A Modified Formulation of Sutaehwan Ameliorates Menopausal Anxiety, Depression and Heart Hypertrophy in the VCD-Induced Menopausal Mouse Model

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Sutaehwan (STH) has been used in Korean medicine for the treatment of abortus habitualis such as fetal restlessness in the uterus. Previously, we reported that a modified formulation of STH, Sutaehwan-Gami, has phytoestrogen-like properties in an ovariectomized menopausal rat model. However, the therapeutic effects of STH and the precise mechanisms by which STH affects various menopausal symptoms remain poorly understood. The current study was designed to explore the effects of a modified form of STH on menopausal anxiety, depression and heart hypertrophy and its mechanisms in 4-vinylcyclohexene diepoxide (VCD)-induced menopausal mouse models. VCD-induced menopausal model mice were fed a modified form of STH, which contained water extract of 3 herbs (called STH_KP17001) at a dose of 100 or 300 mg/kg/d or as a positive control, estradiol at a dose of 0.2 mg/kg/d with standard mouse pellets for 13 weeks. The results show that STH_KP17001 significantly restored the VCD-induced weight reduction of uterine and ovary through the phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B (AKT) in the uterus and ovary. Moreover, STH_KP17001 showed slight proliferative effects and estrogen receptor α phosphorylation in MCF-7 cells. Treatment with STH_KP17001 reversed VCD-induced anxiety and depression through AMP-activated protein kinase (AMPK) activation and brain-derived neurotrophic factor (BDNF) expression in the cerebral cortex, while improving heart hypertrophy through inactivation of inhibitor of kappaB α (IκBα) in the heart. The results indicate that STH_KP17001 improves menopause-induced anxiety, depression and heart hypertrophy, implying its protective role for the management of menopausal symptoms.

Key words  Sutaehwan; menopause; 4-vinylcyclohexene diepoxide (VCD); depression; heart hypertrophy; AMP-activated protein kinase (AMPK)

INTRODUCTION

Menopause is the permanent cessation of menstruation, signaling the end of a woman’s reproductive years. Menopause impacts 25 million women annually. The WHO estimates that 1.2 billion women will be postmenopausal by 2030.13

During menopause, the rapid fall of estrogen production can lead to a decrease in QOL, and women often experience various symptoms such as hot flashes, sweating, anxiety, depression, mood swings, sleep disorders, vaginal dryness and joint pain.23 Moreover, the resistance to hypertension found in premenopausal females is lost after the onset of menopause, resulting in a drastic increase in severity and incidence of hypertension.33 Consequently, women in menopause receive hormone therapy to relieve the menopausal symptoms. However, serious adverse effects such as an increased risk in breast cancer have been reported in hormone therapy.24,34 Therefore, alternative strategies have gained more attention.

Sutaehwan (STH) is composed of four herbs: Cuscuta chinensis Lamarck (Dodder seed) and Dipsacus asperoides C. Y. Cheng et T. M. Ai (Himalayan teasel root), included in STH, and Evodia rutaecarpa Bentham (Evodiae Fructus), which have been used as a major herb to treat sterility caused by irregular menstruation as described in the Dongui Bogam, a representative textbook of Korean Medicine.6 In addition, Evodia rutaecarpa Bentham has been shown to provide protective effects on ovary cells against 4-vinylcyclohexene diepoxide (VCD)-induced ovary toxicity.8 VCD has been shown to gradually induce ovarian failure when repeatedly exposed in mice, which became a mouse model of menopause. In this VCD model, residual ovarian tissue remains intact after ovarian failure (menopause) and continues to be hormonally active secreting androgens, similar to that in humans.8,90 Moreover, VCD-treated mice display variations in the menstruation

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cycle length, increased follicle-stimulating hormone levels, and decreased circulating estrogen levels as the mice progress from perimenopause to ovarian failure, which resembles the progression of menopause in humans.

