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Tetragonia tetragonoides (*Pall.*) *Kuntze* Restores Blood Perfusion from Hind-Limb Ischemic Mice

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Abstract: *Tetragonia tetragonoides* (*Pall.*) *Kuntze* (TTK) is grown for the edible leaves, and can be used as food. And which commonly called Beonhaengcho in Republic of Korea. TTK is found along the seaside of the Jeju-Island and it has long been consumed as a food for women's health. We investigated the effects of TTK on peripheral circulation disorder during menopausal transition and/or menopause in a hind-limb ischemic (HLI) mouse model. Chemotactic motility and tube formation of vascular epithelial cells were evaluated in human umbilical vein endothelial cells (HUVECs). Female C57BL/6 mice were fed a TTK (150 or 450 mg/kg/day) for four weeks and the rate of blood flow was assessed using a laser Doppler after HLI. TTK treatment significantly increased cell migration and the branch interval value of tubular structure in a dose-dependently. In the TTK treatment group, blood flow rate was significant induced at 7, 14, and 28 days after HLI, compared with the vehicle. TTK treatment also an increase in capillary density, and the highest levels of pERK(1/2), pAkt, pPLCγ1 and pFAK proteins compared to the vehicle control. These results suggest that extract of TTK may ameliorate the blood flow via improvement of peripheral angiogenesis under hind-limb ischemic stress in a menopausal mouse model.

Keywords: *Tetragonia tetragonoides* (*Pall.*) *Kuntze*; menopausal syndrome; blood circulation; hind-limb ischemia; ovariectomy; human umbilical vein endothelial cells; angiogenesis

1. Introduction

Symptoms of perimenopause are inconvenient for women and affect their daily lives. Typical symptoms of menopausal syndrome result from hormonal imbalances as especially from a decline in ovarian sex steroid levels [1]. Perimenopause is a physiological process mostly accompanied by aging and other metabolic disorders and lowers quality of life [1,2]. Particularly, Premenopausal and/or postmenopausal women display increased incidence of atherosclerosis and cardiovascular disease (CVD) among symptoms of estrogen deficiency [3–5]. The postmenopausal increase in vascular disease incidence is at least partially due to reduced levels of ovarian hormones, such as estrogen and progesterone [6,7] peripheral vascular disease (PVD) is one of the circulatory diseases that affect blood vessels outside the heart, brain, and limbs, resulting in pain and fatigue [8–10]. Risk factors for CVD and PVD are comparable and include smoking, diabetes mellitus, hypertension, age, and dyslipidemia [11]. Dyslipidemia is another feature of menopause in women, leading to a significant increase in the development of CVD and/or PVD [12,13]. Recent studies also have shown that women that receive hormone replacement therapy (HRT) after menopause have a lower rate of cardiac dysfunction [14–16]. However, some randomized trials have shown that HRT may induce CVD and other risks in postmenopausal women [17–19].

During menopause, estrogen deficiency can lead to many health problems, such as hot flashes, sleep disturbances, joint pain, bone loss, and circulation disorders [20,21]. Estrogen appears to have beneficial effects in various species models of cardiac, brain, and hind-limb ischemia via angiogenesis, limitation of endothelial dysfunction, and anti-inflammatory and anti-apoptotic effects [22–26]. Additionally, estrogen is also an important vascular expansion molecule that activates endothelial nitrogen oxide synthase through estrogen receptor alpha-mediation mechanism, causing the expansion of blood vessels [27]. Estrogen regulates gene transcription via estrogen receptors, and its expression elevates the pathophysiological progress of angiogenesis in endothelial cells [28,29]. Wound healing in postmenopausal women has been shown to be delayed, and the inflammatory response is induced by reduced serum estrogen levels; however, these issues can be recovered through induction of vascular endothelial growth factor (VEGF) or reduction of inflammatory factors by estrogen [1,26,30,31]. The role of estrogen is in the regulation of blood lipid and cholesterol levels, which could affect growth of vascular endothelial cells, and the remedy from ischemic and/or vascular damage leading to various disease models [32–34].

Recently, we studied the efficacy of *Tetragonia tetragonoides* (*Pall.*) *Kuntze* (TTK) therapy on various menopause symptoms [35] in ovariectomized rodents. Ovariectomy is a typical used model for investigation of postmenopausal symptoms due to ovarian hormone deficiency, i.e., estrogen and progesterone [36]. TTK is a halophyte that grows on Jeju Island and the southern coast of Korea. The Korean name of TTK is Beonhaengcho, and it is known as New Zealand spinach in other parts of the world. TTK has similar flavor and texture properties as spinach and is also cooked in a similar manner. Ancient documents, such as the Donguibogam, TTK has been classified as a traditional drug for the treatment of tumors [34], and folk remedies have long prescribed TTK as a food for women's health [34,35]. Herbal medicines are commonly used as treatments for menopausal multi-symptoms, such as dyslipidemia, energy metabolism, diabetes, bone metabolism, and others in perimenopause [35,37]. Further, herbal TTK remedies do not affect the reproductive tract or estrogen receptor (ER-alpha and ER-beta)-mediated mechanisms [37,38]. On the other hand, herbal extracts have been reported to have strong side effects as hormone mimics [39–41], leading to metabolic disorders such as diarrhea, nausea, headaches, allergic reactions, and dizziness [42]. Extract of TTK has been shown to be effective for treatment of symptoms in a menopause-like symptom rat model, although TTK extract did not affect the classical mechanism of estrogen receptors [35]. In particular, TTK extract has been shown regulates the blood LDL-cholesterol and triglycerides in our previous study [35]. However, the detailed mechanisms of angiogenesis and neo-vasculogenesis under surgical and artificial ischemic conditions have not been elucidated.

