on the lower side of membrane were stained with Hematoxylin and eosin (H&E) for 5 minutes, respectively. Migrated cells on the bottom of each well were counted under inverted microscope (Eclipse Ts2; Nikon Corporation, Tokyo, JPN) at 100× magnification. The number of migrated cells was normalized by the number of adherent cells in control.

2.8. Tube formation assay

The effect of COM on HUVECs differentiation was evaluated via *in vitro* tube formation assay. Matrigel (Cat. No. 356231; BD Biosciences, San Diego, CA, USA) was slowly dissolved at 4°C for 24 hours, 300 μL per well was added to a 24-well plate, and the plate was incubated at 37°C for 30 minutes to solidify the matrigel. HUVECs (3×10^4 cells/well) were seeded on matrigel in 24-well plate with or without 50 ng/mL VEGF, 1 pmole Vinblastine (Cat. No. 11762; Cayman Chemical, Ann Arbor, MI, USA), and 10, 50, 75, 100 μg/mL COM were added to the media without the addition of growth factor. Tubular structures of HUVEC were examined using an inverted microscope (Nikon). Number of tube branches, and junctions were measured in three fields (4×) using Image J software version 1.51s (National Institutes of Health, Bethesda, MD, USA).[18]

2.9. Elisa for soluble adhesion molecule (sVCAM-1 and sICAM-1)

Plasma concentrations of sVCAM-1 (Cat. No. E-EL-M0647; Elabscience, Houston, TX, USA) and sICAM-1 (Cat. No. E-EL-M0077; Elabscience) were evaluated by ELISA according to the manufacturer's protocol. Briefly, 100 μL of serum collected from experimental animal groups or standard and 100 μL of biotinylated antibody cocktail was added to each well and allowed to react for 2 hours with 37°C. Subsequently, the wells were treated with 100 μL of HRP conjugate for 1 hours, then reacted with 90 μL substrate for 15 minutes. Finally, 50 μL stop solution was treated and reacted, and then optic density was measured with a microplate reader at 450 nm within 10 minutes. A standard curve of each adhesion molecule was prepared and linear regression analysis was performed using PRISM software (v6.0; Graph Pad, La Jolla, CA, USA).

2.10. Immunohistochemically analysis

Immunohistochemically analysis for endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) was performed with the use of a mouse polyclonal antibody against ET-1 (Cat. No. ab117757; Abcam Ltd, Cambridge, UK) and eNOS (Cat. No. ab76198; Abcam

