Original Research

Ojayeonjonghwan, an oriental medicine composed of five seeds, protects against vasomotor and neurological disorders in estrogen-deficient rats

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Impact statement

Menopausal symptoms impair the quality of life of many women, and although conventional treatments are often effective, their use is limited by adverse effects. Ojayeonjonghwan, OJa, is a traditional Oriental medicine that is used for both male and female reproductive health and has a long history of safe use. We evaluated the effectiveness of two variations of OJa (OJa1 and OJa2) for treating menopausal symptoms in ovariectomized (OVX) rats. Both OJa preparations were effective for relieving indicators of hot flashes and depression, and for preventing loss of bone mineral density and lean body mass. Only OJa 2 prevented memory dysfunction. These results show that the traditional Oriental medicine, Ojayeonjonghwan, has the potential to relieve menopausal symptoms in women and should be further evaluated in human clinical trials as an alternative to convention therapies in women for whom conventional therapies are not indicated or found to be ineffective.

Abstract

The different ojayeonjonghwan remedies all contain five fruit and seed water extracts, and they have been used for reproductive health in men and women. We hypothesized that the two OJa remedies would differently improve the early menopause-related vasomotor and neurological symptoms in estrogen-deficient animals. Ovariectomized (OVX) rats had either 0.5% dextrin (OVX-control), conjugated equine estrogen (150 μg/kg body weight; positive-control), 0.5% ojayeonjonghwan remedy-1 (OJa1), or 0.5% ojayeonjonghwan remedy-2(OJa2) in high-fat diet for 12 weeks. Normal-control rats (sham operation) were fed the same high-fat diet as OVX-control rats. Tail skin temperature, depressiveness, memory function, and body composition were determined. The mRNA expressions of hippocampal serotonin receptor $(5HT)_{1A}$ and $5HT_{2A}$ and brain-derived neurotrophic factor(BDNF) were measured. OJa1 and OJa2 groups had lower tail skin temperatures than OVX-control. Bone mineral density (BMD) and lean body mass (LBM) measured by DEXA increased only in OJa2, and were similar to the positive- and normal-controls ($P < 0.05$). In the forced swim test immobile time, an index of depressiveness was much lower in OJa1 and OJa2 than the control group. Memory as measured by passive avoidance, water maze, and Y maze tests was impaired in the OVX-control group, compared to the normal-control ($P < 0.05$), but normalized in OJa1 comparable to the positive- and normal-control groups. The neurological impairments were associated with serum serotonin levels and $5HT_{2A}$ mRNA expression in the midbrain, and decreased hippocampal BDNF mRNA and protein expressions in the OVX-control group compared to normal-controls ($P < 0.05$). OJa1 increased serum serotonin levels and $5HT_{2A}$ expression in the midbrain, and hippocampal BDNF expression to similar levels as normal-controls ($P < 0.05$). In conclusion, OJa1 and OJa2 improved hot flashes and depression and maintained BMD and LBM. OJa2 prevented the impairment of memory function in OVX rats. OJa1 and OJa2 have the potential to be effective therapies for postmenopausal vasomotor and neurological symptoms.

Keywords: Ojayeonjonghwan, hot flashes, bone mineral density, depression, memory impairment, serotonin receptor

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Introduction

About 85% of women report menopausal symptoms after menopause, and over 50% of post-menopausal women experience vasomotor symptoms (about 40% hot flashes and 18% night sweats) and vaginal dryness/dyspareunia.[1] Estrogen deficiency in post-menopausal women also causes them to experience depression, anxiety, mood changes, and sleeping problems due to decreased release

of neurotransmitters.[2]The changes related to depression eventually induce cognitive dysfunction, such as memory loss, in post-menopausal women.[3,4] Menopause-induced cognitive dysfunction has been widely reported.[5] The combined burden of hot flashes, depression, and cognitive impairment can greatly decrease the quality of life.[4]

The linkage of vasomotor symptoms, depression, and cognitive dysfunction to estrogen deficiency has been studied, but with inconsistent results.[4] These symptoms are associated with estrogen deficiency in the brain.[4,6] The reduction of estrogen levels may decrease serotonin levels.[7] Serotonin secretion activates the serotonin (5-hydroxytryptamine) receptor ($5\text{-}HT_{2A}$) in the brain, which is associated with the control of vasomotor symptoms.[7] Serotonin is also associated with increasing brain-derived neurotrophic factor (BDNF) expression, thereby contributing to cognitive function.[8,9] Estrogen depletion lowers serotonin levels in the brain, which in turn leads to increases in vasomotor symptoms, depression, and cognitive dysfunction. Since estrogen interacts with estrogen receptor connected signaling pathways including insulin/IGF-1 and NF-κB, the promotion of the estrogen receptor-related signaling pathways can prevent the estrogen-deficiency symptoms without increasing estrogen levels.[9,10]

Hormonal replacement therapy (HRT) can alleviate menopausal symptoms. However, potential adverse effects, such as breast and endometrial cancers, have often caused women not to use HRT.[11] About 40–50% of menopausal women have used complementary therapies, mostly herbal medicines, to reduce menopausal symptoms even in Western countries.[1] Herbal medicines contain phytoestrogens, which have similar chemical structures as estrogens in circulation.[12] Phytoestrogens can alleviate menopausal symptoms without stimulating uterine weight by the direct interaction with estrogen receptors, or they modulate other signaling pathways connected with estrogen action. These proposals have been evaluated by epidemiological and experimental studies and some studies have demonstrated that phytoestrogens protect against menopause-related symptoms and metabolic diseases.[12] Estrogen binds to both estrogen receptor-α and -β, which were expressed in tissue-specific manner and differently activated in different tissues. The activation of ER-α is mainly associated with uterine and breast proliferation to induce related cancers but the stimulation of ER-β improves energy, glucose, lipid, and bone metabolisms. Selective estrogen receptor modulators (SERMs) vary in their modulatory effects of ER-α and ER-β.[13] They have been developed for reducing estrogen deficient-related diseases such as osteoporosis. We are interested in exploring the traditional prescriptions used as interventions for menopausal symptoms by exerting SERM-like actions or by otherwise alleviating the symptoms of estrogen depletion by activating the insulin/IGF-1 signaling connected to estrogen signaling.

Dongeuibogam, a traditional clinical medical encyclopedia, describes ojayeonjonghwan (Ojawuziyanzongwan in Chinses), a mixture of herbal remedies using five different fruits and seeds including: *Schisandra chinensis* Baill. (SCB; memory improvement, glucose metabolism),[14,15] *Astragalus complanatus* R. Brown (ACB; improvement of blood pressure and immune modulation),[16,17] *Morus alba* Linn. (MAL; improvement of blood pressure, glucose and lipid metabolism),[18,19] *Broussonetia papyrifera* Vent. (BPV; reduction of nociception and inflammation),[20] *Plantago asiatica* Linn. (PAL; alleviation of glucose and lipid metabolism),[21] *Cassia obtusifolia* Linn. (COL; improvement of memory function),[2] *Lycium chinense* Mill. (LCM; improvement of lipid profiles).[22] All the herbs in the ojayeonjonghwan remedies protect against oxidative stress and inflammation that suppress the release of lipopolysaccharide-induced cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in peritoneal macrophages.[23] Ojayeonjonghwan remedies are traditionally known to be good for the reproductive functions of men and women. It may improve estrogen and/or testosterone secretion and their signaling pathways. Their function can be extended to alleviating menopausal symptoms by modifying the herbal combination. We made two modified ojayeonjonghwan remedies to examine their efficacy for reducing menopausal symptoms. Ojayeonjonghwan-1 included herbs (SCB, ACB, MAL, and BPV) to improve the harmony of yin and yang, consistent with the principles of Chinese medicine. Ojayeonjonghwan-2 contained herbs (SCB, PAL, COL, and LCM) to reduce edema and extra-heat in the body, according to Chinese medicine principles. We hypothesized that the two remedies (ojayeonjonghwan-1 and ojayeonjonghwan-2) might improve autonomic nervous system symptoms such as hot flashes, and neurological symptoms such as depression and memory dysfunction in estrogen-deficient animals. We tested the efficacy of the two remedies, ojayeonjonghwan-1 containing SCB, ACB, MAL, and BPV and ojayeonjonghwan-2 containing SCB, PAL, COL and LCM in ovariectomized (OVX) female rats, and their mechanisms were examined. OVX rats were chosen as the animal model because they experience similar menopausal symptoms as women.[4,24]