In the present study, we used a murine model of ovariectomy- and hind-limb ischemia to investigate whether or not TTK can improve blood flow rate via angiogenesis and inflammatory responses in the context of vascular surgical damage. The flow rate of peripheral blood rate was analyzed using the laser Doppler blood perfusion system [43], and levels of angiogenic and inflammatory cytokines were evaluated by immunoblotting. Additionally, tube formation and cell mobility in human umbilical vein endothelial cells (HUVECs) were determined in order to elucidate the potential mechanisms underlying improvement of blood flow rate by TTK.

2. Materials and Methods

2.1. Preparation of Tetragonia tetragonoides (Pall.) Kunze Extract

TTK was obtained from a mercantile vendor at Gwangmyeong-Dang (Republic of Korea), and which species were identified by the botanist specialist Dr. Byeong-Sub Ko (Korean Institute of Oriental Medicine, KIOM). Dried 4 kg of TTK was extracted at 25–30 °C with 70% ethanol 40 L for three days. The extract was concentrated using a evaporator, and lyophilized in a freezer dryer. The yield of TTK ext. was 992.6 g (22.43% *w/w* yield).

2.2. HUVECs Culture and Cell Proliferation

HUVECs (Lonza, MD, USA) were cultured in endothelial growth medium-2 (EGM-2] with 0.1% VEGF, 0.4% hFGF-2, 0.1% R3-IGF-1, 0.1% hEGF, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100, and 2% fetal calf serum (FCS) at 37 °C, 5% CO_2. EGM-2 containing 2% or 0% FCS was used according to experimental conditions and HUVECs were studied between two and seven passages. HUVECs proliferation was investigated by using the WST assays. 5×10^3 cells/well cultured in 96 well plates were treated with 1, 5, 10, 50, 100 μg/mL TTK for 24 h. 10 μL of EZ-Cytox solution (DoGenBio Co., Ltd., Seoul, Korea) was added to each well to measure the absorbance at 450 nm after 4 h. The cell survival rate for each treatment concentration of TTK was evaluated with the treated optical density formula of untreated control ×100.

2.3. Cell Migration Assay and Tubing Formation Assay

The ability of TTK to enhance HUVEC migration was observed using a corning trans-well system. HUVECs were cultured in the upper chamber with TTK (10, 50 μg/mL) and VEGF (50 ng/mL), 17β-estradiol (1 nM), and Simvastatin (10 μM) in the medium without FCS. The lower chamber containing 2% FCS was used as the chemoattractant. A duration of 6 h later, the trans-well was collected, and the cells at the bottom were stained with H&E solutions (H&E, Sigma-Aldrich, St. Louis, MO, USA) to facilitate observation and cell counting. HUVECs (5×10^4 cells/well) were seeded on matrigel (BD Biosciences, San Diego, NJ, USA) in 24 well plate with TTK (10, 50 μg/mL) and Vinblastine (1 pM), VEGF (50 ng/mL), 17β-estradiol (1 nM), and Simvastatin (10 μM) were supplemented to the cell media without growth factor. After 12 h incubation, the tube structures that formed in the gel were captured at magnification (4×). The branching interval of the tube structure was evaluated using Image J analyzer (angiogenesis ability) [44].

2.4. Experimental Animals and Treatments

The C57BL/6 mice (female, six weeks old, total n = 40) obtained from Dahan Biolink (Eumsung, Korea), and were allowed to adapt to room conditions for 1 week. And the animals were raised in accordance with the following condition (relative humidity: 45 ± 5%, temperature: 20 ± 2 °C). Mice were fed the AIN-76A diet as a vehicle control (ovariectomy and hind-limb ischemia; OH), AIN-76A with 17beta-estradiol (100 μg/kg; Sigma-Aldrich, St. Louis, MO, USA) as a positive control (OH + E_2), and AIN-76A supplemented with TTK (TTK 150; 150 mg/kg or TTK 450; 450 mg/kg) for four weeks. Weight gain and daily intake were measured following the experimental feeding period. Changes in Daily food intake and BW were documented and the estrogenic potency was determined by analyzing uterine weight. All the mice were then anesthetized with Zoletil (30 mg/kg) to collect blood and vessels, and take various tissue samples for further analysis. Animal experimental procedures have been recognized by Ethics Committee of KIOM (approved NO. 16-018).

2.5. Surgeries

The hair of each virgin mice were shaved from dorsal midline or hind-limbs with a clipper and hair removal cream. The skin from both thighs was then incised to expose the vessels (artery, vein). Mice were anesthetized with an intra-peritoneal injection of a Zoletil (30 mg/kg)-Roumpun (5 mg/kg)-saline mixture 4:2:4. The dorsal midline of mice was incised and the abdominal cavity is entered via a blunt puncture through abdominal wall. The fallopian tube (uterine) was ligated, and the ovaries were cut off with surgical scissors. Fat pad and remaining tissue were returned to the abdominal cavity, and the skin incision was sutured by surgical black silk (6-0). The two area of vessels (superficial- and saphenous-; artery and vein) of the femoral area were ligated and excised in the left hind limb (at seven weeks of age), and its surgery was modified in accordance with criteria of [45].

2.6. Blood Perfusion Analysis

The ischemic/non-ischemic blood flow rate were measured using blood perfusion analyzer (PeriScan-PIM3; Perimed AB, Sweden) at the manufacturer's recommendation (Perimed AB). Low or no blood flow indicates black or dark blue; vice versa, yellow or red. The average flow of the ischemic and non-ischemic limb(s) was calculated on the histogram-based colorimetry using by PIMSoft (v1.5; Perimed AB).