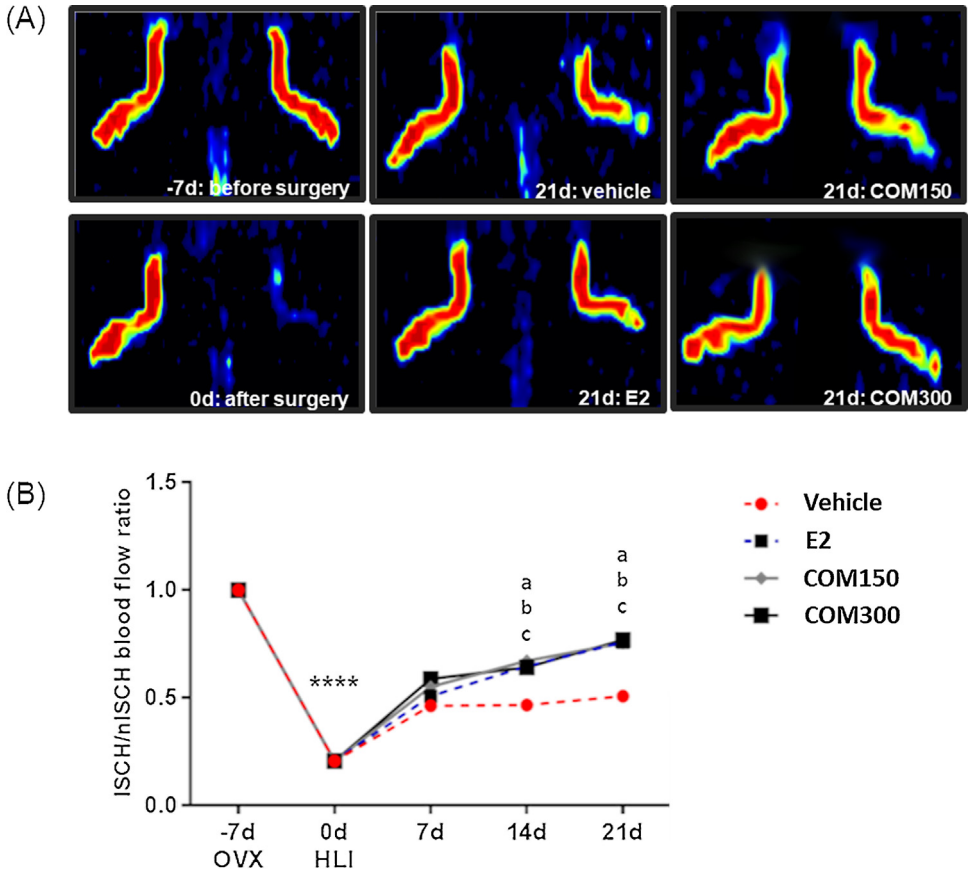

Fig. 1. Laser Doppler scanning of blood flow over hind-limbs on day 21 after surgery. (A) In color-coded images, low to no flow was displayed as black or deep blue, whereas high blood flow was displayed as yellow to red. (B) Quantitative evaluation of ischemic/non-ischemic blood perfusion ratio. Values are presented as the means ± SD ($n = 7$). $^{*}p < 0.05$ vs. −7 d, $^{a}p < 0.05$ vehicle vs. E2, $^{b}p < 0.05$ vehicle vs. COM150, $^{c}p < 0.05$ vehicle vs. COM300. ISCH: ischemia; nISCH: nonischemia; OVX: ovariectomy; HLI: hind-limb ischemic surgery.

Ltd). Cross sections of the thigh and calf muscle tissue were embedded in paraffin and cut in slices 5-μm thick. The sections were deparaffinized with xylene and rehydrated with graded ethanol. The activities of endogenous peroxidase and alkaline phosphatase were blocked by boiling for 20 minutes using 0.01 M citrate buffer (pH 6.0). CAS-BLOCK (Cat. No. 008120; Invitrogen Corporation, Camarillo, CA, USA) were added to inhibit nonspecific protein binding. The primary antibodies were diluted (1:200) and incubated with the tissue sections for 3 hours at room temperature. The sections were then washed three times with phosphate-buffered saline and incubated with a biotin-labeled secondary antibody was applied for 30 minutes, followed by DAKO EnVision system (Cat. No. K5007; Santa Clara, CA, USA) for 30 minutes at room temperature. They were then counterstained with Mayer's hematoxylin stain, dehydrated, and cover slipped.

2.11. Statistical analysis

The results of blood flow, capillary density and gene expression are presented as the means ± SD. The results of cell viability, migration and tube formation are shown as means ± SEM. Paired Student's *t*-tests were used to compare each group, while ANOVA with Tukey's *post hoc* test was used for multiple comparison tests using PRISM software (v6.0; Graph Pad). The p values <0.05 were considered to be statistically significant.

3. Results

3.1. COM improved blood perfusion and animal condition

To determine whether or not COM (150 or 300 mg/kg/day) treatments could stimulate blood reperfusion in the context of hind-limb ischemia, the mice were treated with E2 and COM. Blood reperfusion was determined at 0 (0 d; the time right after ischemia surgery), 7 (7 d; 7 days after ischemia surgery), 14 (14 d; 14 days after ischemia surgery), and 21 days (21 d; 21 days after ischemia surgery) after the COM or E2 treatment. Body weight, weight gain, daily food intake, and uterine weight were unaltered for the estimation of the estrogenic activity of COM under our experimental conditions.

Blood flow rate in the ischemic hind-limb was reduced equally in all surgery groups immediately following ischemic surgery (0 d) (as shown in Fig. 1). At 7 days after ischemic surgery, blood perfusion of the ischemic hind-limb had significantly improved in the E2 and COM 300 mg/kg/day groups compared with the vehicle group, whereas other groups showed no improvement. At 14 days and 21 days, blood perfusion had significantly increased in all treatment groups, although the effects were not dose-dependent in the COM groups. In particular, although no significant differences in blood perfusion were detected between the vehicle and COM 150 mg/kg/day groups at 7 days, a high dose of COM (300 mg/kg/day) significantly ameliorated the signs of hind-limb ischemia earlier than a low dose of COM (150 mg/kg/day).