Materials and methods

Preparation and determination of phenolic and flavonoid contents of ojayeonjonghwan water extracts

The dried herbs included in the ojayeonjonghwan remedies were purchased from Kyung Dong Herbal Market (Seoul, Korea). Ojayeonjonghwan-1 was composed of SCB, ACB, MAL, and BPV and ojayeonjonghwan-2 contained SCB, PAL, COL and LCM (1:1:1:1, based on weights). The herbal remedies for the experiments were prepared according to the traditional preparation method for herbal remedy: the mixed herbs were extracted with hot water. Using traditional method for the preparation gives a merit to avoid the safety issue and some efficacies of the herbal remedies have been confirmed. The herbs were mixed as assigned and powdered in a blender. The 3-h extraction of each remedy used distilled water at approximately 90°C and filtration through filter paper (No. 2). Each filtrate was concentrated using a rotary evaporator and lyophilized. Ojayeonjonghwan-1 and

ojayeonjonghwan-2 yields were 29.6% and 28.3%. Total phenolic compounds in these remedies were determined using Folin–Ciocalteu reagent and expressed as mg gallic acid equivalents·g^{-1}.[25] Total flavonoid contents were determined using a modified[25] method reported by Davis *et al.*,[26] with rutin as the standard.

HPLC analysis

The dried powders of the two preparations (200 mg) were transferred into a 50 mL flask and extracted in 2 mL of water with sonication for 10 min each. The extracts were centrifuged for 10 min at 4000*g* and the supernatant filtered using a disposable syringe filter unit (0.22 μm, 25 mm, CA syringe filter) obtained from Futecs Co., LTD (Daejeon, Korea) and then was subjected to HPLC separation. The peaks on the chromatogram of the samples were identified by comparing their retention times and UV spectra to authentic standards. The external standard method was used to quantify compounds by integration of the peaks obtained from HPLC.

The HPLC system was an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump (G1315B), vacuum degasser (G1379A), autosampler (G1313A), column oven (G1316A), and diode-array detector (G1315B). Chromatographic data were processed by ChemStation software. Chromatographic separation was accomplished using a YMC Hydrosphere C18 column (4.6×150 mm i.d., 5 μm). The column oven was maintained at 40°C and detection was at $\lambda = 242$, 266, 280, 300, and 335 nm, and online UV absorption spectra were recorded in the range of 190 to 400 nm. The gradient elution system was as follows: initial mobile phase acetonitrile/0.1% formic acid in water = 1:99 (v/v); 0–17 min, 1:99 to 3:97(v/v); 17–40 min, 3:97 to 28:72 (v/v); 40–75 min, 28:72 to 55:45 (v/v). The flow rate was maintained at 1.0 mL/min and 10 μL of sample was injected into the column.

Nine chemical compounds 1–9 were identified with purities of ≥ 95%. The stock solutions of the nine chemicals mixture were prepared at concentrations of 0.5 mg/mL in 70% ethanol and 30% water. The working solutions were serially diluted with 70% ethanol to obtain a concentration of 0.0005 mg/mL and were stored at −4°C until used for analysis. Standards of geniposidic acid, mulberroside A, astragalin, complanatoside A, marmesin, aurantio-obtusin, and obtusifolin were purchased from ChemFaces (Wuhan, China). Piceid was acquired from the Tokyo Chemical Industry (Tokyo, Japan) and schzandrin A was acquired from Sigma-Aldrich (St. Louis, MO).

Animal care and feeding

Sixty female Sprague–Dawley rats weighing 227 ± 18 g (average 8 weeks old) from Daehanbio (Eum Sung, Korea) were separately accommodated in stainless steel cages in the animal facility with a controlled environment (23°C and with a 12/12-h light/dark cycle. All experimental procedures followed the guidelines for Animal Care and Use of Laboratory Committee of Hoseo University (Asan, Korea). Approval for the study was obtained from the Hoseo University Animal Care and Use Review Committee (HTRC-16–52).

High-fat diets are known to induce obesity, and both exacerbate menopausal symptoms.[27–29] Therefore, a high-fat, modified AIN-93 semi-purified diet was prepared.[30] The macronutrient content of each groups diet consisted: carbohydrate 40% energy (En%); protein 20 En%; and fat 40 En%. The high-fat experimental diets also contained 0.5% water extract powder of ojayeonjonghwan-1 or 0.5% water extract powder of ojayeonjonghwan-2. Since the water extracts of ojayeonjonghwan-1 and ojayeonjonghwan-2 mainly contained soluble dietary fiber, 0.5% dextrin was used to replace the extracts in OVX-control, positive-control and normal-control diets. The major sources of carbohydrate, protein and fat were starch plus sugar, casein, and lard (CJ Co., Seoul, Korea).

Experimental design

Rats were subjected to ovariectomy or a sham operation under anesthesia by intramuscular injection of ketamine and xylazine (100 and 10 mg/kg bw), which was used in all subsequent procedures requiring anesthesia. A mid-ventral incision exposed the ovary which was ligated and surgically removed at the most-proximal portion of the oviduct.[24] This procedure was repeated on the contralateral side. Identical surgeries were used for the sham group, but without removing the ovaries.[24] Following surgery, 48 OVX rats were assigned to groups of 12 rats each. Each group was randomly and blindly allocated into (1) OJa1 group provided the diet containing ojayeonjonghwan-1 water extract powder, (2) OJa2 group given the diet containing ojayeonjonghwan-2 water extract powder, 3) OVX-control given the diet containing dextrin and (4) positive-control fed the diet containing conjugated equine estrogen (CEE) 150 μg/kg BW and dextrin. The normal-control group consisted of 12 sham-operated rats fed the diet containing 0.5% dextrin. The rats were given free access to water and their respective diets for 12 weeks. Each Tuesday at 10 a.m., food consumption and body weights were recorded after 16-h fasting. Skin temperature was also measured by a non-contact infrared thermometer (D186; Dickson, Addison, IL, USA) every week. At the 11th-week, energy expenditure, memory function by Y maze, passive avoidance, and water maze tests, and depression state by forced swimming test (FST) were consecutively conducted. The detailed methods are explained below. Urine was collected for 24 h using the metabolic cage.

After the 12-week treatment, rats were anesthetized as with the OVX surgery and body composition was measured by dual-energy X-ray absorptiometry (DEXA). Rats were then sacrificed and blood for preparing serum was collected by cardiocentesis. Peri-uterine and retroperitoneal fat pads and uteri were excised, weighed and the sum of peri-uterine and retroperitoneal fat considered the visceral fat mass. Hippocampi dissected from the brain were then stored at −70°C for biochemical analysis.

Serum 17β-estradiol and osteocalcin levels and cerebrospinal fluid (CSF) serotonin were measured by ELISA (Enzo Life Sciences, Farmingdale, NY). Serum and urine

Ca and P levels were determined by colorimetry (Asan, Seoul, Republic of Korea). Serum osteocalcin was assayed using an ELISA kit (Osteocalcin EIA; Biomedical Technology Inc., USA). Serum concentrations of glucose and insulin were determined using a Glucose Analyzer II (Beckman-Coulter, Palo Alto, CA, USA) and radioimmunoassay kits (Linco Research, Billerica, MA, USA). The homeostasis model assessment estimate of insulin resistance (HOMA-IR) (fasting insulin (μIU/mL) × fasting glucose (mM)/22.5) was used to assess insulin resistance. The hippocampus was lysed by RIPA buffer and centrifuged at $3000 \times g$ for 10 min at 4°C. The contents of BDNF and acetylcholinesterase in the hippocampal supernatants were quantified using ELISA kits (Biocompare, South San Francisco, CA, USA).

Y maze test

The next day after measuring energy expenditure, rats were subjected to the Y maze test which consisted of a horizontal maze with three arms at 120° angles. Each arm was 50.5 cm in length, 20 cm in width, and 20 cm in height. Initially, rats were located in one arm and then the sequences of entering into the other arms were monitored for 8 min. A right alternation was defined as a rat consecutively entering into all three arms. When each arm in the Y maze was assigned as A, B, and C, the right consecutive alternation was ABC, BCA, or CAB, but not CAC, BAB, or ABA. The spontaneous alternation (%) was calculated from the following equation: % alternation = [(Number of right alternations)/(Total number of arm entries − 2)] × 100.