2.7. Histopathological Analysis

The tissue was inflated with 10% neutral buffer formalin to fix the tissue. These tissues were embedded with paraffin and sliced into 5–7 micron thick sections, and these sections were stained with H&E. All tissue samples were evaluated, scored and photographed in the way of the blind under a light microscope (BX43; Olympus, Tokyo, Japan). The images were captured under a microscope using Olympus DP-73 (Olympus) and software (CellSens standard, Olympus). The number of follicles was also calculated by microscopy.

2.8. Immunohistochemical Stain

Endothelial cell marker, cluster of differentiation 31 (CD31), were observed by immunohistochestry. Alkaline phosphatase and endogenous peroxidase activities were blinded by BLOXALL (Vector Lab. Inc., Burlingame, CA, USA) for 0.5 h. Non-specific reactions were prevented by placing the sections with CAS-BLOCK (Thermo-Fisher Scientific, Pittsburgh, PA, USA) for 1 h. Blocked sections were incubated for antigen-antibody reactions at room temperature for 4 h with antibodies against CD31 (1:250; rabbit polyclonal, Abcam) diluted with CAS-BLOCK solution. After washing with PBS-T (Phosphate-buffered saline containing 0.5% Tween-20), the sections were subsequently incubated with a Dako REAL^{TM} Envision^{TM}/HRP (Rabbit/Mouse; Dako, Carpinteria, CA, USA) for 0.5 h at room temperature. After washing with PBS-T, the sections were reacted with Dako REAL^{TM} DAB + Chromogen (Dako) and sections were stained with Mayer's hematoxylin (Sigma-Aldrich). The sections were mounted with mounting solution (Thermo-Fisher Scientific).

2.9. Immunoblot Assay

Proteins were extracted using T-PER^{TM} reagent (Thermo-Fisher Scientific) in accordance at the manufacturer's recommendations. Protein (20 μg) were loaded and separated on a SDS-PAGE gel under 80 V and transferred to a PVDF (polyvinylidene fluoride transfer membrane; Perkin Elmer Co., Foster City, CA, USA) in a Mini Trans-Blot® Cell (Bio-Rad, Hercules, CA, USA). The blot was blocked with PBS-T include 5% skim milk for 0.5 h, the incubated with 1st antibody: phosphor-ERK(1/2) or ERK(1/2) (1:2000, rabbit-monoclonal, Cell Signaling Technology Inc., Beverly, MA, USA), phosphor-Akt or Akt (1:2000, rabbit-monoclonal, Cell Signaling Technology), phosphor-FAK (1:2000, rabbit-monoclonal, Cell Signaling Technology) or FAK (1:2000, rabbit-monoclonal, Thermo-Fisher Scientific), phosphor-PLCγ1 or PLCγ1 (rabbit-monoclonal, Cell Signaling Technology) or GAPDH (mouse-monoclonal, Sigma-Aldrich). The membrane was incubated with the appropriate HRP-conjugated secondary antibodies (anti-rabbit, Cell Signaling Technology; anti-mouse, Santacruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The blots were developed by incubation in EzWestLumi One reagent (WSE-7110; ATTO, Japan) and subsequently exposed to the Fusion SL-4 imaging system (Vilber Lourmat, Marne La Vallée, France), after which images were captured using the Fusion SOLO software (Vilber Lourmat).

2.10. Statistical Analysis

Results of blood perfusion, weight(s) and capillary density are indicated by the means ± SD. And the results of HUVECs proliferation, tube formation and migration presented as means ± SEM.

For multiple comparison tests with two-way ANOVA using Tukey by PRISM software (v6.0; Graph Pad, La Jolla, CA, USA). p values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of TTK on Proliferation of HUVECs

To examine the angiogenic activity of TTK, the optimum doses of TTK with regards to HUVECs growth were evaluated. A concentration range of 0, 1, 5, 10, 50, 100 μg/mL of TTK was applied to HUVECs. As a result, the growth increase of HUVECs was observed dependent on the TTK treatment concentration, and significant endothelial cell proliferation was observed in the 100 ug/mL treatment group from TTK 10 μg/mL. TTK at a concentration of 10 μg/mL significantly increased cell proliferation by 26%, and the proliferative effect further increased to 44.1% at 100 μg/mL of TTK (Figure 1).

