Original Research

Tetragonia tetragonioides (Pall.) Kuntze protects estrogen-deficient rats against disturbances of energy and glucose metabolism and decreases proinflammatory cytokines

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Impact statement

Menopause decreases the quality of life in middle-aged women and herbal remedies are sometimes used as alternatives for hormone replacement therapy, which may have detrimental side effects. Although several herbal extracts have been studied, no remedies improve all the menopausal symptoms. In this study, the 70% ethanol extract of *Tetragonia tetragonioides* (Pall.) Kuntze (TTK) reduced the symptoms of hot flushes and improved energy, glucose, and lipid metabolism in estrogen-deficient animals without increasing serum 17β-estradiol levels. This extract acts like a selective estrogen receptor modulator and it may be a useful intervention for alleviating menopausal symptoms. This is the first study to show that the 70% ethanol extract of TTK has the potential to treat menopause-associated symptoms and metabolic disturbances. It may be a useful intervention for alleviating the symptoms of menopause in women if its efficacy can be confirmed in human studies.

Abstract

Tetragonia tetragonioides (Pall.) Kuntze (TTK) and JakYakGamCho-Tang (JGT) have been used for improving women's health and treating inflammatory diseases. We determined that the long-term consumption of these herbal extracts alleviates the progression of postmenopausal symptoms in high-fat-diet fed ovariectomized (OVX) rats, and further explored the mechanisms involved. Five groups of OVX rats were fed high fat diets that were supplemented with either 2% dextrin (control), 2% TTK (70% ethanol extract), 2% JGT (water extract), 1% JGT + 1% TTK (JGTT), or 30 μg/kg body weight/day of 17β-estradiol (positive control). After eight weeks of dietary intervention, the herbal treatments did not change the serum concentrations of 17β-estradiol or uterine weight in control rats, but they were higher in the positive-control group. TTK rats exhibited higher daily energy expenditure, particularly fat oxidation, without modifying the energy intake than the controls. TTK lowered the fat mass but lean body mass of the abdomen and leg were increased. JGT decreased periuterine fat mass and lean body mass more than the control but the decrease was not as much as TTK. TTK resulted in substantially lower serum concentrations of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1, than the control and JGT had lesser effect than TTK. Insulin resistance, determined by homeostasis model assessment estimate for assessing insulin resistance (HOMA-IR) and insulin tolerance test, was reduced in the decreasing order of control, JGT, JGTT, and TTK and the HOMA-IR of TTK was similar to the positive control. TTK, but not JGT, enhanced glucose tolerance compared with the control, although the serum insulin levels in TTK were lower compared to the control. Interestingly, the β-cell masses were much greater in the TTK and JGTT groups than in the control, and they were comparable to the positive control. The increases in β-cell masses in TTK and JGTT groups were associated with enhanced β-cell proliferation and suppressed apoptosis, which was related to the decreased TNF-α and interleukin-1β expressions. In conclusion, JGTT did not improve menopausal symptoms better than TTK itself. TTK itself prevented the OVX-induced impairments in energy, lipid, and glucose metabolism, similar to the positive control, without changing serum 17β-estradiol levels and potentiating insulin signaling and decreasing proinflammatory cytokines. TTK may be a useful intervention to alleviate some menopausal symptoms similar to selective estrogen receptor modulators and should be investigated with further human study.

Keywords: *Tetragonia tetragonioides* (Pall.) Kuntze, JakYakGamCho-Tang, energy, glucose intolerance, β-cell, dyslipidemia

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Introduction

Estrogen is an important factor in the regulation of energy, glucose, lipid, and bone metabolism, particularly in young and middle-aged women.[1] As they age, women exhibit reduced fertility and symptoms of menopause due to a decrease in sex hormone synthesis, which also increases

their risk of osteoporosis, dyslipidemia, and type 2 diabetes.[2,3] Hormone replacement therapy (HRT) effectively decreases the symptoms of menopause and prevents bone mineral loss. However, HRT induces adverse effects, such as increasing the susceptibility to breast and endometrial cancers, and various cardiovascular diseases.[4,5] Therefore, HRT is not generally recommended for menopausal women.