3.2. Capillary density, CD31 immunohistochemical staining

To investigate the angiogenic effects of COM on microcirculation, we assessed the capillary density in sections of immunostained tissue from ischemic thigh and calf muscles using an anti-CD31 antibody. Five different microscopic fields from one section as well as capillaries were counted under 400× magnification to determine the capillary density (the mean number of capillaries/mm^2). Immunohistochemistry showed that the E2 had a higher capillary density in ischemic muscles than the vehicle control. Additionally, quantitative analysis showed that the capillary

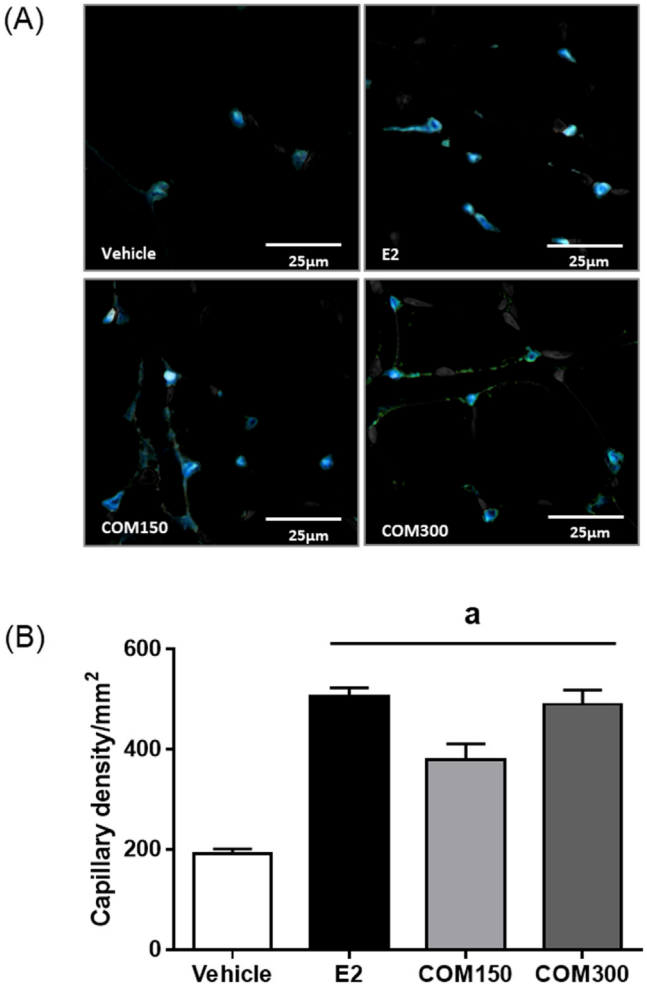

Fig. 2. COM increased capillary density. (A) Representative microphotographs of the section of ischemic hind-limb muscles stained histochemically for CD31 (400×). (B) Quantitative analysis of capillary density in ischemic hind-limb muscles. Data are presented as the mean ± SD (n = 7). [a] p < 0.05 vs. vehicle. E2: estradiol.

density significantly increased in both the low and high dose COM groups. In particular, a high dose of COM significantly increased the number of capillaries compared to a low dose of COM. These findings are in agreement with those of the blood perfusion analysis (as shown in Fig. 2).

3.3. HUVECs viability assay

First, we evaluated the effect of COM on cell viability of HUVECs. Cell viability was confirmed via MTT analysis in the presence of various concentrations of COM (0, 10, 25, 50, 75 μg/mL). As shown in Supplementary Fig. 1, compared with control (non-treated group), HUVECs viability was not significantly changed by any concentration of COM (10–75 μg/mL). Therefore, in the experiments based on these results, the treatment concentration range of COM was determined.