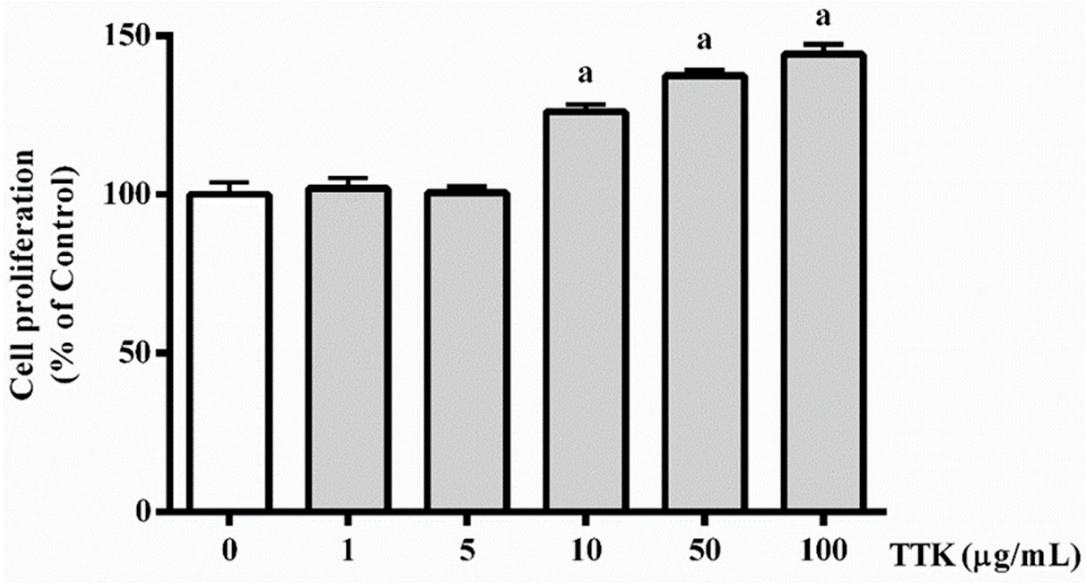

Figure 1. Effect of TTK on proliferation of HUVECs. Cells were plated to the 96-well plate and treated at the marked TTK concentration for 24 h. Cell growth rates were evaluated using WST assay. The experiments were repeated four times. The results are presented as the means ± SEM. [a] $p < 0.001$ vs. 0 (untreated cells).

3.2. Effect of TTK on Migration of HUVECs

Effect of TTK on endothelial cell migration was determined. In the control, the number of migrated cells was 1 ± 0.82 cells/field, and TTK induced cell migration in a dose-dependent manner (Figure 2A). TTK at 50 μg/mL elevated cell migration to 106.33 ± 4.19 cells/field, which was 106-fold higher compared with that of the control (Figure 2B). In the positive control, 50 ng/mL of VEGF and 1 nM 17β-estradiol induced cell migration to 122.33 ± 4.92 and 105.33 ± 4.11 cells/field, respectively (Figure 2B). In the case of simvastatin treatment for peripheral vascular disease, HUVEC migration showed a 58-fold increase compared with the control, whereas TTK treatment induced 1.8-fold higher HUVEC migration compared to simvastatin (Figure 2B).

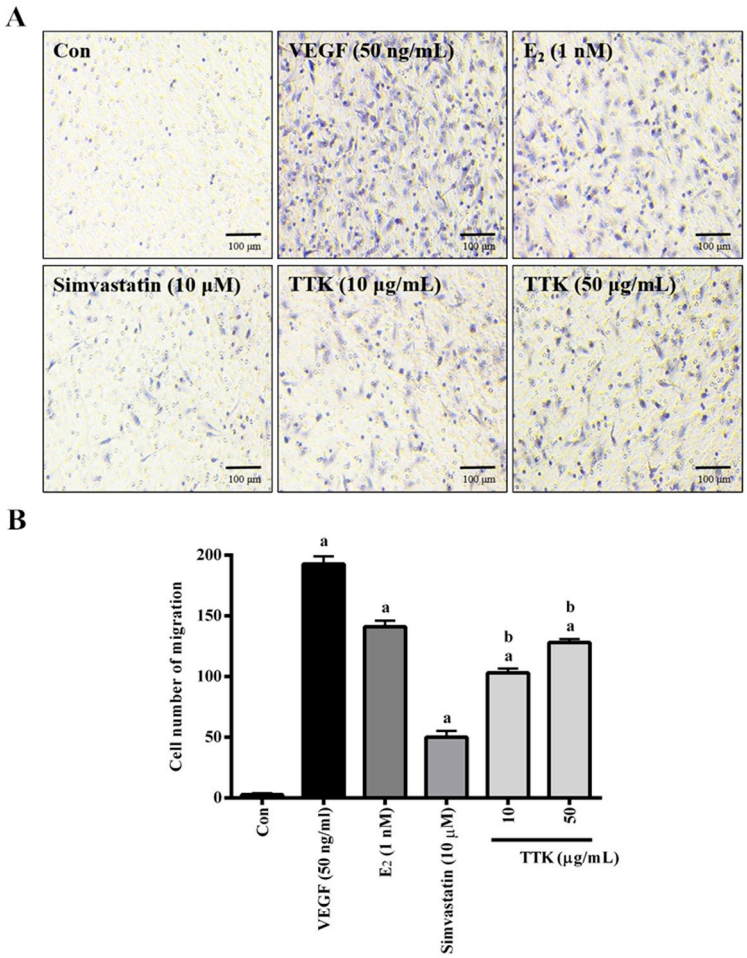

Figure 2. Effect of TTK on migration of HUVECs. (**A**) The cells were treated with TTK (10, 50 μg/mL), 50 ng/mL VEGF, or 1 nM 17β-estradiol, or 10 μM Simvastatin. Cell migration was evaluated by observing each section under a microscope using the tran-swell chamber. (magnification, 200×). (**B**) The migrated cells were numbered in at least three fields after each examination, and the data are expressed in the number of cells per field. Data present means ± SEM. of three experiments. [a] $p < 0.001$ vs. control (untreated cells), [b] $p < 0.001$ vs. Simvastatin.

3.3. Effect of TTK on In Vitro tube Formation

The effect of TTK on differentiation of HUVECs into tubes in vitro was investigated. Compared with the branching interval of the HUVEC tubular structure formed under control conditions, the branching interval value of the tubular structure significantly increased in a dose-dependent manner upon TTK treatment (Figure 3A). The branching interval value of the tube formed in the 50 μg/mL of TTK group was 222.39 ± 1.56, which was 2.2-fold developed than that of the control (Figure 3B). In addition, a significant increase in tube formation was observed in the TTK group compared to the simvastatin group (Figure 3B). These results suggest that TTK may play an important role in the induction of angiogenesis via vascular endothelial cell activation as well as have a stronger effect on vascular endothelial cell activity than simvastatin.