Herbal remedies have been used as alternatives to reduce menopausal symptoms and sometimes mitigate the metabolic disturbance associated with menopause.[6,7] Dong quai and ginseng stimulate proliferation of the Michigan Cancer Foundation-7 (MCF-7) Human Breast Adenocarcinoma cell line but without activating estrogen receptor (ER)-α and ER-β. The uterine weights were not altered in OVX mice that were treated with these herbal extracts for four days.[8] In our preliminary study, we screened more than 20 herbal extracts for their efficacy to stimulate or inhibit the growth of MCF-7 cells to evaluate their effects on estrogen-sensitive cells. The 70% ethanol extract of *Tetragonia tetragonioides* (Pall.) Kuntze (TTK) (*Aizoaceae* family) suppresses cell growth, but the water extract of JakYakGamCho-Tang (JGT), a mixed herbal formula of Paeoniae Radix and Glycyrrhizae Radix, increases cell growth in MCF-7 cells. Because it has not been determined if either ERs were activated, both herbs may influence menopausal symptoms. Recent studies have shown that ER agonists and antagonists are used to alleviate the negative symptoms of menopause, based on the types of ERs expressed in various tissues.[9,10] Selective estrogen receptor modulators (SERMs) exhibit different activities against ER-α and ER-β.[10] Most SERMs activate ER-β and inhibit ER-α in breast cancer cells to improve metabolic activities, such as glucose and bone metabolism, without increased risk of cancers of the breast and endometrium.[11] Thus, herbal extracts may modulate menopausal symptoms by acting as SERMs or by otherwise normalizing the effects of estrogen deprivation.

JGT is composed of equal amounts of Paeoniae Radix, *Paeonia lactiflora* Pall. root, and Glycyrrhizae Radix, *Glycyrrhizae uralensis* Fischer (Chinese Liquorice) root. JGT has been used clinically and pharmacologically to improve women's health and treat inflammatory diseases.[12] TTK has demonstrated efficacy for preventing sedative drug-induced ulcers.[13] Thus, both have a common anti-inflammatory mechanism. Menopause-induced obesity significantly increases the expression of adipose monocyte chemoattractant protein-1 (MCP-1), as well as the circulating MCP-1 levels. MCP-1, an important proinflammatory cytokine is critical for the recruitment of immune cells. Thus, increased inflammation is related to metabolic disturbances after menopause. Because they exhibit anti-inflammatory activities, TTK and JGT may improve the postmenopausal symptoms in the menopausal state. However, no studies have evaluated their effects on reducing the menopausal symptoms.

For this study, we hypothesized that the long-term consumption of TTK and/or JGT would prevent and/or delay the progression of postmenopausal symptoms in estrogen-deficient obese rats. This research tested the hypothesis and attempted to identify the mechanisms involved in ovariectomized (OVX) rats with diet-induced obesity that were treated with the herbal medicines.

Materials and methods

Total phenol and flavonoid concentrations of the 70% ethanol extract of TTK and water extract of JGT

TTK and JGT were obtained from Kyung Dong Herbal Market (Seoul, Korea) and extracted with 70% ethanol and water, respectively, at 70°C for 2 h in an ultrasonic extractor. The extracts were concentrated by 50% using a rotary evaporator, centrifuged at 8000 × *g* for 30 min, and finally lyophilized. The yields of TTK and JGT were 16.8 and 20.7%, respectively. JGTT was made with the mixture of equal amounts of TTK and JGT. Total phenolic compounds were determined by using Folin–Ciocalteu reagent and were expressed as milligram gallic acid equivalents·per gram.[14,15] Total flavonoids were also assayed using the methods reported by Association of Official Analytical Chemists (AOAC) International,[16] with some modifications. Rutin was used as a standard.