Passive avoidance test

The day following the Y maze test, passive avoidance test was used to assess short-term memory.[31] A two-compartment dark/light shuttle box was set up, and in the acquisition trial, the rat recevied an electric shock (75V, 0.2 mA, 50 Hz) for 5 s, immediately entering the dark chamber. Five seconds later, the rat was transferred back to its cage. After 24 h, the retention latency time was measured using the same method as the acquisition trial, except there was no shock; latency time was recorded up to 600 s. The shorter the latency time, the greater the memory deficit.

Water maze test

The Morris water test was used to assess spatial memory as previously described[31,32] at the day after the passive avoidance test. Morris water maze tests are used to assess hippocampal-dependent learning, including spatial, long-term, and long-term spatial memory. A video tracking system monitored the location of each swimming rat relatively to the start position and platform (Ethovision system; Noldus, Wageningen, Netherlands). Each day for three days, the rats were given three trial sessions 15 min apart, and escape latency times were recorded. The locations of the entry point and the platform were not changed during the three trial days. On day 5, the platform was removed for the probe test, with a 60 s cut-off time.

Forced swimming test

At two days after the water maze test, forced swimming test (FST) was conducted to measure depression state.[33] Rats engaged in swimming exercises by placing them in small glass tubs (46 cm height × 20 cm diameter) containing 39 cm of water at 23–25°. Each rat exercised while videotaping for 10 min in a pre-test and then for 5 min 24 h later. When a rat just floated with struggling or trying to keep its head above water during the FST, it was considered to be in a state of immobility. The presence of immobility was scored at every 5 s. Results were represented as mean of immobility time for 6 min. Inter- and intra-rater reliabilities for scoring FST activity by two observers were at least $r = 0.87$.

Lean body mass, fat mass, and bone mineral density determinations

At the study conclusion, prior to sacrificing the rats, a densitometer calibrated with a factory supplied phantom was used to determine body composition. Anesthetized rats were placed in a prone position, with their hind legs held in external rotation by tape. Hip, knee, and ankle articulations were fixed at 90° flexion. When the scanning was completed, an absorptiometer (pDEXA Sabre; Norland Medical Systems Inc., Fort Atkinson, WI, USA), set up for use in small animals, was used for BMD determination in the right femur and lumbar spine. During the same session, LBM in the hip and leg and fat mass in the leg abdomen were measured and amounts calculated by DEXA and its included software.[24]

Generation of cDNA and real-time PCR (polymerase chain reaction)

At the conclusion of the experiment, brain tissues were powdered using a cold-steel mortar and pestle, and blended into a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent; Gibco-BRL, Rockville, MD) for total RNA extraction, by following the instructions of the manufacturer. Equal amounts of total RNA were used to synthesize cDNA using superscript III reverse transcriptases. A high-fidelity Taq DNA polymerase was used for PCR. cDNA in equal amounts was added to SYBR Green mix (Bio-Rad, Richmond, CA, USA) and a real-time PCR instrument was used for amplification (Bio-Rad, Hercules, CA, USA). mRNA expression of BDNF, $5HT_{1A,}$ and $5HT2_A$ in the hippocampus was assessed using the following primers ($n = 4$). The primers were forward: 5′-CACACACA GCGCTCCTTA-3′ and reverse: 5′-AGTGGTGGTCT GAGGTTGG-3 for BDNF, forward 5′-GGGCAACTCCAAAGAGCA-3′ and reverse 5′-CGGGG GCATAGGAGTTAGAT-3 for serotonin receptor $(5\text{-HT})_{1A}$, forward 5′-CATGTATGCCATCCTCAACG-3′and reverse 5′-GGGATGGACAATTTGGTGAC-3′ for 5-HT_{2A}, and forward 5′-TGG AAT CCT GTG GCATCC ATG AAA C-3′ and reverse: 5′-AA AAC GCA GCT CAG TAA CAGTCC G-3′ for β-actin, which was used as the "housekeeping" gene for normalizing the expressions of the genes of interest. The comparative CT method was used for

determining the amounts of the target gene by normalizing it to the housekeeping gene and relative to a calibrator (ΔΔCT). All data are shown as fold-changes in comparison to the control group.

Statistical analysis

SAS software (ver. 7.0; SAS Institute, Cary, NC, USA) was used for all statistical analyses. Results are expressed as means ± SD. The distribution of the data was checked by univariate analysis and all the results exhibited a normal distribution. One-way ANOVA was used to test the significance of the metabolic effects among OVX-control, OJa1, OJa2, positive-control, and normal-control. When the F-tests indicated significant differences among the groups, the differences were identified by Tukey's tests. Statistical significance was accepted at $P < 0.05$.

Results

Ingredients of OJa1 and OJa2

The contents of total phenols in OJa1 and OJa2 were 69.5 ± 1.2 and 78.7 ± 0.6 mg/g extract, respectively, whereas OJa1 and OJa2 contained total flavonoids of 23.0 ± 0.8 and 30.5 ± 0.3 mg/g extract, respectively. HPLC chromatograms of the standard mixture, OJa1, and OJa2 are presented in Figure 1. The contents of nine compounds 1–9 were verified in the two different complex formulae OJa1 and OJa2. We found that the mulberroside A (2) from MAL, astragalin (4) and complanatoside A (5) from ACB, and Schizandrin A (8) from SCB were major constituents and their contents were as following: (2) 0.9890 ± 0.0148; (4) 0.1686 ± 0.0014; (5) 0.7774 ± 0.0033, and (8) 0.7598 ± 0.0032 mg/g in OJa1 containing SCB, MAL, ACB, and BPV. OJa2 was a blend of SCB, PAL, COL, and LCM and major bioactive compounds were identified as geniposidic acid