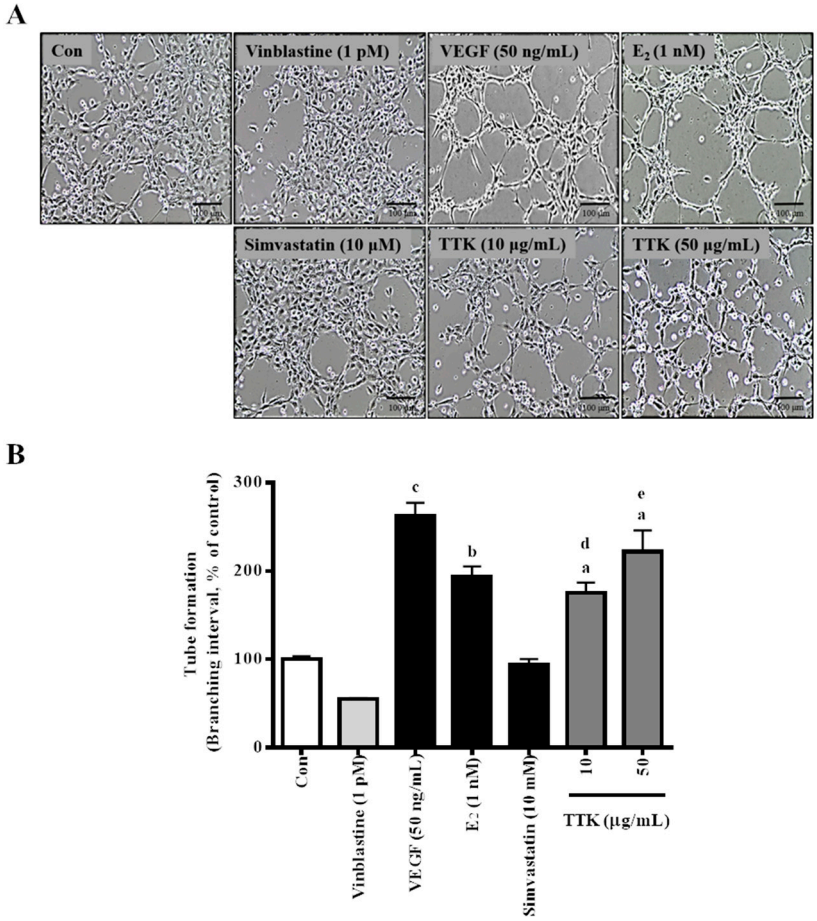

Figure 3. Effect of TTK on in vitro tube formation in HUVECs. (**A**) Cells were treated with different concentration of TTK (10, 50 μg/mL), 50 ng/mL VEGF, or 1 nM 17β-estradiol, 10 μM Simvastatin on matrigel. After 12 h of incubation, tube formation was observed using a microscope (magnification, 4×) and photographed. (**B**) The tube formation was analyzed by measuring each branch interval value using Image J analyzer (angiogenesis ability). Data are expressed as the mean ± SEM. of three experiments. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs. control, [d] $p < 0.01$, [e] $p < 0.001$ vs. Simvastatin.

3.4. Blood Perfusion and Animal Condition

To determine whether dietary consumption of TTK (150 or 450 mg/kg/day) could stimulate vascular endothelial cells to blood circulation in the context of hind-limb ischemia. No abnormal weight gain, daily food intake and other findings in our animal model have been observed, and uterine weight were not changed to estimate estrogen activity of TTK under our animal condition (Table 1). However, uterine weight was higher in E_2 treatment group compared the other groups.

Table 1. Body weight and daily food intake of hind-limb ischemic mice.

Groups	Body Weight (g)		Body Weight Gain (g)	Daily Food Intake (g)	Uterine Weight (mg)
	Baseline	After 28 Days			
OH	19.80 ± 0.49	23.06 ± 1.22	3.27 ± 1.36	3.72 ± 0.41	17.48 ± 3.61
OH + SV	20.11 ± 0.88	23.00 ± 1.68	2.89 ± 1.99	3.91 ± 0.96	16.34 ± 3.31
OH + E2	19.98 ± 0.73	22.51 ± 0.82	2.53 ± 0.63	3.84 ± 0.62	265.10 ± 176.02 [a]
TTK 150	20.48 ± 0.63	23.36 ± 1.69	2.88 ± 0.88	3.39 ± 0.40	14.79 ± 3.09
TTK 450	20.41 ± 1.26	24.19 ± 1.63	3.78 ± 1.86	3.73 ± 0.52	15.83 ± 2.62

[a] $p < 0.05$ vs. Vehicle.

The flow rate of peripheral Blood was observed at the lesion of hind-limbs in all surgical groups after ischemic surgery (0 d) (Figure 4). At day 7 after surgery, the rate of blood perfusion in ischemic hind-limbs had significantly ameliorated in the E_2 and TTK 450 groups than vehicle group, however, there was no therapeutic effect in other experimental groups. At 14 d and 28 d (after ischemia surgery), blood perfusion rate was significantly improved in all treatment groups. Although no significant difference in blood flow between the vehicle and the TTK 150 group was found in 7 d following ischemic surgery, however, the high dose of TTK 450 significantly improved the signs of hind-limb ischemia (Table 2).