The Agilent 1100 series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) used for all assays utilized an autosampler (G1313A), binary pump (G1312), DAD detector (G1315B), column oven (G1316A), and degasser (G1379A). The contents of the indicated compounds in TTK (caffeic acid) and JGT (albiflorin, paeoniflorin, liquiritigenin, and glycyrrhizinic acid) were analyzed with an Atlantis C18 column (4.6 nm × 250 nm, 5 μm, Waters). The mobile phase was acetonitrile (A) in distilled water and 0.1% TFA (B) and the gradients used were 0 min, A:B 10:90 (v/v); 30 min, A:B 15:85; 40–55 min, A:B 100:0 for TTK and 0 min, A:B 15:85 (v/v); 5 min, A:B 15:85; 30 min, A:B 60:40, 50 min A:B 100:0 for JGT. Flow rate of the mobile phase was 1.0 mL/min under the following conditions: column temperature 30°C, injection volume 10 μL, and UV detection at 230 nm. Standards use to identify compounds were caffeic acid (0–100 μg/mL), albiflorin (2.5–250 μg/mL), paeoniflorin (2–250 μg/mL), liquiritigenin (2–150 μg/mL), and glycyrrhizinic acid (1–600 μg/mL; ChromaDex, USA).

Ovariectomy operation

Female Sprague-Dawley rats aged about 10 weeks (231 ± 20 g) were housed in individual stainless steel cages in a dedicated animal facility and were maintained at 23°C and a 12 h light/dark cycle. This study conformed with the NIH Guide for the Care and Use of Laboratory Animals with the approval of the Hoseo University Animal Care and Use Committee (2014-03). Female Sprague-Dawley rats obtained from DBL (Yeumsung-Kun, Korea) were allowed to acclimate for one week before undergoing ovariectomy. Prior to the OVX surgery, anesthesia was induced by subcutaneously injecting ketamine/xylazine mixture (100 and 10 mg/kg body weight).[17] After a midventral incision, each ovary was isolated by ligating the most proximal part of each oviduct. Both ovaries were removed with scissors. The OVX rats were then randomly divided into five groups of 12 each.

Diet preparation

All groups were fed high fat diets, which are known to exacerbate the menopausal symptoms as compared to diets low in fat.[17–19] Diet energy percentages were 37% from carbohydrate, 20% from protein, and 43% from fat. The major carbohydrate, protein, and fat sources of the modified AIN-93 formulation[20] were starch plus sugar, casein, and lard (CJ Co., Seoul, Korea). In our preliminary study, treatment with JGT (10 μg/mL) in inactivated media increased MCF-7 cell proliferation by 123.3% compared with the control (DMSO treatment), whereas the TTK treatment (10 μg/mL) decreased proliferation by 51.8%. Based on the cell-based study, 2% lyophilized water extracts of JGT and 70% ethanol extract of TTK, 1% JGT + 1% TTK (JGTT), 2% dextrin (control), or 30 μg/kg body weight of 17β-estradiol+2% dextrin (the positive control) were supplemented into a high fat diet. Equal amounts of total herbal extracts were maintained by using half the amounts of TTK and JGT to make the JGTT blend, since using higher dosages of total herbs might improve the effectiveness without synergistic effects. The JGT and TTK powders thoroughly blended into the vitamin, mineral, and sugar ingredients which were then sifted to remove lumps and was mixed with the designated amounts of starch, casein, and lard, resifted and refrigerated at 4°C. The nutrient composition of the diets was the same. Food consumption records were used to calculate the amounts of each supplement consumed.

Experimental design

Sixty OVX rats were divided into the following treatment groups using a randomized block design: (1) 2% JGT, (2) 2% TTK, (3) JGTT, (4) positive control (30 μg/kg body weight of 17β-estradiol+2% dextrin), or (5) OVX control (2% dextrin, placebo). After the OVX operation, all rats were provided free access to water and experimental diets. After eight weeks of dietary intervention, the rats were fasted overnight and serum glucose concentrations were determined. Food and water intakes, and body weights were measured 10 am each Tuesday.