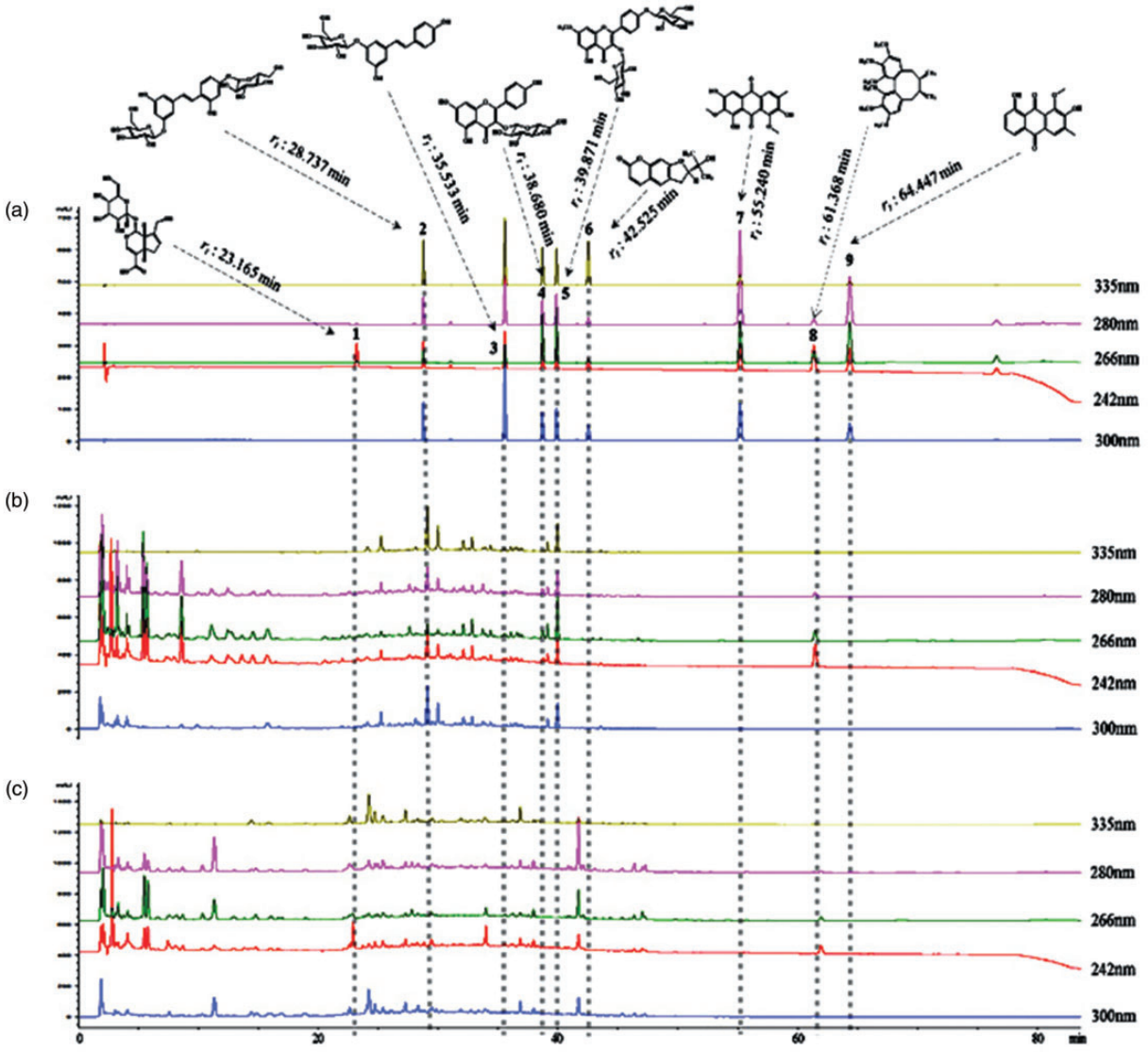

Figure 1. HPLC chromatograms. Standard mixture (0.05 mg/mL) at λ = 242, 266, 280, 300, and 335 nm of Geniposidic acid (1), Mulberroside A (2), Piceid (3), Astragalin (4), Complanatoside A (5), Marmesin (6), Aurantio-obtusin (7), Schizandrin A (8) and Obtusifolin (9) 0.05 mg/mL (a). The water extracts of OJa1 (b) and OJa2 (c) were measured in 100 mg extracts/mL.

(1, 0.8550 ± 0.0053 mg/g) from PAL, and schizandrin A (8, 0.3228 ± 0.0008 mg/g) from SCB. The rest of the compounds included piceid (3) 0.0060 ± 0.0005 and marmesin (6) 0.0033 ± 0.0000 in OJa1 and aurantio-obtusin (7) 0.0069 ± 0.0002 and obtusifolin (9) 0.0002 ± 0.0001 mg/g in OJa2 were minor constituents (Table 1). The linear regression data of nine standard compounds were confirmed as shown in Table 1, and they showed good linearity with a correlation coefficient of the equation value (r^2) over 0.99.

Serum 17β-estradiol concentrations and uterine weights

Serum concentrations of 17β-estradiol differed significantly among the groups. OVX-control rats lower serum 17β-estradiol concentrations by about four folds in compared to normal-controls, and positive-control rats had similar serum 17β-estradiol levels as normal-controls ($P < 0.05$; Table 2). However, serum 17β-estradiol levels in the OJa1 and OJa2 groups were similar to OVX-control rats. Uterine weights exhibited the same patterns as serum 17β-estradiol concentrations, indicating that 17β-estradiol is associated with the proliferation of the uterine. OJa1 and OJa2 did not increase the uterine weight (Table 2).

Tail skin temperature

Estrogen deficiency induces hot flashes, which are one of the major menopausal vasomotor disorders.[7] Tail skin temperature in animals is an established model for studying hot flashes in women.[24] Tail skin temperatures did not differ among the groups until the second week, but were significantly different for the remainder of the experiment. As compared to normal-l and positive-control groups, the OVX-control group's tail skin temperatures were significantly higher after three weeks and throughout the remainder of the study ($P < 0.05$). Tail skin temperature was lowered in OJa1 and OJa2 to as much as the positive-l and normal-controls ($P < 0.05$; Figure 2).

Table 1. Linearity and contents (mg/g) of compounds 1–9 in samples 2(J) and 3(K).

Compounds	Equation (Linear Model)	Linear range (mg/mL)	r^{2b}	Contents of samples (mg/g)	
				2(J)	3(K)
Geniposidic acid (1)	y=15653x−8.6413[a]	0.0005–0.3	0.9994	N.D.[d]	0.8550[c]± 0.0053[e]
Mulberroside A (2)	y=17678x−2.2325	0.0005–0.3	0.9996	0.9890 ± 0.0148	N.D.
Piceid (3)	y=45033x + 9.3566	0.0005–0.3	0.9996	0.0050 ± 0.0005	N.D.
Astragalin (4)	y=23600x−0.1268	0.0005–0.3	0.9998	0.1686 ± 0.0014	N.D.
Complanatoside A (5)	y=21095x + 75.464	0.0005–0.2	0.9919	0.7774 ± 0.0033	N.D.
Marmesin (6)	y=27912x + 1.3803	0.0005–0.3	0.9996	0.0033 ± 0.0000	N.D.
Aurantio-obtusin (7)	y=75897x + 58.075	0.0005–0.1	0.9986	N.D.	0.0069 ± 0.0002
Schizandrin A (8)	y=23828x + 3.1979	0.0005–0.3	0.9996	0.7598 ± 0.0032	0.3228 ± 0.0008
Obtusifolin (9)	y=53012x + 13.024	0.0005–0.05	0.9982	N.D.	0.0002 ± 0.0001

[a]y is the peak area and x is the concentration (mg/mL) of the component.
[b]r^2 is the correlation coefficient of the equation with 12 indicated points except 5 (11points), 7 (10points) and 9 (9points).
[c]All values are mean obtained by quadruple analysis ($n = 4$).
[d]N.D: not detected.
[e]Mean ± SD.

Table 2. Metabolic parameters at the end of the experimental period.

	Normal-control	OVX-control	Positive-control	OJa1	OJa2
Serum 17β-estradiol levels (pg/mL)	6.7±0.9[a]	1.8±0.6[b]	7.4±1.0[a]	1.7±0.5[b]	1.8±0.6[b*]
Uterine weight (g)	1.02±0.14[a]	0.24±0.04[b]	0.93±0.06[a]	0.21±0.07[b]	0.23±0.08[b*]
Food intake (g/day)	23.9±2.9	24.7±3.5	23.7±2.8	24.2±2.4	23.2±2.8
Body weight gain (g)	68.7±5.8[c]	110.7±7.9[a]	62.7±5.6[c]	81.7±6.3[b]	112.7±8.4[a**]
Peri-uterine fat (g/kg body weight)	13.1±1.5[b]	16.9±1.5[a]	6.3±0.9[d]	10.3±1.2[c]	12.3±1.3[b**]
Retroperitoneum fat (g/kg body weight)	8.4±1.1[bc]	11.5±1.2[a]	7.7±1.2[c]	9.4±1.0[ab]	8.8±0.9[b*]
Visceral fat[1] (g/kg body weight)	21.5±2.8[bc]	28.4±3.7[a]	14.0±2.5[d]	19.7±2.8[c]	21.1±2.5[bc*]
Fasting serum triglyceride (mg/dL)	93.8±11.1[b]	113±12.3[a]	92.6±10.0[b]	86.8±10.5[b]	69.8±6.8[c]
Fasting serum glucose (mg/dL)	90.1±6.5[b]	98.7±7.8[a]	94.0±4.5[ab]	92.5±5.9[b]	97.4±4.8[a*]
Fasting serum insulin (mg/dL)	0.91±0.13[c]	1.52±0.24[a]	0.96±0.12[bc]	1.19±0.18[b]	1.09±0.17[b*]
HOMA-IR	4.6±0.6[c]	8.3±0.9[a]	5.0±0.6[c]	6.1±0.7[b]	5.8±0.6[b*]

Note: Each value represents the mean ± SD ($n = 12$).
OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed 150 ug conjugated equine estrogen/kg bw + 0.5% dextrin; OJa1: the OVX rats consumed the 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); OJa2: the OVX rats consumed 0.5% extract of SCB, PLA, COL and LCM (1:1:1:1, based on weights); Normal-control: the sham rats consumed 0.5% dextrin.
[1]Peri-uterine fat + Retroperitoneum fat.
*Significantly different among the groups at each time point by one-way ANOVA at $P < 0.05$.
** at $P < 0.05$.
[a,b,c,d]Means with different letters were significantly different among the groups by Tukey test at $P < 0.05$.