In the present study, we investigated the effect of STH_KP17001 on the cardiovascular hypertrophy and anxiety-like behavior associated with menopause and its mechanism of actions in the VCD-induced menopausal animal model.

MATERIALS AND METHODS

Preparation of STH_KP17001 STH_KP17001, a dried decoction of a mixture of three herbs, *Cuscuta chinensis* Lamark (800 g, Voucher number: 2016. 12. 05, CK16-C116-1-077, China), *Dipsacus asperisoides* C. Y. Cheng et T. M. Ai (800 g, CK16-C083-994, Voucher number: 2016. 12.13, China) and *Evodia rutaecarpa* Benthem (400 g, CK16-C063-1-927, Voucher number: 2016. 07. 11, China), was obtained from the College of Korean Medicine of Dongguk University. A decoction was prepared by adding a total of 2 kg of herbs to 1.6 L of water and boiling at 80°C for 4 h. Then, the decoction was pulverized (100 mesh) and used for activity evaluation and other analyses. Finally, 400 g of the extract was obtained from a total of 2 kg of herbal raw materials (yield: 20%).

HPLC/UV Condition HPLC analyses were performed using the Agilent 1260 HPLC system with a UV detector and Agilent autosampler (Agilent, Santa Clara, CA, U.S.A.). Reverse-phased columns were used for separation and detection of target compounds, including Waters Xbridge C18 column (250×4.6 mm, 5 μm) (GL Sciences Inc., Shinjuku, Japan) for Dodder seed (neochlorogenic acid, chlorogenic acid, caffeic acid and hyperoside). Isocratic separation of Evodiae Fructus was performed using 50% acetonitrile at a flow rate of 1 mL/min for 15 min with detection at 254 nm. Linear gradient separations were carried out for the analysis of Himalayan teasel root using 100% acetonitrile (A) and water (B) as mobile phase at a flow rate of 1 mL/min for 15 min with detection at 212 nm (gradient condition: 0–60 min, 15–35% A), and Dodder seed using 100% acetonitrile (A) and water (B) as mobile phase at a flow rate of 1 mL/min for 60 min with detection at 212 nm (gradient condition: 0–60 min, 15–35% A), and Dodder seed using 100% acetonitrile (A) and water (B) as mobile phase at a flow rate of 1 mL/min for 90 min with detection at 254 nm (gradient condition: 0–5 min, 5% A; 5–30 min, 5–11.2% A; 30–90 min, 11.2–30% A). The mobile phase was filtered under vacuum using a 0.45 μm membrane filter and degassed before use. Throughout the HPLC analyses, column temperature was maintained at 30°C.

Cell Culture and Cell Viability Assay Human breast cancer cell line, MCF-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL streptomycin (WelGENE, Daegu, Korea). The cells were incubated in a humidifier containing 5% CO₂ at 37°C. Cell cytotoxicity was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, U.S.A.) assay. First, MCF-7 cells were seeded on 24-well plates at a density of 5×10⁴ cells/well and mixed with varying concentrations of STH_KP17001 (25, 50, 100, 200 and 400 μg/mL) or estradiol (0.001, 0.01, 0.1, 1, 10 μM) (Sigma-Aldrich) for 24 h. Then, the cells were incubated with 2 mg/mL MTT solution for 4 h at 37°C. After incubation, the supernatants were collected, and dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added. Finally, the optical density (OD) was measured at 590 nm with a microplate reader (UV max, Molecular Devices, Sunnydale, CA, U.S.A.). For Western blotting, MCF-7 cells were incubated with 50 μg/mL of STH_KP17001 for the indicated time (5, 10, 30, 60 and 120 min).