3.4. Effect of COM on HUVEC migration

To determine the effect of COM on HUVECs migration, we performed a migration assay using the trans-well system as described in Section 2. Angiogenesis is tightly regulated by angiogenic factors such as vascular endothelial growth factor (VEGF) which stimulate endothelial cells to migrate to form new vessels.[19,20] We firstly treated HUVECs with VEGF (50 ng/mL) or COM (10, 50, 75 μg/mL)

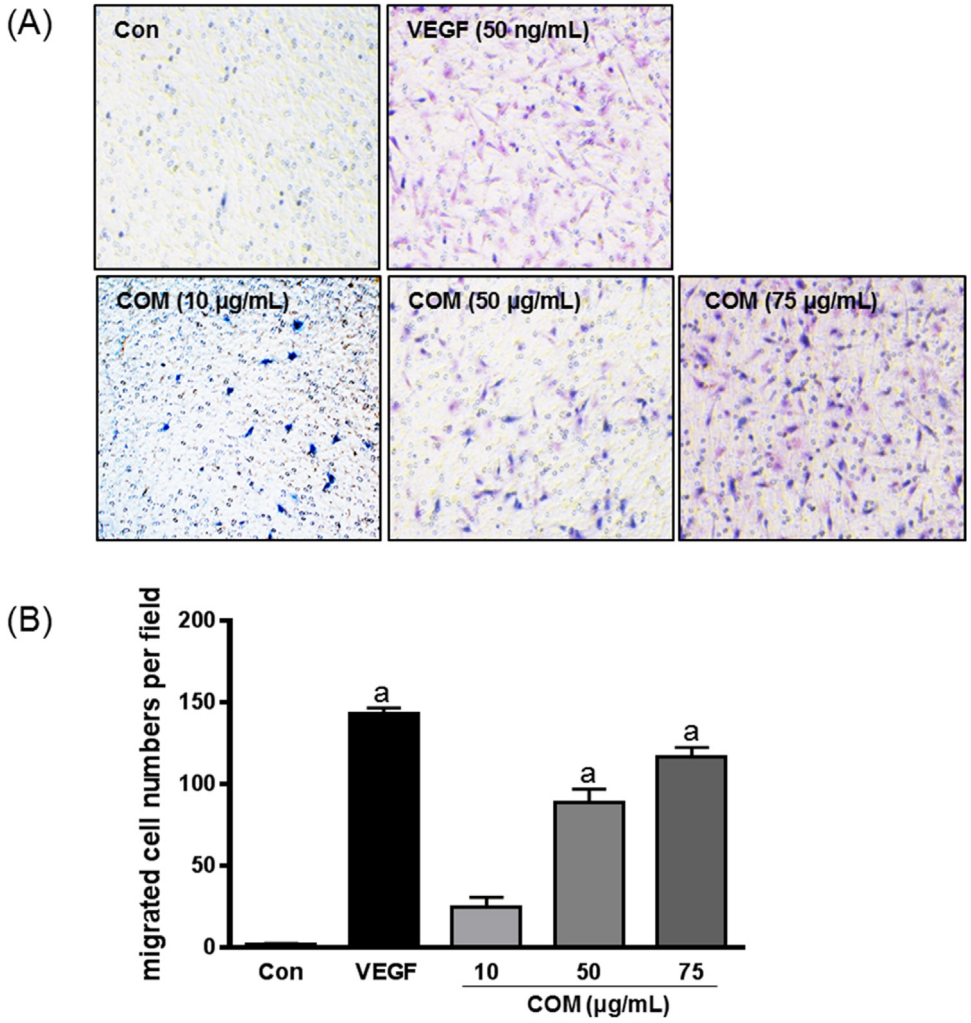

Fig. 3. Effect of COM on HUVECs migration. (A) Cells were treated with different concentration of COM (10, 50, 75 μg/mL), 50 ng/mL VEGF for 6 h. Trans-well chamber was used to assess migratory activity of photomicrographs (H&E stained, 200×). (B) Representative experimental result was shown. Migrated cells were counted in at least three fields after each assay, and data are expressed as the number of cells per field. Data present means ± S.E.M. of three experiments. [a] $p < 0.05$ vs. control.

for 6 hours, then examining the level of HUVECs migration. As shown in Fig. 3, the number of migrated cells in the 50 ng/mL VEGF-treated group was significantly higher than control group (143 ± 4.90 cells/field). In the case of the COM treatment groups, compared with the control, HUVECs migration was significantly increased in a dose-dependent manner (until 75 μg/mL) within 6 hours. The number of migrating cells was 116.33 ± 8.34 cells/field, a significant 106-fold induction compared with control.