Table 3. Parameters related to bone metabolism at the end of the 12-week treatment.

	Normal-control ($n=12$)	OVX-control ($n=12$)	Positive-control ($n=12$)	OJa1 ($n=12$)	OJa2 ($n=12$)
Serum Ca (mg/dL)	10.8±1.3	11.4±1.3	10.7±1.2	11.2±1.3	10.8±1.1
Serum P (mg/dL)	7.08±0.93^{b}	8.32±0.98^{a}	7.17±0.95^{b}	8.24±0.97^{a}	7.05±0.89^{b*}
ALP (IU/L)	151±20^{b}	211±24^{a}	157±18^{b}	192±23^{a}	142±18^{b*}
Serum osteocalcin(ng/mL)	6.3±0.8^{b}	9.4±1.2^{a}	6.5±0.8^{b}	8.8 ±1.3^{a}	6.8±0.9^{b*}

Note: Each value represents the mean ± SD ($n=12$).
OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed 150 ug conjugated equine estrogen/kg bw + 0.5% dextrin; OJa1: the OVX rats consumed the 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); OJa2: the OVX rats consumed 0.5% extract of SCB, PLA, COL and LCM (1:1:1:1, based on weights); Normal-control: the sham rats consumed 0.5% dextrin.
*Significantly different among the groups at each time point by one-way ANOVA at $P<0.05$.
[a,b]Means with different letters were significantly different among the groups by Tukey test at $P<0.05$..

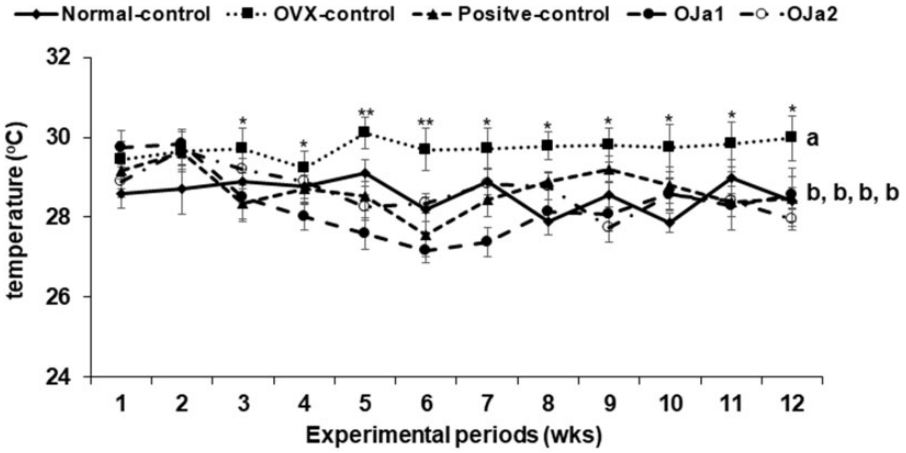

Figure 2. Changes in tail skin temperature during the experimental treatment. OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed conjugated equine estrogen 150 μg/kg BW + 0.5% dextrin; Normal-control: the sham rats consumed 0.5% dextrin; OJa1: the OVX rats consumed a 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); and OJa2: OVX rats consumed 0.5% extract of SCB, PAL, COL and LCM (1:1:1:1, based on weights). Tail skin temperature was measured by a non-contact infrared thermometer every week and each dot represents the mean ± SD ($n=12$).
*Significantly different among the groups at each time point by one-way ANOVA at $P<0.05$.
**at $P<0.01$.
[a]Bars with different letters were significantly different among the groups by Tukey test at $P<0.05$.

Food intake and body weight changes

Food consumption did not differ significantly among the groups. However, the groups did gain significantly different amounts of body weight ($P<0.01$). OVX-control rats gained much more weight than the normal-control rats, but it was normalized in the positive-controls ($P<0.05$). OJa1 and OJa2 did not change food intake (Table 1). However, increases in body were much greater in OVX-controls than normal- and positive-controls ($P<0.05$). OJa1 rats exhibited significantly less increases in body weight than OVX-controls, but it was still more than the positive-control. Body weight gain was not affected by OJa2 (Table 2). There were significant differences in visceral fat among the groups ($P<0.05$). Visceral fat (peri-uterine fat plus retroperitoneal fat) was much greater in the OVX-control rats than in the normal-controls ($P<0.05$). Unlike total body weight gain, visceral fat in both OJa1 and OJa2 was similar to the normal-control, but was lower in the positive- and normal-control groups ($P<0.05$; Table 2).

Body composition and bone metabolism

As expected, lumbar spine and femur BMDs were different among the groups ($P<0.01$). BMD in the lumbar spine and femur did not increase in the OVX-control rats to the same degree as seen in normal-controls, whereas the positive-control (conjugated equine estrogen treatment) elevated their BMD more than OVX-control group ($P<0.05$), but it was less than the normal-control. BMD was elevated by OJa2 in the lumbar spine and femur and was similar to the positive control ($P<0.05$), and femur densities were almost similar to normal-controls (Figure 2(a)). Femur BMD was higher in OJa1 but lumbar spine BMD was not. (Figure 3(a)). Hip and leg LBMs decreased in OVX-controls compared to normal-controls, whereas the decrease in their LBM was prevented by the positive-control, but not equivalent to the normal-control ($P<0.05$; Figure 3(b)). OJa1 prevented the LBM decrease in the hip but not in the leg ($P<0.05$). However, OJa2 protected against LBM loss in the hip as effectively as the normal-control, and leg LBM was higher than normal controls in the OJa2 group ($P<0.05$; Figure 3(b)). This explained why the body weight was higher in OJa2 than in normal-controls even though visceral fat mass was similar. The fat mass, as assessed by DEXA, showed the same results as visceral fat. Abdominal and leg fat masses were higher in OVX-controls than normal-controls ($P<0.05$; Figure 3(c)).