Experimental Animals and Treatment Immature female C57BL/6 mice (21 d old) were obtained from Koatech (Pyeongtaek, Korea). Animals were maintained at constant temperature of 22 ± 1°C, relative humidity of 55 ± 1%, and 12:12 h light-dark cycle (light on at 9:00 a.m.). Animals were treated according to the protocols approved by the Institutional Animal Care and Use Committee at Dongguk University (no. IACUC-2015-056) and the National Institute of Health guidelines. VCD (Sigma-Aldrich) dissolved in sesame oil (Sigma-Aldrich) was administered by intraperitoneal injection (i.p.) in 28-d old mice. Mice were randomly selected for each group and treated with VCD (160 mg/kg/d, i.p.) (VCD group, n = 7) or sesame oil (vehicle control, i.p.) (Sham group, n = 7) for 17 d. Low (100 mg/kg/d) (Low group, n = 7) or high level (300 mg/kg/d) (High group, n = 7) of STH_KP17001, or estradiol (0.2 mg/kg/d, Sigma-Aldrich) (Estradiol group, n = 7) were given to mice with food. To examine the nutritional status, weight gain and food intake were recorded weekly at the same time for 13 weeks. At the end of the treatment schedule, all animals were sacrificed, and the blood, heart, ovaries and uterus were collected. Feed efficiency ratio (FER) was calculated using the following equation: FER = total weight gain/total food intake.

Behavior Test Open field test apparatus had a white floor of 40×40 cm and white walls of 40 cm high. Each mouse was placed in the center of the open field and allowed to freely explore the apparatus for 10 min. Time in the central square (10×10 cm) and total traveled distance were automatically recorded by ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, U.S.A.).

Biochemical Analysis of Blood Blood samples were collected and centrifuged at 3000×g for 15 min at 4°C, and serum was stored at −70°C until further analysis. Blood glucose, total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, glutamate oxaloacetate transaminase (GOT) or glutamate pyruvate transaminase (GPT), triglyceride (TG) (Asan Pharmaceutical, Seoul, Korea) and cortisol (Enzo life sciences, NY, U.S.A.) levels were analyzed by service providers specified above.

Western Blotting The heart, uterus and prefrontal cortex were removed and homogenized in lysis buffer containing 50 mM Tris-base (pH 7.5), 1% glycerol, 1% NP-40, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium pyrophosphate, 10 mM NaF, and protease inhibitors (0.1 mM phenylmethylsulfonylfluoride, 5 μg/mL leupeptin and 5 μg/mL aprotinin). The tissue lysates or cell lysates (30 μg) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. Then, the membranes were incubated with anti-protein kinase B (AKT), anti-extracellular signal-regulated kinase (ERK), anti-AMP-activated protein kinase (AMPK)α,
anti-phospho-ERK, anti-phospho-AMPKa, anti-phospho-ERKa (Ser118), anti-ERKa and anti-β-actin (Cell Signaling Technology, Danvers, MA, U.S.A.), anti-brain derived neurotrophic factor (BDNF), anti-α-actin (Abcam, Cambridge, U.K.), anti-glycogen phosphorylase, anti-Troponin T type 2 (Novus Biologicals, Centennial, CO, U.S.A.) or anti-myomesin2 (Bioss Antibodies, Woburn, MA, U.S.A.) antibodies for 16h at 4°C. Following washing in 0.05% Tris-buffered saline-Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG). The signals were visualized using the enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Finally, images were obtained by a Molecular Imager ChemiDoc XR+ (Bio-Rad, Hercules, CA, U.S.A.), and signal intensity was quantitated by Image Lab™ software version 2.0.1 (Bio-Rad).