3.5. Effect of COM on HUVECs tube formation

Through previous experiments, we confirmed that COM is effective in migration of HUVECs, and based on this result, angiogenesis of HUVECs was evaluated by tube formation assay. This analysis is one of the most common *in vitro* angiogenesis testing methods.[21–23] Tubule branches and junctions were counted after HUVECs began forming capillary tubes. As shown in Fig. 4, tube formation increased in the treated group of VEGF (50 ng/mL) compared to the control group (36.5 ± 1.5 per field). COM markedly enhanced tube formation at 50 μg/mL (tubule branches) and 75 μg/mL (tubule junctions) treated group compared to the control group. These data indicate that COM can promote tube formation of HUVECs.

3.6. Inhibitory effect of COM on serum sVCAM-1 and sICAM-1

We further assessed whether COM has the potential to prevent surgical damage *in vivo*. E2 group showed lower levels of sVCAM-1 (Fig. 5A) and sICAM-1 (Fig. 5B) compared with vehicle. In addition, COM also decreased the levels of compared with vehicle, however, its lowering effect was did not appeared to be dose-dependent.

3.7. Immunohistochemistry analysis for eNOS and ET-1

eNOS is the constitutively expressed isoform in the vascular endothelium and serves as the predominant source of nitric oxide (NO) for the regulation of vascular tone.[24] Therefore, eNOS-generated NO may play an important role in the physiologic regulation of the basal vasomotor tone and blood flow.[25] Immunohistochemical staining showed that E2-treated group had a greater eNOS expression levels than vehicle. Administration of COM showed a protective effect against surgical induced eNOS down-regulation to be increased to 1.02 ± 0.08 AU and 0.56 ± 0.08 AU, 1.00 ± 0.12 AU and 0.56 ± 0.08 AU in the muscle blood vessels of the COM treated groups (150 and 300 mg/kg), respectively. Furthermore, the ET-1 is an important vasoconstrictor. ET-1 and NO are endothelium-derived mediators essential for maintaining vascular homeostasis.[25,26] ET-1 protein expression showed a significant