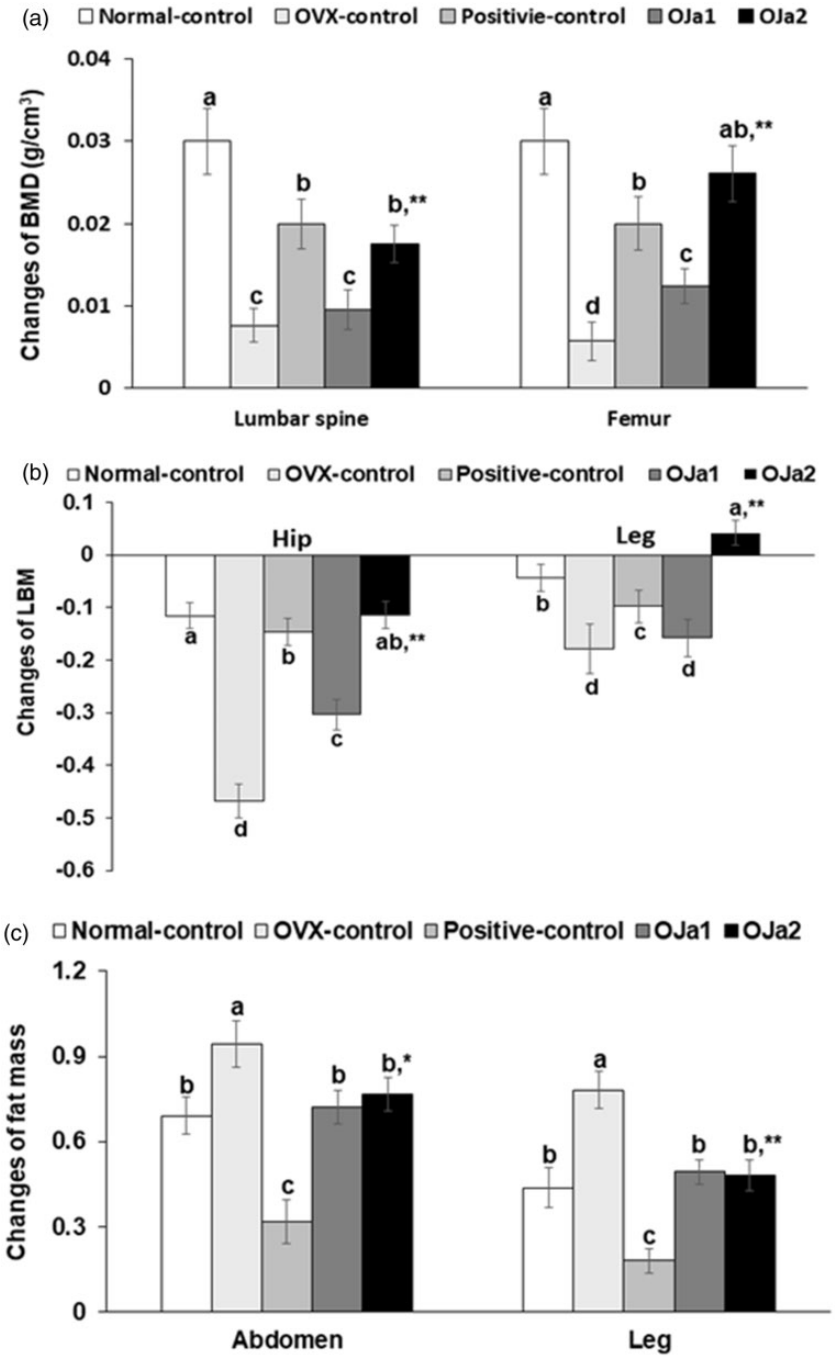

Figure 3. Bone mineral density (BMD), lean body mass (LBM), and fat mass (FM) at the end of the experiment. OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed conjugated equine estrogen 150 μg/kg bw + 0.5% dextrin; Normal-control: the sham rats consumed 0.5% dextrin; OJa1: the OVX rats consumed a 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); and OJa2: OVX rats consumed 0.5% extract of SCB, PAL, COL and LCM (1:1:1:1, based on weights). At the end of the experimental period, BMD in the lumbar spine and femur (a), LBM in the hip and leg (b) and FM in the abdomen and leg regions (c) were measured by DEXA. Each bar represents the mean ± SD (n=12).
*Significantly different among the groups at each time point by one-way ANOVA at $P<0.05$.
**at $P<0.01$.
[a,b,c]Bars with different letters were significantly different among the groups by Tukey test at $P<0.05$.

Conjugated equine estrogen treatment in the positive-control group decreased fat mass to below that for normal-controls ($P < 0.05$). OJa1 and OJa2 treatments protected against accumulation of abdominal and leg fat mass maintaining them at levels similar to normal-controls (Figure 3(c)). Therefore, higher body weights of OJa2 were caused by increased LBM, not fat mass.

Serum Ca concentrations did not differ among groups (Table 3). However, serum P levels were higher in the OVX-group than the normal- and positive-controls. Serum P concentrations were lower in OJa2, but not in OJa1, and OJa2 was similar to the normal-controls ($P < 0.05$; Table 3). Serum ALP and osteocalcin levels, bone turnover markers, were greatly elevated in the OVX-controls compared to

normal-controls, but the increase was prevented in positive-control and OJa2 ($P < 0.05$; Table 3). Thus, OVX increased bone turnover and CEE and OJa2 suppressed the increase to reduce BMD in OVX rats (Table 3).

Glucose metabolism

At the fasting state, serum glucose concentrations were greatly elevated in OVX rats compared to normal-controls, and they were lower in OJa1 as similar to the concentration of the normal controls (Table 2). Serum insulin concentrations were also elevated in the OVX rats compared to the normal-control and positive-control rats, indicating that OVX modulated the insulin resistance. Serum insulin concentrations decreased in OJa1 and OJa2 as compared to the OVX-controls (Table 2). As expected, the insulin resistance index, HOMA-IR, was highly elevated in the OVX-rats in relation to normal- and positive-controls. OJa1 and OJa2 decreased HOMA-IR compared to OVX-controls (Table 2). Serum triglyceride concentrations were lower in OJa1 than the OVX-control and were similar to normal- and positive-controls (Table 2). OJa2 lowered the concentrations to less than the normal-control.

Depressiveness

The forced swim test showed signficant differences in immobile times in the first and second day among the groups ($P < 0.05$; $P < 0.01$). OVX-control rats had longer immobile times than did normal controls in the first trial, and CEE treatment (positive-control) reduced the immobile time (Figure 4(a); $P < 0.05$). OJa1 and OJa2 partially prevented the increase in immobile time in the first trial ($P < 0.05$; Figure 4(a)). The immobile time was increased much more in the OVX-control rats in the second trial than in the first trial ($P < 0.05$). Normal-control rats also had increased immobile times in the second trial, more than the first trial but the increment was not as much as the OVX-control group ($P < 0.05$; Figure 4(a)), indicating that the OVX-control rats had greater depressiveness than normal-controls. However, OJa1 and OJa2 rats did not experience increased immobile time in the second trial compared to the first trial, and there was much less immobile time OJa1 and OJa2 than in the OVX-control group ($P < 0.05$; Figure 4(a)). OJa1 exhibited similar immobile times as normal controls in the second trial.

The depressiveness was associated with serotonin levels in the brain including CSF,[34] and it is known to be related to 5-HT_{1A} and 5-HT_{2A} expressions.[8,9,35], Hippocampal 5-HT_{1A} mRNA expression was significantly associated among the groups ($P < 0.05$). Its expression was the same between the control and normal-control groups and OJa1, OJa2, and positive-control did not affect 5-HT_{1A} mRNA expression (Figure 4(b)). However, the OVX-control group did have greater 5-HT_{2A} mRNA expression than the normal-control group ($P < 0.05$). Its expression was lowest in OJa1 and positive-control, and was equivalent to the normal-controls, but not OJa2 ($P < 0.05$; Figure 4(b)). The OVX-controls had lower CSF serotonin levels than the normal-controls ($P < 0.05$), but the levels were higher in the positive-control and OJa1 groups, and were similar to the normal-control group. However, OJa2 did not increase the CSF serotonin levels (Figure 4(b)).

Memory function

OVX-control rats exhibited a significant decrease in a spontaneous alteration in the Y maze test as compared to normal- and positive-controls ($P < 0.05$), and its decrease was inhibited by the positive-control and OJa1 groups and was similar to normal-controls. OJa2 increased spontaneous alterations to more than OVX-control, but they were less than those of normal-controls ($P < 0.05$; Figure 5(a)).

All rats in the passive avoidance test entered the lighted room in the first trials and administered the electric shock. The latency to enter the dark room was differed significantly among the groups ($P < 0.05$). They learned to associate pain from the electric shock with the lighted room, and not to enter into the lighted room. In the second and third trials, the retention times were shorter among the OVX-controls than the normal-controls. In the second trial, OJa1 increased retention times to enter the lighted room compared to OVX-controls, but not as long as positive-controls and OJa2 ($P < 0.05$). Retention times in the third trial were increased in OJa2 and positive-control to as much as the normal-control ($P < 0.01$). These results suggested that the OVX-control group had more short-term memory loss than did normal-controls, and OJa1 and OJa2 prevented its impairment equally as much as positive-controls (Figure 5(b)).

OVX-control rats found the zone 5, the location of the platform, in the Morris water maze test, much more slowly than the normal-control. Latency to find zone 5 was shorter for positive control, OJa1, and OJa2 groups than for the OVX-control group, and OJa1 was most effective ($P < 0.05$; Figure 5(c)). The duration in zone 5 was less for the OVX-controls than for normal-controls. Positive-control and OJa1 had longer durations, equal to normal-controls, whereas OJa2 also increased it, but as long as normal-controls ($P < 0.05$; Figure 5(c)). Therefore, OJa1 and OJa2 improved spatial memory impairment, whereas OJa1 was more effective for improvement than OJa2.