### Two-Dimensional Gel Electrophoresis

Heart or brain tissues were homogenized in sample lysis solution containing 7M urea, 2M thiourea containing 4% (w/v) 3-(3-cholamidopropy)dimethyammonio)-1-propanesulfonate (CHAPS), 2% (v/v) pharmalyte and 1% (w/v) dithiothreitol (DTT), and 1 mM benzamidine using motor-driven homogenizer (PowerGen125, Fisher Scientific, Hampton, NH, U.S.A.). Proteins were extracted for 1h at room temperature with vortexing. Insoluble materials were separated by centrifugation at 15000 × g for 1h at 15°C, and the soluble fraction was saved for the following analysis. Protein concentration was determined by Bradford method. Immobilized pH gradient (IPG) dry strips (4–10NL IPG, 24 cm, Genomine, Korea) were equilibrated for 12–16h with 7M urea, 2M thiourea containing CHAPS, 1% pharmalyte and 1% DTT, and loaded with 200 µg of the sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit equipped with EPS 3500 XL power supply (Amersham Biosciences). The voltage was linearly increased from 150 to 3500V for 3h during sample entry, followed by constant 3500V until IEF is completed after 96 kVh. Next, strips were incubated in equilibration buffer (50mM Tris-Cl, pH 6.8, 2% SDS, 30% glycerol and 6M urea) for 10min, first with 1% DTT and with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 × 24cm, 10–16%) for the second dimension. SDS-PAGE was performed at 20°C for 1700 Vh using a Hoefer DALT 2D system (Amersham Biosciences). 2D gels were then stained with Colloidal Coomassie Brilliant Blue (CBB). The fixing and sensitization step with glutaraldehyde was omitted. Digitized images were quantitated using the PDQuest software (version 7.0, BioRad) following the manufacturer’s instruction. The quantity of each spot was normalized by the total intensity of valid spots. Protein spots were identified as the significant expression variation when their expression level deviated over two-fold compared with control or normal sample.

### Peptide Mass Fingerprinting (PMF)

To identify the proteins, selected spots were excised and digested with trypsin (Promega, Madison, WI, U.S.A.). After mixing with α-cyano-4-hydroxycinamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA), matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis was performed with Microflex LRF 20 (BrukerDaltonics, Billerica, Massachusetts, U.S.A.). Spectra were collected from 300 shots per spectrum over m/z range 600–3000, followed by two-point internal calibration using Trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). The Flex Analysis 3.0 software was used to generate a peak list. The following threshold was used for peak-picking: 500 for a minimum resolution of monoisotopic mass, 5 for signal-to-noise ratio. Finally, each protein was identified using MASCOT, a search program developed by Matrix Science (Marylebone, London, U.K.). The parameters used for the database search are as follows: trypsin as the cleaving enzyme, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, a maximum of one missed cleavage, monoisotopic masses, and a mass tolerance of ±0.1 Da. PMF acceptance criteria are probability scoring.

### Data Analysis

Results were shown as the mean ± standard error (S.E.) Data were statistically compared using one-way ANOVA and Tukey’s post hoc test for multiple comparisons. A p-value of 0.05 or less was considered as indicative of a significant difference.

### RESULTS

#### HPLC Analysis of Herbal Extracts

HPLC analysis of each herbal extract was successfully performed, establishing the chemical concentrations of key active compounds known in herbal extracts used in the present study. The content of evodiamine and rutaecarpine in 1 g of Evodiae Fructus extract was 0.065 and 0.07 mg, respectively. The content of akebia saponin in 1 g of Himalayan teasel root extract was 35.31 mg. The content of neochlorogenic acid, chlorogenic acid, caffeic acid and hyperoside in 1 g of Dodder seed extract was 0.95, 2.78, 1.30 and 0.26 mg, respectively.

#### Effects of STH_KP17001 on Cell Proliferation

MCF-7 cells are the estrogen receptor-positive cell line, which is regulated by estrogens that promote proliferation. To compare the estrogenic effect of STH_KP17001 with estradiol, MCF-7 cells were treated with various concentrations of STH_KP17001 or estradiol for 24h and examined by MTT assay. Estradiol treatment significantly increased cell proliferation in a dose-dependent manner, over two-fold at 10µM compared with the control, while STH_KP17001 treatment resulted in a less pronounced increase in cell proliferation throughout the range from 25 to 400µg/mL (Fig. 1A). In order to investigate the estrogenicity of STH_KP17001, the phosphorylation level of estrogen receptor (ER) α was measured by Western blotting after treatment with 50µg/mL of STH_KP17001 in MCF-7 cells. STH_KP17001 gradually increased ERα phosphorylation and reached the maximum level at 30min (Fig. 1B).