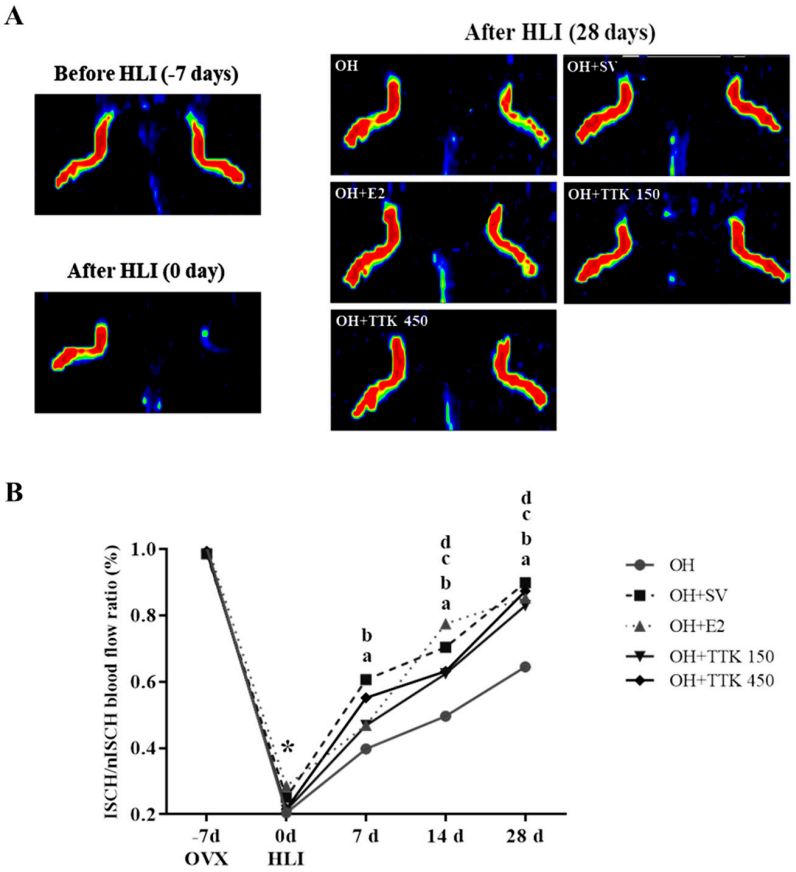

Figure 4. Effects of TTK on the blood perfusion in ischemic limb. (**A**) in color-coded images, low to no flow was displayed as black or deep blue whereas high blood flow was as yellow to red. Isch-L = ishchemic limb. nIsch-L = Non ischemic limb. (**B**) Quantitative evaluation of ischemic/non-ishcemic blood perfusion ratio. Values are presented as means ± SD (n = 7). * $p < 0.05$. vs. −7d, [a] $p < 0.05$ vs. OH, [b] $p < 0.05$ vs. OH + SV, [c] $p < 0.05$ vs. OH + E_2, [d] $p < 0.05$ vs. OH + TTK 150.

Table 2. Ischemic/non-ischemic blood flow ratio.

Group	Surgery I (OVX)	Surgery II (HLI)	After Surgery II (HLI)		
	−7d	0d	7d	14d	28d
OH	1.01 ± 0.04	0.21 ± 0.05	0.40 ± 0.12	0.50 ± 0.07	0.64 ± 0.08
OH + SV	0.99 ± 0.03	0.25 ± 0.06	0.61 ± 0.07 [a]	0.70 ± 0.11 [a]	0.90 ± 0.06 [a]
OH + E2	1.02 ± 0.02	0.29 ± 0.08	0.47 ± 0.08 [b]	0.78 ± 0.09 [a]	0.85 ± 0.08 [a]
TTK 150	1.00 ± 0.02	0.21 ± 0.04	0.47 ± 0.07 [b]	0.62 ± 0.06 [ac]	0.83 ± 0.07 [a]
TTK 450	0.99 ± 0.04	0.22 ± 0.04	0.55 ± 0.10 [a]	0.63 ± 0.09 [ac]	0.87 ± 0.09 [a]

[a] $p < 0.05$ vs. Vehicle, [b] $p < 0.05$ vs. Positive I, [c] $p < 0.05$ vs. Positive II.

3.5. Capillary Density

To investigate the angiogenesis effect of TTK on microcirculatory environment, the capillary density of the immunostained calf and thigh muscles was evaluated using anti-CD31 antibody. The five different fields of microscopic area as well as capillary vessels were scored under 200× magnification (mean number of capillaries/mm^2). Immunostained cells showed that the positive control had higher capillary density in ischemic muscles than vehicle control (Figure 5). Additionally, quantitative analysis also showed significant increases in capillary density in both low and high TTK groups. In particular, the high dose of TTK showed a significant increase in the number of capillary vessels compared to the low dose TTK (Figure 5). These results are consistent with the results of blood perfusion analysis (Figure 4).