Cognitive function was associated with the proliferation of neuronal cells and decreased apoptosis, and it is known to be associated with BDNF-5HT_{2A} signaling that is impaired in estrogen deficiency.[8,9] Neuronal cells are associated with BDNF and insulin signaling in the hippocampus. Hippocampal BDNF mRNA expression and protein levels were lower in the OVX-controls than normal-controls, but they did not inhibit the decrease observed in positive-controls ($P < 0.05$; Figure 5(d)). OJa1 and OJa2 increased BDNF levels compared to the OVX-control and even higher levels were observed in the normal-controls group (Figure 5(d)). The expression of acetylcholinesterase, which decreases acetylcholine levels in the brain, was also elevated in the OVX-control rats compared to the normal-controls, and OJa1 and OJa2 suppressed the increase of acetylcholinesterase expression more than did the positive-control ($P < 0.05$; Figure 5(d)). Glycogen levels in the hippocampus were lower in the OVX-controls than the normal- and positive-controls; OJa2 increased the hippocampal glycogen ($P < 0.05$; Figure 5(e)). However, there

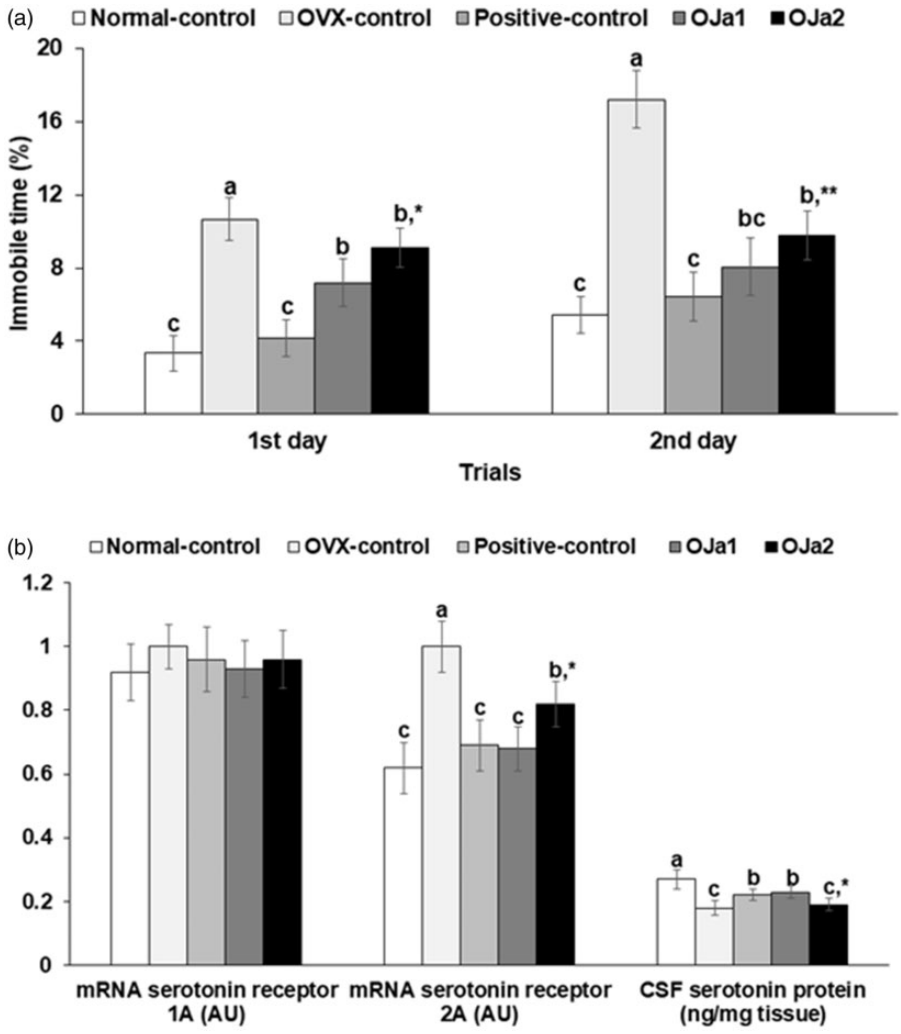

Figure 4. The immobile time during forced swimming test and hippocampal serotonin receptor 1A and 2A mRNA expression at the end of the experiment. OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed conjugated equine estrogen 150 μg/kg BW + 0.5% dextrin; Normal-control: the sham rats consumed 0.5% dextrin; OJa1: the OVX rats consumed a 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); and OJa2: OVX rats consumed 0.5% extract of SCB, PAL, COL and LCM (1:1:1:1, based on weights). An immobile time during forced swimming test (a). The mRNA expression of serotonin receptor 1A and 2A in the hippocampus was measured by realtime PCR and serotonin protein levels in the cerebrospinal fluid were determined by ELISA kit (b). Each bar represents the mean ± SD (n=12).

*Significantly different among the groups at each time point by one-way ANOVA at $P<0.05$.

**at $P<0.01$.

[a,b,] [c]Bars with different letters were significantly different among the groups by Tukey test at $P<0.05$.

were no differences in hippocampal triglyceride among the groups.

Discussion

The two ojayeonjonghwan remedies, each contained as much SCB as commonly used alone as well as ACB, MAL, and BPV in ojayeonjonghwan-1 and PAL, COL, and LCM in ojayeonjonghwan-2. These remedies have traditionally been used for improving the reproductive health of men and women. The ingredients included in the remedies have anti-oxidant and anti-inflammatory activities.[36] The ingredients in ojayeonjonghwan remedies (OJa1 and OJa2) have also been reported to improve the metabolic disturbances involved in menopausal complications, such as dysregulation of glucose and lipid metabolism, depression, and cognitive dysfunction.[14,16,18,23] SCB, MAL, COL, and LCM have hypoglycemic effects and improve memory impairment by potentiating insulin sensitivity.[2,14,18,19,22] The present study showed that OJa1 and OJa2 attenuated memory impairment, depression, and vasomotor symptoms in menopausal women without stimulating the uterine proliferation. The results indicated that OJa1 and Oja2 improved the menopausal symptoms, not by direct activation of ER-α and they might alleviate them by activation of the downstream of ER signaling like insulin/IGF-1 signaling and/or stimulation of ER-β in the brain tissues. Estrogen signaling is connected to insulin/IGF-1 signaling through activation of phosphoinositide 3-kinase to phosphorylation of Akt.[37] The herbs improve the menopausal symptoms by activation of insulin/IGF-1 signaling without activation of ER-α.