#### Effects of STH_KP17001 on Uterine, Heart and Ovary Indices in VCD-Treated Mice

VCD-induced ovarian failure mice were fed with low (100 mg/kg) or high (300 mg/kg) concentration of STH_KP17001 for 13 weeks to examine the effects of STH_KP17001 on nutritional status by measuring weight gain, food intake and FER. As shown in Fig. 2, the treatment of STH_KP17001 did not alter the level of total weight gain, food intake or FER significantly. However, the reduction in the relative weight (index) of reproductive tissues observed in the VCD group was restored in both STH_KP17001 (Low and High) and positive control group (Estradiol) (Figs. 2E, F). In addition, a significant increase in the relative weight of heart found in the VCD group decreased in the STH_KP17001-Low group to a level compa-
Effects of STH_KP17001 on the Serum Biochemical Markers in VCD-Treated Mice

Compared to the sham group, the VCD group displayed significantly elevated levels of serum TC and TG (p < 0.001), but lower level of serum HDL cholesterol (p < 0.05). However, the TC level was significantly attenuated in the STH_KP17001-High group (p < 0.01), while the TG level was reduced in both STH_KP17001 and the sham group (Figs. 3A, C). The level of HDL cholesterol was lowered in the VCD group but restored moderately by the treatment of STH_KP17001 (Fig. 3B). The levels of serum glucose, GOT and GPT were similar in all experimental groups (Figs. 3D–F).

Anti-anxiety and Depressant Effect of STH_KP17001 in VCD-Treated Mice

To test the effect of STH_KP17001 on anxiety-like behavior, open field tests were carried out in VCD-induced menopausal model mice. The time spent in the center and the total traveled distance were significantly decreased in the VCD group compared to the sham group (p < 0.05 and p < 0.001, respectively). However, both the time in the center and traveled distance were significantly improved in STH_KP17001-Low and STH_KP17001-High groups (Figs. 4A, B, D). In addition, the increase in blood cortisol level observed in the VCD group was lowered in the STH_KP17001-Low group (Fig. 4C) (p < 0.05), indicating that STH_KP17001 reduces VCD-induced depression.

Effects of STH_KP17001 on the Phosphorylation of ERK and AKT in the Uterus and Ovary of VCD-Treated Mice

To explore the mechanisms by which STH_KP17001 exerts its protective effects such as the recovery of uterus and ovary weight, ERK and AKT signaling pathways were examined using the tissue lysates. The results show that the phosphorylation levels of ERK and AKT decreased in the uterus and ovary in the VCD group. The decrease was suppressed by
both STH_KP17001 \( (p < 0.01) \) and estradiol \( (p < 0.01) \) treatments (Fig. 5).

**Differential Protein Expression Profiles and Western Blotting in the Heart of Sham, VCD-Treated and STH_KP17001-Treated Mice** 2D PAGE with colloidal CBB staining revealed approximately 350 polypeptide spots in the pH 4-10 interval from the hearts of sham, VCD-treated and STH_KP17001-treated mice (Figs. 6A, B). After matching the replicated maps, top 21 proteins that showed differential changes in intensities between VCD-treated and STH_KP17001-treated mice were selected; 4 proteins showed increase and 17 proteins showed decrease in intensity. Among 21 proteins, 6 proteins were identified: Myosin light chain 3, α-actin, serpina1b protein, troponin T2, and myomesin-2 were down-regulated in the heart of STH_KP17001-treated mice compared to VCD-treated mice, while glycogen phosphorylase was up-regulated (Fig. 6C). To further validate the proteomics analysis results, alpha-actin, troponin T2, myomesin-2, and glycogen phosphorylase were selected and analyzed by Western blotting (Figs. 7A, B). The results of Western blotting analysis were consistent with those of two-dimension electrophoresis. To understand the mechanisms by which STH_KP17001 mediates the protection of heart hypertrophy, the phosphorylation of inhibitor of κB (IκB) α, a proinflammatory signal, was examined in the heart tissue lysates. Notably, VCD treatment substantially increased the phosphorylation of IκBα, which was restored to the original level by STH_KP17001 and estradiol (Fig. 7C).
Effects of STH_KP17001 on the Phosphorylation of AMPK and Production of BDNF in the Prefrontal Cortex