Tail skin temperature

Tail skin temperatures were measured weekly during the sleep cycle using an infrared thermometer for small rodents (BIO-152-IRB, Bioseb, Chaville, France).[19] Each data point was the mean of three measurements 10 min apart.

Body composition

A dual-energy X-ray absorptiometer (DEXA) was calibrated using a phantom supplied by the manufacturer (Norland Medical Systems Inc., Fort Atkinson, WI, USA) and used to determine body composition of the rats at the seventh week of the experiment. The animals were anesthetized with the ketamine/xylazine mixture as above and placed in a prone position with the rear legs held in external rotation with tape. The articulations of the hips, knees, and ankles were in 90° flexion. After the scanning, hip and leg lean body masses were also determined using the DEXA instrument equipped with software for assessing bone mineral density in rodents.[21] Fat mass was also measured in the leg and abdominal areas using the DEXA instrument.

Energy expenditure by indirect calorimetry

Two days' post-DEXA analysis, indirect calorimeter was used to assess energy expenditure. During the early dark phase of the light/dark cycle 6 h fasted rats were moved into metabolic chambers with airflow = 800 mL/min which had a computer-controlled O_2 and CO_2 monitoring system (BIOPAC Systems, Inc., Goleta, CA) to measure mean oxygen utilization (VO_2) and carbon dioxide production (VCO_2) over periods of 30 min. The rats were then removed from the chamber and means of the data were calculated over 1 min intervals. VO_2 and VCO_2 were corrected for the metabolic body size ($kg^{0.75}$) of the rats and calculated respiratory quotient (RQ). Resting energy expenditure (RER) was also determined as previously reported.[7,22] Non-protein oxygen consumption was used to estimate carbohydrate and fat oxidation, RQ, RER, and oxygen consumed per gram of energy substrate oxidized.[18,19]

Glucose homeostasis and sample collection at the end of experiment

At the eighth week, overnight fasted animals were conducted to an oral glucose tolerance test (OGTT) by oral administration of 2 g of glucose/kg body weight. At 10 min intervals from 0 to 120 min postglucose loading, tail blood was collected for serum glucose measurements using a Glucose Analyzer II (Beckman, Palo Alto, CA, USA). At 0, 20, 40, 90, and 120 min serum insulin concentrations were assessed using a radioimmunoassay kit (Linco Research, Billerica, MA). The trapezoidal rule was used for calculating means of the total AUCs for serum glucose and insulin concentrations. Three days' post-OGTT, a 6 h fasting intraperitoneal insulin tolerance test (IPITT) was performed. Serum glucose concentrations were determined every 15 min for 90 min following an intraperitoneal (IP) insulin injection (0.75 U/kg body weight).

Two days' post-IPITT, the rats were anesthetized with the ketamine/xylazine used earlier in the study and epididymal and retroperitoneal fat masses and uteri were excised and weighed. The uterus index is uterus weight divided by body weight. Serum was prepared from blood that was collected by cardiac puncture by centrifuging at 3000 rpm for 20 min. Human insulin (5 U/kg body weight) was then injected into the inferior vena cava for determining hepatic insulin signaling. Serum and tissues were then stored at −70°C for future use.

The homeostasis model assessment estimate for assessing insulin resistance (HOMA-IR) was calculated as previously reported.[21] Serum glucose and insulin concentrations were assayed using the Glucose Analyzer II and Linco Research radioimmunoassay kits. Serum 17β-estradiol levels were measured by ELISA kits (Enzo Life Sciences, NY, USA). Serum lipid profiles were measured by using colorimetry kits for total cholesterol, HDL cholesterol (HDL-C), and triglycerides (Asan Pharmaceutical, Seoul, Korea). Serum LDL-C was calculated using the

Friedewald equation (LDL-C = TC– HDL-C – triglyceride/5). ELISA kits were used for serum tumor necrosis factor (TNF)-α and MCP-1 determinations (R & D Systems, Minneapolis, MN, USA).