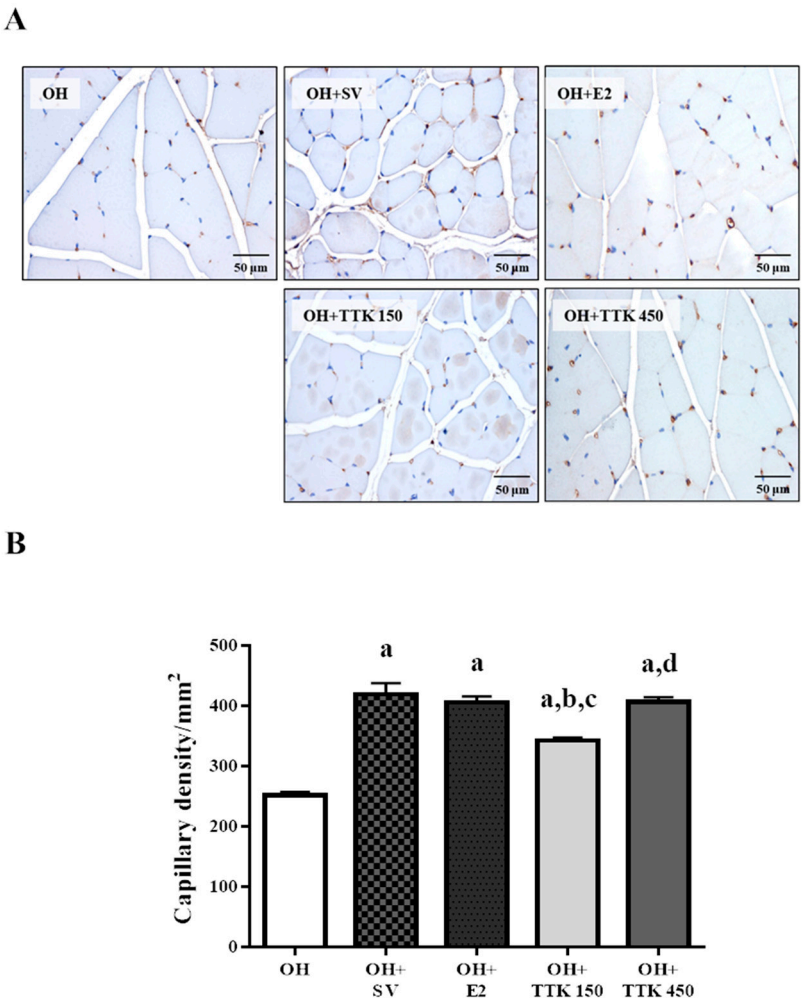

Figure 5. Effects of TTK on vascular formation in ischemic limb. (**A**) Representative micro-photographs of the section of ischemic hind-limb muscles stained histochemically for CD-31, magnification 400×. (**B**) Quantitative analysis of capillary density in ischemic hind-limb muscles. Data are presented as mean ± SD (n = 7). [a] $p < 0.05$ vs. OH, [b] $p < 0.05$ vs. OH + SV, [c] $p < 0.05$ vs. OH + E_2, [d] $p < 0.05$ vs. OH + TTK 150.

3.6. Expression of Angiogenic Factors In Vivo

To further elucidate the mechanisms underlying TTK-induced angiogenesis in hind-limb ischemic mice, we measured the expression levels of pERK(1/2), pAkt, pPLCγ1, and pFAK proteins in local tissues from all five groups of mice at 14 d after induction of ischemia. According to the results, expression levels of all proteins were significantly elevated in both TTK groups compared with

the vehicle control (Figure 6). The expression of pERK(1/2), pAkt, pPLCγ1, and pFAK were also upregulated in E2-treated group, but not significantly.

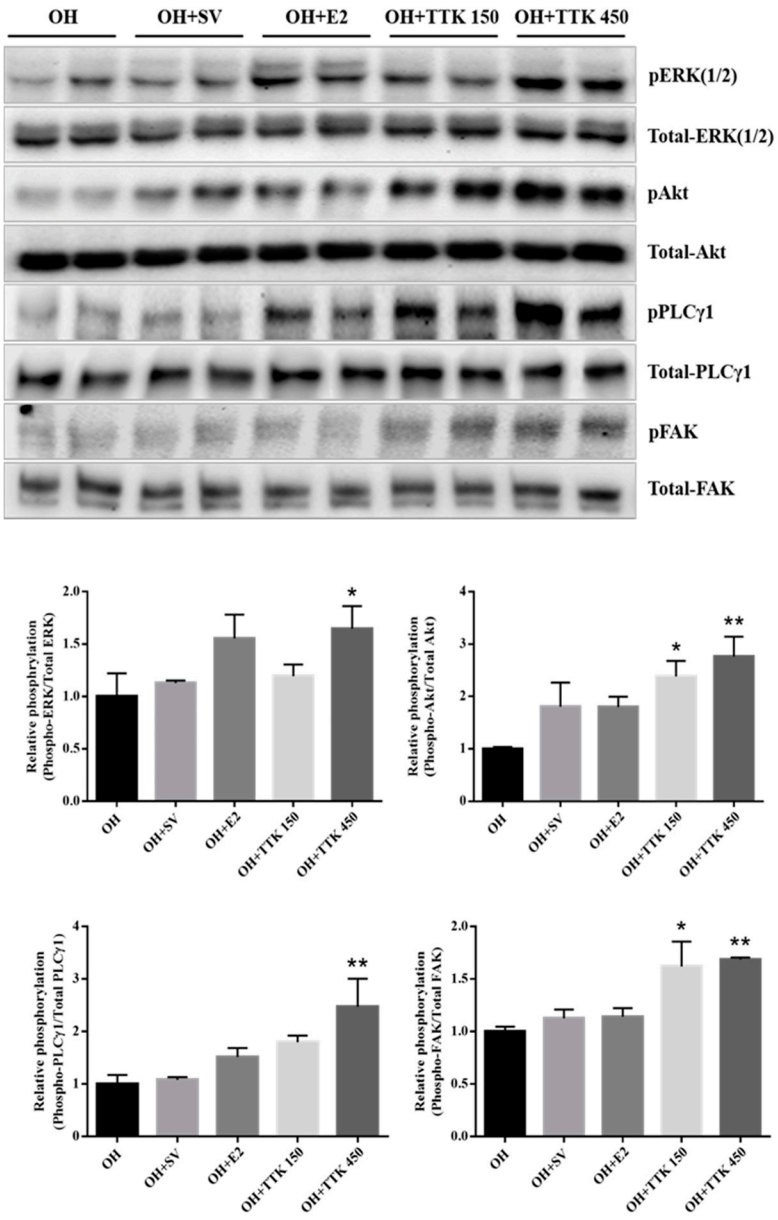

Figure 6. Angiogenic effects of TTK in ischemic limb. Western blotting assay to determine the expression of phosphor-ERK(1/2), ERK(1/2), phosphor-Akt, Akt, phosphor-FAK, FAK, phosphor-PLCγ1 and PLCγ1 contained in the ischemic hind limb on day 28 after surgery. * $p < 0.05$, ** $p < 0.01$ vs. OH.