To study the mechanisms by which STH_KP17001 exerts anti-anxiety and anti-depressive effect, the activation of AMPK was assessed in the prefrontal cortex using phospho-specific antibody. As shown in Fig. 8, VCD treatment reduced the phosphorylation of AMPK, which was restored by STH_KP17001 and estradiol ($p < 0.01$). Next, we examined the expression of brain-derived neurotrophic factor (BDNF), a downstream target of AMPK, in the cortex. The expression of BDNF was decreased by VCD treatment, but restored by STH_KP17001 (Fig. 8), suggesting that STH_KP17001 may induce BDNF expression through AMPK activation in the prefrontal cortex.

DISCUSSION

Women in menopause face numerous psychological and physical challenges, some of which are insomnia, tiredness, hot flashes, sweating, and hair loss. An earlier clinical study showed that postmenopausal women are more vulnerable to anxiety and depression. Depression is a crippling condition that leads to heavy societal, personal and economic burdens. In this study, we demonstrated that STH_KP17001 attenuate VCD-induced depression and anxiety behavior and stabilize the cortisol level in mice (Fig. 4). Moreover, we found that STH_KP17001 induces AMPK activation and BDNF expression in the VCD-treated group (Fig. 8).

Estrogen plays multiple biological functions via mainly estrogen receptors (ERs) encoded by ER$\alpha$ and ER$\beta$, which are required for the growth and maintenance of reproductive tissues, energy homeostasis, bone remodeling and neuroprotection. Upon binds with estrogen, activated receptors trigger various protein kinase pathways including the mitogen-activated protein kinase (MAPK)/ERK and phosphatidylinositol 3-kinase (PI3K)/AKT pathways. The estrogen-induced activation of ERK and AKT led to mitogenesis in MCF-7 cells, which was blocked by PI3K inhibitors and PD98059. In concert with that, estradiol significantly proliferated MCF-7 cells in a dose-dependent manner in vitro and STH_KP17001 also induced a significant ($p < 0.05$) MCF-7 cells proliferation at the broad range of concentration from 25 to 400 $\mu$g/mL as compared to control (Fig. 1A). Moreover, STH_KP17001 induced ER$\alpha$ phosphorylation in MCF-7 cells (Fig. 1B) and estradiol or STH_KP17001 activates the ERK and AKT pathways in the VCD-induced uterus and ovary, resulted in recovering the uterus and ovary index (Figs. 2, 5), implying that STH_KP17001 has phytoestrogen-like properties through the activation of ERK and AKT. However, in this study, STH_KP17001 did not show any dose dependency of MCF-7 cell proliferation. These can be explained that STH_KP17001 is an herbal mixture including complex components showing multiple effects. Thus, due to the interactions of each component, MCF-7 cell proliferation may not be increased in a dose-dependent manner.

Estrogen is reported to regulate AMPK in many cells and rodents. AMPK is a pivotal enzyme regulating energy metabolism. Altered functionality of AMPK has been associated with multiple metabolic disorders, such as obesity, type-2 diabetes, and cardiovascular disorders. Moreover, estrogen-evoked neuron survival is mediated by AMPK pathway and neuronal AMPK plays a significant role in neurogenesis that AMPK activator enhances neurogenesis as well as cognitive function in rodents. Notably, neuronal AMPK is essential for the signaling pathways to express BDNF, an essential mediator of neurogenesis, and has been extensively studied.