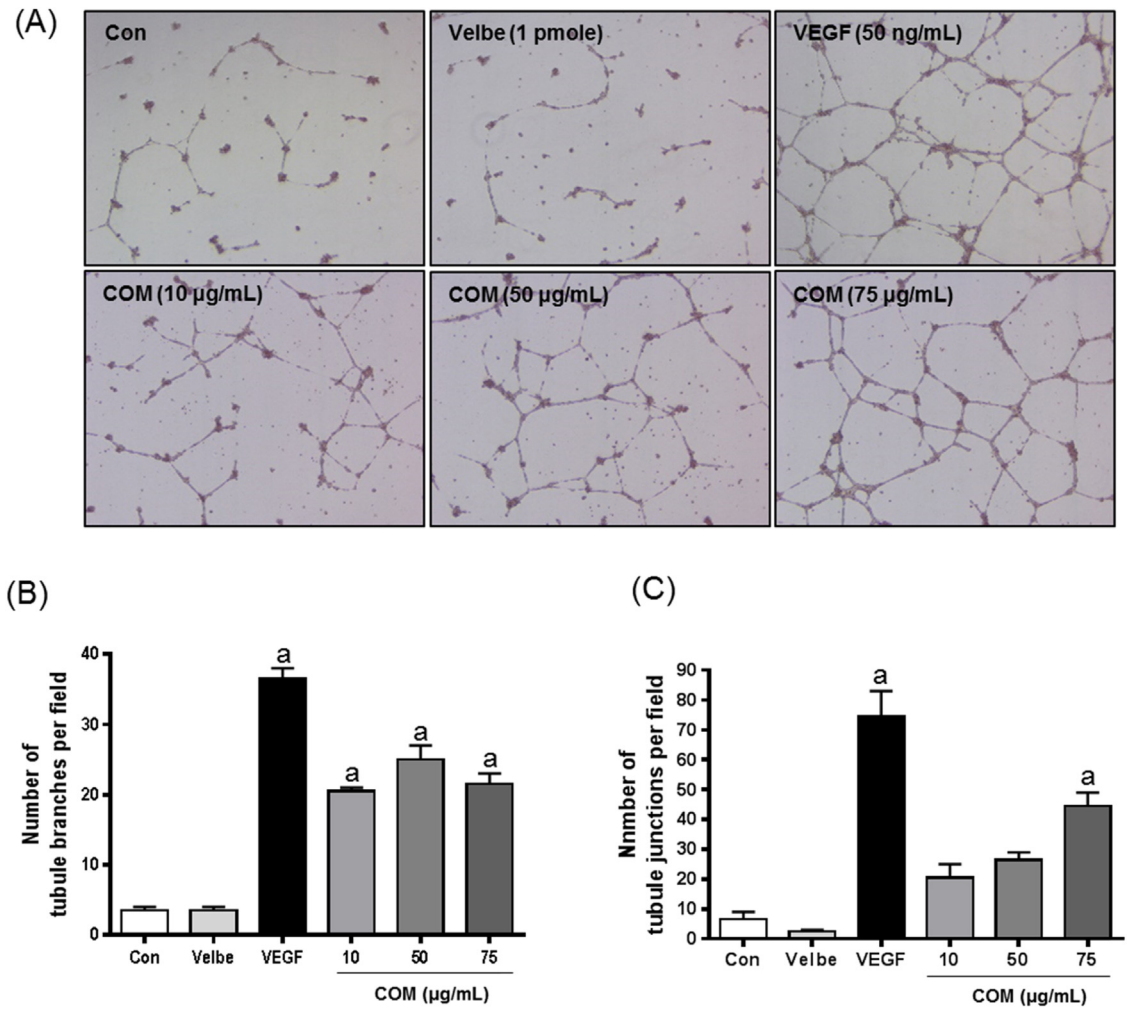

Fig. 4. Effect of COM on HUVECs tube formation. *In vitro* endothelial cells tube formation assays employed Matrigel as a three-dimensional extracellular matrix. (A) HUVECs tube formation was evaluated by Matrigel with COM (10, 50, 75 μg/mL), 50 ng/mL VEGF, and 1 pmole Vinblastine (Velbe) in EBM medium for 24 h. (B) The number of tubule branches and (C) tubule junctions per field, was calculated using angiogenesis analyzer of Image J software. Data are expressed as the mean ± S.E.M. of two experiments. $^{a}p < 0.05$ vs. control.

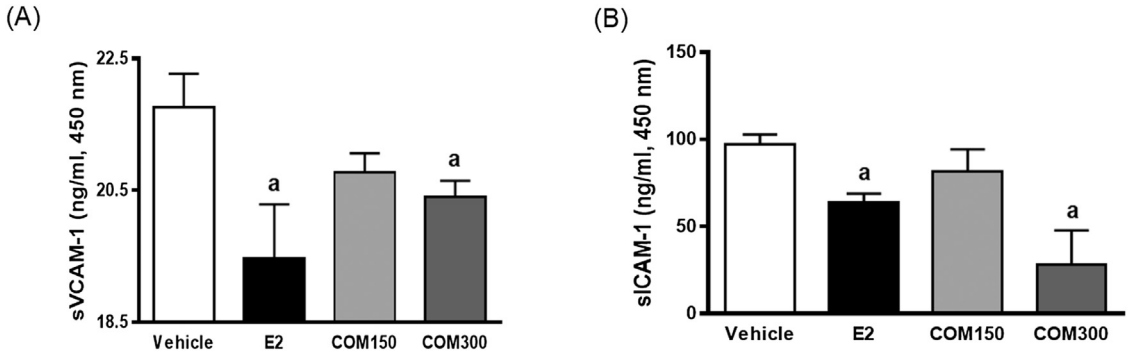

Fig. 5. Inhibitory effects of COM on serum sVCAM-1 and sICAM-1 levels. Adhesion molecule, (A) sVCAM-1 and (B) sICAM-1, was analyzed by ELISA kit. Values are presented as the means ± SD ($n = 7$). $^{a}p < 0.05$ vs. vehicle. E2: estradiol.

decrease in the muscle blood vessels of COM-treated groups (150 and 300 mg/kg) compared to the non-treated group (Fig. 6).