4. Discussion

Perimenopause is a medical condition that develops in middle-aged females and is characterized by irregular or lack of estradiol and follicular-stimulating hormone levels. During the menopausal transition, imbalances in levels of ovarian hormones such as E_2 and P4 can lead to health problems, including hot flashes, night sweats, sleeping difficulty, osteoporosis, and heart disease [46,47]. In the present study, we exposed ovariectomized rats to hind-limb ischemic stress in order to induce conditions similar to those of PVD during the menopausal transition. We then examined blood perfusion and capillary density in the hind-limb and evaluated the angiogenic effects of TTK extract based on changes in endothelial cell migration, tube formation activities, and angiogenic factors.

In the case of PVD, risk factors indicating inadequate blood perfusion, perimenopause, postmenopause, or lipid metabolism disorders contribute to peripheral ischemic symptoms such as intermittent claudication [15,20]. In other study, in vivo models of hind-limb ischemia in ovariectomized rats were developed, including ones for examining blood perfusion, capillary density, and angiogenic factors in the presence or absence of estradiol [5,24,48]. In these studies, estrogen deficiency exacerbated blood perfusion by attenuating angiogenesis or neovascularization in a mouse model of hind-limb ischemia. In our previous study, treatment with TTK extract improved risk factors for CVD or PVD, including high-fat mass, overweight status, and lipid profiles [35]. Thus, we hypothesized that TTK extract could have a beneficial effect on blood circulation by decreasing risk of PVD in a murine hind-limb ischemic model.

We focused on improvement of blood perfusion by evaluating the blood flow ratio between ischemic (left hind-limb) and non-ischemic (sham-operated; right hind-limb) lesions. TTK treatment resulted in marked increases in blood perfusion, and a significant difference was first observed at 7d after ischemic surgery in the high TTK group. Additionally, blood perfusion improvements were observed at 14 to 28d after surgery in all treatment groups (positive control, low TTK, and high TTK groups).

Notable sexual dimorphism was previously reported in a rodent model of liver ischemic injury [49]. This result suggests that the protective mechanisms in hind-limb ischemic mice are dependent upon the up-regulation and activation of angiogenic signaling mediated by estradiol [49–51], and growth factors for angiogenesis are mediated via active-kinase signal pathways, including PI3K/Akt, ERK1/2, FAK, and p38/MAPK. Kinases regulate endothelial cell functions such as cell migration, survival, and vascular cell permeability [52,53]. Therefore, in our model, expression of angiogenic factors might have initially been stimulated in ischemic tissues of thigh and calf muscles by excision of femoral vessels. Expression levels of pERK(1/2), pAkt, pFAK, and pPLCγ1 were elevated by TTK treatment in a dose-dependent manner. Mitogen-activated protein kinases ERK1 and ERK2 (ERK1/2) are critical for angiogenesis and endothelial cell function [54,55]. FAK, also known as cytoplasmic tyrosine kinase, is important for cell migration and integrin-related signal production [56]. The serine-threonine kinase Akt is activated downstream of PI3K and mediates survival of endothelial cells [57], phospholipase Cγ 1 (PLC-γ1) activity at Ser1248, and enhancement of cellular motility [58]. However, the current in vivo studies had limitations lacking in inhibitor studies, such as siRNA and kinase inhibitors, in pERK(1/2), pAkt, pFAK, and pPLCγ1 expression. Therefore, further studies need to use the inhibitors to conduct experiments on the expression(s) of pERK(1/2), pAkt, pFAK, and pPLCγ1. In addition, the effects of the TTK's active compounds need to be tested.

The most striking phenotypic change observed in the present study was an increase in the number of blood vessels present in the hind-limb ischemic areas of experimental groups. These changes related to capillaries were expected given the well-known effects of VEGF on endothelial cell proliferation, migration, and tube formation [31,52,59]. Additionally, it is also reported that interactions between endothelial cells are involved in the promotion of various angiogenic pathway [23,24,44]. In vascular, the anti-apoptotic proteins as Bcl-2 and Bcl-xL, are may regulate arterial functions, and which are also regulates inflammatory response and angiogenic response under ischemic stress [25,60]. However, current studies have not been performed the correlation between ischemic stress in hind-limb ischemic lesions. In this study, we observed that TTK dramatically enhanced tube formation as well as HUVEC migration in an in vitro trans-well assay. Further, TTK treatment induced blood reperfusion of hind-limb ischemic lesions, suggesting that TTK-activated pERK(1/2), pAkt, pFAK, and pPLCγ1 pathways are involved in the recovery of ischemic damage via the angiogenic response.

In conclusion, we generated a model of hind-limb ischemia in estrogen-deficient mice that shares clinical similarities with humans suffering from clinical limb ischemia or other ischemic diseases. We proved the effect of improving blood perfusion through the improvement of peripheral microvasculature in ischemic lesions upon TTK treatment. Based on the results, TTK is likely to be a valid therapeutic compound for the treatment of ischemic injury by effectively controlling levels of

pERK(1/2), pAkt, pFAK, and pPLCγ1 in hind-limb ischemic lesions, including the angiogenic response in endothelial cells.

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