Fig. 5. Effects of STH_KP17001 on the Expression of p-AKT and p-ERK in the Uterus and Ovary of VCD-Treated Mice

Relative levels of p-AKT and p-ERK in VCD-treated mice was measured in (A) ovary and (B) uterus by immunoblotting, and the signal intensity was quantitated by densitometry. The phosphorylated form was normalized against the total amount of each protein. Data represents the mean ± S.E.M. $^{* * }p < 0.01$ vs VCD; $^{† † † }p < 0.001$, $^{† † }p < 0.01$ vs Sham ($n = 7$).
with regard to the prediction of anti-depressant response.\(^{25}\)

In this study, we examined that estradiol and STH_KP17001 restored AMPK activation but STH_KP17001 only recovered the expression of BDNF in the VCD-treated mice frontal cortex. In addition, STH_KP17001 significantly reduced VCD-induced anxiety behavior and cortisol level but estradiol slightly recovered the locomotors activity and reduced cortisol level. Estradiol has been known to reduce anxiety and depressive behavior along with restoration of BDNF levels in ovariectomized female rats.\(^{26}\) Conversely, although estradiol improved the behavior of anxiety and depression in postmenopausal animal models, but it did not increase the expression of BDNF in the brain.\(^{27}\) In addition, they compared the administration methods via subcutaneous (SC) and intracerebroventricular (ICV) route to examine therapeutic effect of estrogen on anxiety and depressive phenotypes in the postmenopausal mice model. In the study, ICV administration of estradiol shows a significant increase in the number of crosses into the central area but not SC, implying that depending on the concentration of estrogen that reaches the brain, the results of behavioral testing may vary. Thus, the results of this study provide evidence for the neuroprotective role and therapeutic effects of STH_KP17001 on the menopause-induced depression and anxiety, which are likely mediated by AMPK activation and

![Fig. 6. Differential Protein Expression Profiles in the Hearts of Sham, VCD-Treated or STH_KP17001-High-Treated Mice](image-url)
of IxB kinase activity.

STH_KP17001 has many therapeutic components showing anti-menopausal symptom such as chlorogenic acid, akebia saponin and hyperoside. Among them, hyperoside prevents age-associated renal injury through inhibition of autophagic activity by mammalian target of rapamycin (mTOR)-independent and AMPK-autophagy activating kinase 1 (ULK1) signaling pathways. Moreover, hyperoside improves cardiac function and prevents the development of cardiac hypertrophy, suggesting that STH_KP17001 may suppress VCD-induced cardiac hypertrophy via hyperoside-induced AMPK inhibition.

Chlorogenic acid increases estrogen biosynthesis and follicle-stimulating hormone receptor (FSHR) expression in ovarian granulosa cells. In the present study, chlorogenic acid was main component of Dodder seed extract. Although we did not examine the estrogen level in the serum of STH_KP17001-treated mice, these data suggest that STH_KP17001 shows the recovery of uterine and ovary functions through estrogen-stimulating effect of chlorogenic acid and which is resulted in the improvement of brain and heart function as indirect mechanisms.

Akebia saponin D is a major component of Himalayan teasel root extract, which is a typical bioactive triterpenoid saponin isolated from the rhizome of Dipsacus asper Wall, has been known to have various therapeutic properties, including cardioprotective through reducing inflammation, anti-Alzheimer’s disease through MAPK signaling, and AKT/NF-kappa B pathway, anti-osteoporosis through PI3K/AKT signaling, and anti-hyperlipidemia effects through several metabolic pathways and modulation of the microbial community, suggesting that akebia saponin of STH_KP17001 may be involved in the reduction of VCD-induced cardiac inflammation, hyperlipidemia and depression.