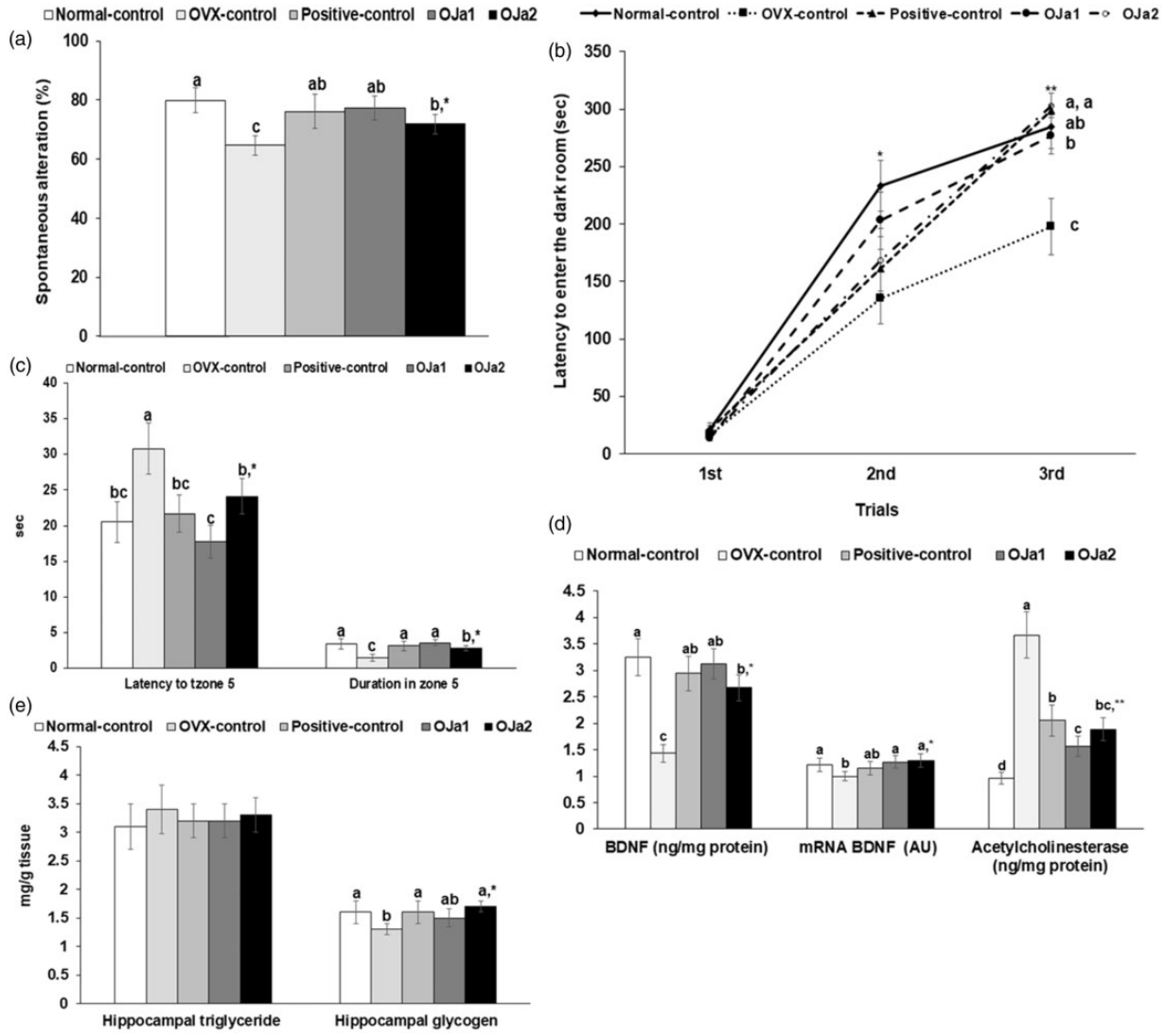

Figure 5. Memory deficit of rats with the amyloid-β infusion. OVX-control: the OVX rats consumed 0.5% dextrin; Positive-control: the OVX rats consumed conjugated equine estrogen 150 μg/kg bw + 0.5% dextrin; Normal-control: the sham rats consumed 0.5% dextrin; OJa1: the OVX rats consumed a 0.5% extract of SCB, ACB, MAL, and BPV (1:1:1:1, based on weights); and OJa2: OVX rats consumed 0.5% extract of SCB, PAL, COL and LCM (1:1:1:1, based on weights). The percentage of the consecutive alternations in the Y maze (a), latency time to enter the dark room in 3 trials in passive avoidance test (b) and the latency to locate zone with the platform during the water maze test and the period to stay in the platform zone on day 5 during the water maze test (c) were provided. Hippocampal BDNF mRNA expression and protein levels and acetylcholinesterase levels, which affect memory function, were determined (d). Hippocampal contents of triglyceride and glycogen involved in brain function were measured (e). BDNF: brain-derived neurotrophic factor. Each bar represents the mean ± SD (n=12).
*Significantly different among the groups at each time point by one-way ANOVA at $P<0.05$.
** at $P<0.01$.
[a,b,c]Bars with different letters were significantly different among the groups by Tukey test at $P<0.05$.

Ojayeonjonghwan-1 and ojayeonjonghwan-2 were both high in polyphenols, flavonoids, and anthocyanins in the present study, suggesting that the remedies have antioxidant and anti-inflammatory properties. The mechanism of hot flashes, a major symptom of menopausal women, remains uncertain although it is related to estrogen deficiency. Estrogen deficiency reduces brain serotonin concentrations, an inhibitor of noradrenaline activity, which is one of the stimulators of hot flashes.[38] Selective serotonin reuptake inhibitors, which increase serotonin levels, are popular for relieving hot flash symptoms.[35] Venlafaxine, a selective serotonin reuptake inhibitor, has been approved by FDA to treat hot flashes since 2013.[39] In addition, estrogen deficiency elevates 5-HT_{2A} expression in the hippocampus and estrogen treatment lowers its expression in rats and humans.[8,40] The present study showed that serum serotonin levels and hippocampal 5-HT_{2A} mRNA expressions were higher in OVX-control rats than in normal-controls, and OJa1 decreased the same levels as positive- and normal-control groups. OJa2 also lowered the levels, although not to normal-control levels. Increased 5-HT_{2A} may decrease the brain serotonin levels due to increased reuptake of serotonin. Estrogen deficient rats had lower serotonin levels in the present study. Serotonin in the brain is known to be involved in depression, OJa1 reduced the immobile time to as short as the normal- and positive-controls by the second day, indicating that OJa1 decreased depression to as much as the positive-control. OJa2 also

decreased the depression, but it was less than the normal- and positive-control groups. Previous studies showed that MAL specifically ameliorates the physical stress and depression by increasing the monoamine oxidase, and possibly by increasing the serotonin synthesis.[41,42]

Menopause is associated with not only depression but also memory impairment. Memory dysfunction is a late symptom of estrogen deficiency and is a consequence of decreased acetylcholine and phosphorylation of cAMP responding element binding protein which regulates neuronal cell survival.[30] Estrogen deficiency also decreases hippocampal expressions of BDNF mRNA and protein, and the decrease is reversed by estrogen replacement and exercise.[5,43] Memory impairment is also associated with depression, and the deterioration of BDNF-serotonin signaling in the hippocampus may be associated with the exacerbation of memory dysfunction. The present study also demonstrated that estrogen deficiency decreased BDNF mRNA expression and increased 5-HT_{2A} mRNA expression in the hippocampus, which might be associated with the impairment of memory function as observed during the passive avoidance and water maze testing sessions. Both OJa1 and OJa2 improved memory function, but OJa1 was more effective than OJa2. The greater decrease in 5-HT_{2A} expression by OJa1 may have made a critical contribution to the improved memory function. However, previous studies have demonstrated that some ingredients of OJa2 such COL and LCM improve the cognitive function by reducing the amyloid-β accumulation. COL ameliorates the memory impairments by amyloid-β accumulation by improving Akt/GSK-3β signaling.[2,22,44] LCM also improves the cognitive dysfunction and fatty liver and reduces menopausal induced obesity through modulation of PPAR-γ and estrogen receptor expression.[45–47] Thus, OJa2 might improve the cognitive function in Alzheimer's disease by potentiating the insulin signaling in the hippocampus but OJa1 might be better for alleviating the menopause-related memory impairment.

Menopause is known to result in gains in body weight, especially visceral fat. Body weight gain in estrogen deficient animals and women is a result of both increased food intake and decreased energy expenditure. However, the present study demonstrated that daily food intake was essentially the same among the groups, although it was slightly higher in the OVX-group compared to normal-controls. The accumulated food intake during experimental periods might be higher in the OVX- than the normal-control rats. In addition, daily energy expenditure, especially fat oxidation, is reported to be lower in OVX- than normal-control rats.[24] Thus, decreased energy expenditure might be an important factor for increasing the body weight, especially visceral fat mass.

Menopause also changes body composition.[48] BMD loss is a major problem with estrogen deficiency, but LBM is also decreased and fat mass is increased.[29] Menopausal metabolic disturbances can lead to sarcopenia with mobility restrictions, bone fractures, functional disabilities, and physical impairments and increased body fat, especially abdominal fat.[49] Interestingly, OJa2 protected against the decrease in BMD and LBM better than OJa1, and the efficacy of OJa2 was similar to the positive-control. SCB, a common herb in OJa1 and OJa2, is reported to ameliorate osteoporosis by activating the estrogen receptors.[50] LCM is demonstrated to improve the osteoporosis and increase bone formation in OVX animal models.[51,52] The combination of SCB and LCM in OJa2 might have been responsible for the greater effectiveness for increasing BMD and LBM in the OVX rats in the present study. However, the mechanism needs to be examined in further studies. An estrogen-deficient animal model used in this study had a sudden blockage of estrogen, although women have gradually decreased estrogen concentrations. However, previous studies have demonstrated that the OVX animals are an appropriate model for researching estrogen-deficiency symptoms.[24]

Conclusion

OVX rats experienced hot flashes, depression, and memory impairment, as well as decreased BMD and LBM as compared to the sham-operated rats. These symptoms were prevented by oral treatment with low-dose CEE. OJa1 and OJa2 contained SCB as a common ingredient, and three additional herbs were included in the formulations. Both OJa1 and OJa2 were beneficial for improving vasomotor menopausal symptoms and protecting against the decrease in BMD and LBM in an estrogen-deficient animal model. OJa1 and OJa2 or both combined, depending on the symptoms, may be effective for preventing or treating neurological and metabolic symptoms of menopause.

Authors' contributions: BSK and SP designed research; JAR, JTH and conducted biochemical assays and analyzed the data. ZT and HX conducted animal study; SP and BSK wrote the paper. SP had primary responsibility for final content. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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