4. Discussion

Peripheral arterial disease is a serious medical condition that is affected by both age and common atherosclerosis risks (i.e., smoking, diabetes mellitus and dyslipidemia).[27,28] Menopause is one of the most common physical symptoms of sensory abnormalities in the limbs.[29] Descending estrogen production reduces the structural effect of collagen and makes skin thinner; this leads to decreased blood flow to surface nerves and symptoms of tingling and numbness.[30] Paresthesia are a common signs of central and/or peripheral pathological progress and is due to the ectopic stimulatory activity in dermal afferents.[31] Paresthesia is usually related to peripheral blood pressure and the inflammation

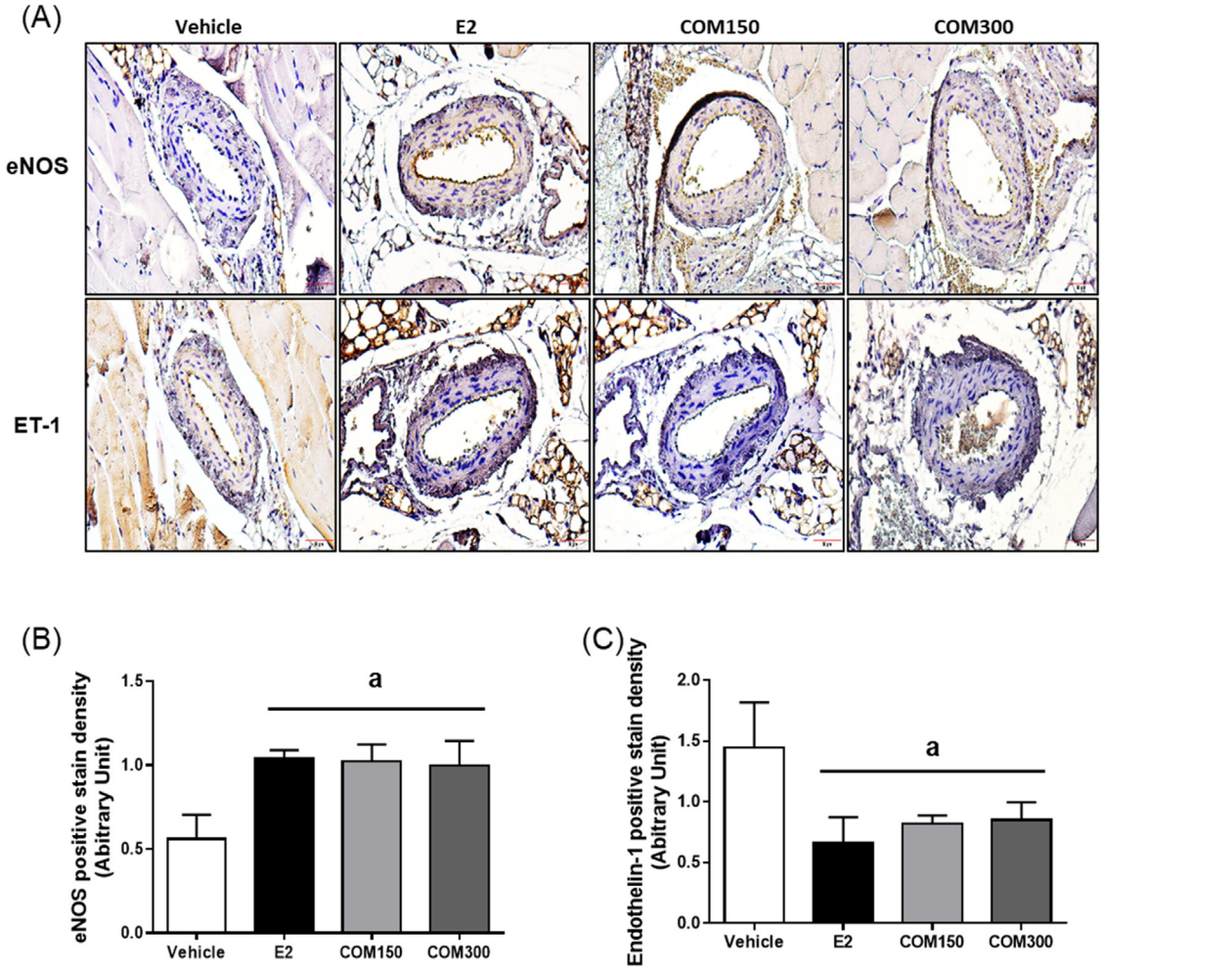

Fig. 6. Immunohistochemistry analysis for ET-1 and eNOS. (A) Immunohistochemistry (hematoxylin counterstained) in the muscle blood vessels of different experimental groups (400×). (B) The eNOS and (C) ET-1 immune positivity expressed as area %. Values are presented as the means ± SD ($n = 7$). [a] $p < 0.01$ vs. vehicle.