Immunohistochemistry and islet morphometry

Following the experimental period, nine or 10 rats from each group were given IP injections of BrdU (100 μg/kg body weight). Six hours later, the rats were given ketamine/xylazine anesthesia IP and the pancreas immediately excised, fixed with 4% paraformaldehyde, and embedded in paraffin as previously described.[23] To avoid counting the same islets twice, two serial 5 μm, paraffin-embedded sections were chosen from seven or eight sections for measuring β-cell area, uptake of BrdU, and apoptosis β- and α-cells were identified by treating the sections with guinea pig anti-insulin and rabbit antiglucagon antibodies (Abcam, Cambridge, MA, USA). BrdU incorporation was assessed by staining the paraffin sections with anti-insulin and anti-BrdU antibodies.[23] Apoptotic β-cells were stained and quantified using a TUNEL kit (Roche Molecular Biochemicals, Indianapolis, IN), and the islets were visualized by counterstaining the sections with hematoxylin and eosin.[23]

Pancreatic β-cell area was microscopically (10x) measured in non-overlapping images from two insulin-stained sections from each rat using a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Thornwood, NY, USA). The area was expressed as the percentage of insulin-positive cells in the total section area examined using IP Lab Spectrum software (Scanalytics Inc., Fairfax, VA, USA).[24] β-cell mass of the pancreatic portion was calculated as the weight of the portion multiplied by the percentage that was insulin positive.[23,24] Individual β-cell sizes were calculated by dividing the insulin-positive area by the number of nuclei contained within it. Enlargement of a β-cell indicates β-cell hypertrophy.[23,24] Beta-cell proliferation was defined as the total number of $BrdU^+$ nuclei per pancreas section.[23,24] β-cell apoptosis was quantified by counting apoptotic bodies in the β-cell nuclei of each pancreatic section.[23,24]

Islet isolation, total RNA generation, and gene expression by real-time PCR

The pancreatic islets from the excised pancreata of five rats from each group were isolated using collagenase digestion.[25] Collagenase P (3 mg; Roche Molecular Biochemicals, Indianapolis, Indiana, USA) in DMEM-high glucose was infused through the pancreatic ducts into pancreata of rats under ketamine/xylazine anesthesia. The pancreata were immediately excised and incubated for 15 min at 37°C. The pancreata were then washed on ice four times with DMEM-high glucose, and the islets were isolated using a separation medium (Ficoll reagent gradient, Sigma). Islets from two or three rats from each group were pooled.

Total RNA was extracted by mixing the islets with a phenol/guanidine isothiocyanate monophasic solution (TRIzol reagent; Gibco-BRL, Rockville, MD, USA), following the manufacturer's instructions. cDNA was synthesized using equal amounts of total RNA using Superscript III reverse transcriptase. Polymerase chain reaction (PCR) was implemented using high-fidelity Taq DNA polymerase. The cDNAs were added equally to the SYBR Green mix (Bio-Rad, Richmond, CA, USA) along with primers for specific genes which were amplified using a real-time Bio-Rad PCR instrument under optimal conditions for thermal cycling. The expressions of the genes of interest were normalized to that of the β-actin gene. Inflammatory cytokine, TNF-α, and interleukin (IL)-1β expressions were determined using corresponding primers, as previously described.[21] Cycle of threshold (CT) for each sample was assessed in the real-time PCR. Expression levels of the islet genes were quantitated using the comparative CT method (ΔΔCT method). ΔCT was calculated as subtracting CT of target gene and CT of endogenous reference gene (β-actin). Relative fold changes in gene expression were calculated as $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$. Changes in expression were expressed as $2^{-\Delta\Delta CT}$.