In this study, we examined ERα phosphorylation by STH_KP17001 treatment in MCF-7 cells (Fig. 1B) and it has been reported that *Cuscuta chinensis* Lamarck that constitute STH_KP17001 exhibits a phytoestrogenic effect in MCF-7 cells, suggesting that STH_KP17001-induced ERα phosphorylation may be through the extract of *Cuscuta chinensis* Lamarck, which is involved in the direct mechanism of it on the recovery of ovary and uterus.

According to the recent analysis, women who enter menopause before the age of 45 are more likely to develop cardiovascular problems and die younger than those who enter menopause later in life. A possible cause for increased cardiovascular risk in the menopause is the loss of antioxidant effects of endogenous estrogens. Oxidative stress induced by well-known risk factors (e.g., hypertension, hypercholesterolemia and smoking) increases the risk of cardiac hypertrophy. Cardiac (ventricular) hypertrophy is caused by the addition of new sarcomeres, which occurs when the rate of myofibrillar protein synthesis is greater than that of degradation (proteolysis), resulting in the increased myofibrillar assembly. In this study, we showed that myofibrillar proteins such as myomesin 2, α-actin, troponin 2 and myosin light chain 3 were up-regulated (Fig. 7). In addition, the major proteolytic pathways in the heart, such as the calpain/calpastatin and ubiquitin/proteasome systems, play an essential role in protein turnover and cardiac remodeling. Our results showed that the VCD-induced increase in serpinA1, which belongs to the α1-antiproteinase/α1-antitrypsin gene family and

![Fig. 7. Effects of STH_KP17001 on the Expression of Myomesin-2, Glycogen Phosphorylase, Alpha-Actin, Troponin T2, p-IxBα and p-AMPK in the Hearts of VCD-Treated Mice](image-url)

(A) The protein levels of myomesin-2, glycogen phosphorylase, alpha-actin, troponin T2 and β-actin was measured in the hearts of VCD-treated mice by immunoblotting and shown as (B) the relative expression level normalized by β-actin. (C) The relative levels of p-IxBα and p-AMPK in VCD-treated mouse hearts was measured by immunoblotting, and the signal intensity was quantitated by densitometry. The phosphorylated form was normalized against the total amount of each protein. Data represent the mean ± S.E.M. **p < 0.01, *p < 0.05 vs VCD; †† p < 0.01 vs Sham (n = 3).
forms stable inhibitory complexes with trypsin, chymotrypsin and elastase,\textsuperscript{45} was suppressed by STH_KP17001 in the heart (Fig. 7B). These results provide evidence that STH_KP17001 prevents menopausal cardiac hypertrophy.

Hyperlipidemia is a common symptom in perimenopause and has been associated with ovarian dysfunction during perimenopause.\textsuperscript{46} In light of this, we also examined whether STH_KP17001 could improve lipid regulation in VCD-treated mice. We observed that the serum concentrations of TC and TG were increased only in the VCD-treated group, but not in the STH_KP17001-treated or the sham group (Fig. 3). Moreover, the concentration of HDL was increased in the STH_KP17001-treated groups, suggesting that STH_KP17001 could modulate lipid metabolism. Lastly, GOT and GPT levels remained similar upon STH_KP17001 treatment, implying that the herbal medicine STH_KP17001 may not induce liver toxicity.

CONCLUSION

In summary, the findings in the present study demonstrated the protective effects of STH_KP17001 in VCD-induced menopausal mouse model. STH_KP17001 administration suppressed anxiety behavior and cortisol level, possibly through AMPK activation and BDNF expression in the cerebral cortex (Fig. 9). In addition, STH_KP17001 activated cell survival signaling pathways such as AKT and ERK in the uterus and ovary, resulting in the improvement of uterus and ovary weight. Moreover, STH_KP17001 inhibited menopause-induced heart hypertrophy, possibly through the suppression of inflammatory response (Fig. 9). Thus, the use of the herbal formula, STH_KP17001, may be a potential therapeutic intervention for the treatment of menopausal symptoms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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