associated with ischemic injury.[32,33] The purpose of this study was to investigate whether COM extracts alleviate the blood flow disorder in ischemic injury of the hind-limb of ovariectomized mice. In the present study, we exposed female mice to surgeries for the induction of estrogen deficiency and hind-limb ischemia, which was hypothesized to induce conditions similar to those of peripheral arterial disease observed during menopause. In this study, we then determined the activities of cell migration and tube formation (tubule branches and junctions) of vascular endothelial cells. In addition, we examined the effect of the COM extract by measuring changes in the blood flow and capillary density in the hind-limb of the ovariectomized mice. Given the well-known angiogenic effects of endothelial cell proliferation, migration, and tube formation, changes related to the capillary blood vessels are expected.[34–36]

The activity of migration in HUVECs showed a significant increase following treatment with 10, 50 and 75 μg/mL of the COM extract. In addition, the tubule branches and junctions also showed an increase following treatment with all doses of the COM extract. The wound healing process involves angiogenesis, tissue formation and tissue remodeling; the inflammatory response is also known to play an important role in wound healing.[4,37,38] Previously, the increased movement of vascular endothelial cells during the angiogenic process is closely linked to the formation of new blood vessels in existing blood vessels for various types of wounds.[35,39] Ashcroft et al reported that the acceleration of wound healing through the reduction of inflammation and pro-inflammatory cytokines plays a crucial role during the healing response, and neovascularization occurs during pathological conditions, such as ischemic and inflammatory responses.[12,34] In our mouse model, we observed the induction of blood flow with treatment of the COM extract after hind-limb ischemic surgery in ovariectomized mice, compared to the nontreated group. Overall, this study demonstrates a more pronounced angiogenic response and improves the capillary density of hind-limb muscle following treatment of the COM extract. These results suggest that the effect of COM in our animal model is examined for therapeutic potential of COM after hind-limb ischemic surgery, and its improving effect may have potential for recovery effects rather than protective effect.

Recently, several studies have been accomplished to allecviate the side effects of chemicals and hormones therapy by using natural substances. Additionally, there have been an increasing number of studies examining the use of herbal medicine to treat symptoms in pre-/postmenopausal women.[40,41] One such herbal medicine, COM, is known as a medicinal herb that protects the liver and kidneys, and it has been reported to effectively protect against bone disorders, such as rheumatoid arthritis and osteoporosis.[42–44] Moreover,

COM containing prescriptions are known to reduce some common symptoms of menopausal women, such as obesity and hormonal disturbances.[42,44]

We also examined the effect of the COM extract by changes of inflammation-associated markers, sVCAM (soluble vascular cell adhesion molecule), sICAM (soluble intracellular adhesion molecule) in our animal model. Significant reductions in sVCAM and sICAM serum levels observed in COM treated groups, and which indicates stimulation of vascular endothelial cells and further development of angiogenesis of peripheral blood vessels. These data suggest that COM has anti-inflammatory effects *in vivo*, given that the secretion of adhesion molecules plays an important role in the robust adhesion of monocytes to activated endothelial cells and induction of subsequent endothelial dysfunction.[45] We also found that induction of the protein levels of angiogenic factor (eNOS; endothelial NO synthase) or reduction of anti-angiogenic factor (ET-1; endothelin-1) in the hind-limb lesion of the ovariectomized from COM-treated mice.

In conclusion, we demonstrated improvement of blood perfusion and the peripheral density via the regulation of angiogenic responses by COM treatment using *in vivo* and *in vitro* models. Based on the current findings, COM may be used as a potent therapeutic for the treatment of ischemic injury by effectively controlling the functions of vascular endothelial cells under the ischemic condition of hind-limb in mice of the ovariectomized. Additionally, based on the results, COM has the possibility for clinical use as recovery medicine after plastic surgery, disturbance of peripheral blood circulation, punctured wound, and also applicable for use as a postmenopausal women's health functional food.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Data availability

Data will be made available on request.

Supplementary

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imr.2019.04.010.

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