Immunoblot analysis

Livers were lysed in 20 mM Tris buffer as previously reported.[18] Lysates equilibrated to equal amounts of protein (30–50 μg) were immunoblotted with specific antibodies against protein kinase B (PKB/Akt), glycogen synthase (GSK)-3β, AMP kinase (AMPK), phosphoenol-pyruvate carboxykinase (PEPCK), and β-actin, and phosphorylated forms of PKB^{Ser473}, GSK-3β, and AMPK (Cell Signaling, Danvers, MA), as previously described.[18] Intensities of protein expression were measured using Imagequant TL (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

SAS software version 7 (SAS Institute) was used for statistical analysis. Required sample size was estimated using a G power program (power = 0.90 and effect size = 0.5) and a sample size 12 per group was required. When the results were normally distributed as confirmed by using Proc univariate, results are given as mean ± standard deviation (SD). Variables spanning multiple time points were analyzed using two-way repeated measures ANOVA, with independent variables being time and group and the interaction term being between time and group. Measurements were statistically analyzed by one-way ANOVA. Significance of differences among the multiple groups was assessed by Tukey's test at the level of $P < 0.05$.

Results

Total polyphenolic compounds and flavonoids in TTK and JGT

The total polyphenolic and flavonoid contents in TTK and JGT were similar. Because no good indicator compounds for TTK are known, caffeic acid was used as its indicator compound (Table 1). In JGT, paeoniflorin and glycyrrhizinic acid were measured as indicator compounds for Paeoniae Radix and Glycyrrhizae Radix, respectively. JGT contained

substantial amounts of paeoniflorin and glycyrrhizinic acid (Table 1).

Serum 17β-estradiol levels and uterine weight

The control rats had 4.4-fold lower serum 17β-estradiol levels than the positive controls (Table 2). A sham-operated group of rats was not included in this study since the positive controls maintained the similar concentrations of serum 17 β-estradiol to the Sham rats. When used in our previous studies serum 17β-estradiol levels of Sham rats were approximately 6.0 pg/mL,[19,22] and the control rats had approximately 3.5-fold less 17β-estradiol than the sham-operated rats. TTK, JGT, and JGTT did not cause significant changes in the 17β-estradiol concentrations in comparison to the control rats (Table 2). The uterine weight of the control rats was lowered by 2.6-fold compared with the positive control. The uterine weights were also not altered by the TTK, JGT, and JGTT treatments as compared to controls (Table 2). Thus, TTK, JGT, and JGTT did not stimulate uterine proliferation, indicating that they may not activate ERs in the uterus.

Table 1 The bioactive compounds in 70% ethanol extract of *Tetragonia tetragonioides* (Pall.) Kuntze and water extract of JakYakGamCho-Tang

	Tetragonia tetragonioides (Pall.) Kuntze	JakYakGam Cho-Tang
Total polyphenol (mg/g extract)	5.88 ± 0.32	6.0 ± 0.43
Total flavonoids (mg/g extract)	4.34 ± 0.23	4.67 ± 0.33
Caffeic acid (μg/g extract)	0.32 ± 0.01	ND
Albiflorin (μg/g extract)	Non-detectable	1.22 ± 0.03
Paeoniflorin (μg/g extract)	Non-detectable	24.7 ± 0.14
Liquiritigenin (μg/g extract)	Non-detectable	0.12 ± 0.04
Glycyrrhizinic acid (μg/g extract)	Non-detectable	40.2 ± 0.42

Each value represents mean ± SD (n = 3).

The serum levels of TNF-α and MCP-1 were higher in the controls than in the positive-control rats. TTK decreased the serum TNF-α levels to similar level as the positive control. Although JGT reduced the level to that of the positive control, it did not differ significantly from the control. Serum MCP-1 concentrations were higher in the control than the herbal extract treatment groups and the levels were as low in both the TTK and JGTT groups as in the positive controls (Table 2).

Tail skin temperature

Tail skin temperature is elevated in rats with estrogen deficiency due to a vasomotor disturbance, which is called a hot flash. In animals, this symptom can be monitored by checking the tail skin temperature. The control rats showed a higher tail skin temperature than the positive controls from the third week onward. None of the treatment affected tail skin temperature during the first and second weeks, but TTK, JGT, and JGTT lowered the tail skin temperature below that of the control and skin temperature was similar to the positive-control group during the final five weeks of treatment (Figure 1).

Body composition

Body weight as well as visceral fat masses (periuterine and retroperitoneal) was higher in control rats (Table 2). TTK, JGT, and JGTT tended to decrease the body weight compared to the control but not significantly. However, periuterine and retroperitoneal fat pad weights masses were lower in the rats fed TTK, JGT, and JGTT than in controls (Table 2). As with the visceral fat mass, the fat contents in the abdomen and leg areas were much lower in control rats than in positive controls and decreased in the descending order of control, JGT, JGTT, and TTK as measured by DEXA (Figure 2(a)). Rats in the TTK group exhibited similar fat mass in the abdomen as the positive control (Table 2). DEXA revealed that the lean masses of the hips and legs were

Table 2 Metabolic parameters at the end of the experimental period

	Control (n = 12)	TTK (n = 12)	JGT (n = 12)	JGTT (n = 12)	Positive control (n = 12)
Serum 17β-estradiol levels (pg/mL)	1.7 ± 0.5^{b}	1.8 ± 0.6^{b}	1.8 ± 0.6^{b}	1.9 ± 0.7^{b}	7.5 ± 1.0^{a}
Uterine weight (g)	0.23 ± 0.07^{b}	0.23 ± 0.07^{b}	0.19 ± 0.06^{b}	0.21 ± 0.11^{b}	0.61 ± 0.22^{a}
Body weight (g)	404 ± 27^{a}	389 ± 20^{a}	393 ± 30^{a}	388 ± 24^{a}	369 ± 26^{b}
Body weight gain (g)	99.6 ± 14.2^{a}	83.2 ± 12.3^{b}	90.4 ± 12.9^{ab}	88.7 ± 14.6^{ab}	70.0 ± 11.5^{c}
Periuterine fat (g)	15.1 ± 2.6^{a}	11.3 ± 2.1^{c}	13.1 ± 2.0^{b}	11.8 ± 2.3^{bc}	7.2 ± 1.6^{d}
Retroperitoneum fat (g)	9.9 ± 1.8^{a}	6.4 ± 1.8^{b}	8.9 ± 2.3^{a}	8.0 ± 2.1^{ab}	5.7 ± 1.6^{b}
Visceral fat (g)	25.0 ± 4.1^{a}	17.7 ± 3.8^{b}	19.9 ± 3.2^{b}	21.0 ± 3.7^{ab}	12.9 ± 2.6^{c}
Serum TNF-α (pg/mL)	50.5 ± 4.4^{a}	43.1 ± 4.1^{b}	46.4 ± 4.7^{ab}	45.8 ± 3.8^{b}	43.3 ± 3.9^{b}
Serum MCP-1 (pg/mL)	59.4 ± 6.1^{a}	43.2 ± 4.8^{c}	52.0 ± 5.0^{b}	47.5 ± 5.7^{bc}	42.2 ± 4.9^{c}

The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1%TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. At the end of the experimental period, visceral fat (periuterine and retroperitoneum fat) mass and serum levels of 17β-estradiol and inflammatory indicators were measured. Values represent mean ± SD.

[a,b,c]Means on the same row with different superscripts were significantly different at $P < 0.05$.

reduced lower in the control than in the positive control (Figure 2(b)). JGT and JGTT elevated their lean body mass in the hip and leg areas more than the control (Figure 2(b)). TTK increased the lean body mass more than the control and other herbal groups and the increase in the leg was even higher than the positive control (Figure 2(b)).

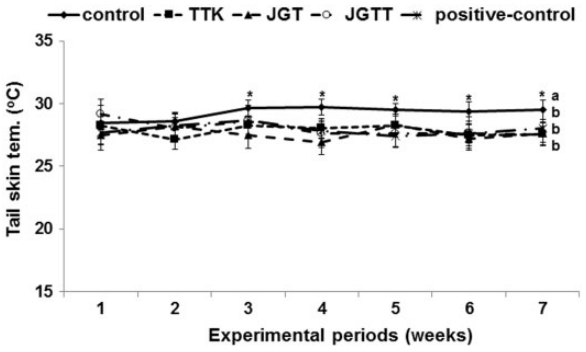

Figure 1 Tail skin temperatures measured every week during the experimental period. The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1% TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive-control) for eight weeks. The tail skin temperature was measured every week. Dots and error bars represent the mean ± SD (n = 12). [a, b] The different letters at the dots represent significant differences among the groups by Tukey's test at $P < 0.05$

Energy balance

The energy balance was the combination of calorie intake and expenditure on a daily basis. The daily calorie intake was not significantly different among the groups, but energy expenditure was suppressed in the controls compared to positive controls; however, JGTT and TTK increased daily energy expenditure (Table 3). Surprisingly, the increase in the TTK group was similar to the positive control. Indirect calorimetry revealed that control rats had higher carbohydrate oxidation than positive controls, but fat oxidation exhibited the opposite results compared to carbohydrate oxidation (Table 3). The TTK group had similar carbohydrate and fat oxidation as the positive control. The JGT and JGTT groups had lower carbohydrate oxidation than controls, but not as low as the positive controls (Table 3). Both treatments elevated fat oxidation above that of the control but were still less than the positive control.

Glucose metabolism

The overnight-fasted serum glucose and insulin concentrations were higher in the control than positive-control rats (Table 4). TTK, but not JGT, significantly lowered serum

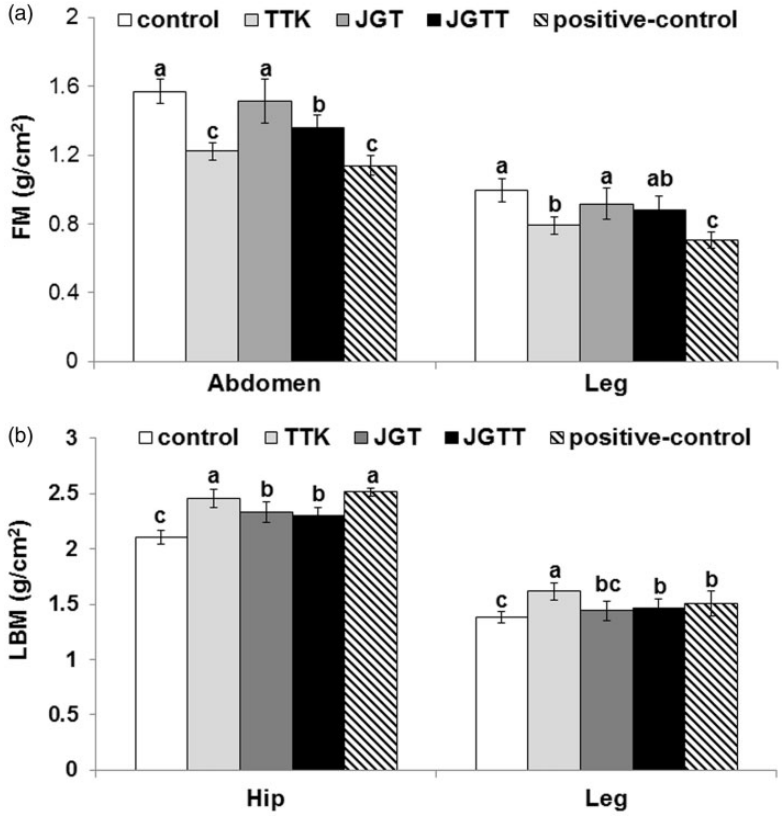

Figure 2 Fat mass (FM) and lean body mass (LBM) measured by DEXA. The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1% TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive-control) for eight weeks. At the end of the experimental period, FM in the abdomen and leg regions (a) and LBM in the hip and leg regions (b) were measured by DEXA. The values are expressed as means ± SD (n = 12). [a, b] The different letters on the bars represent significant differences among the groups by Tukey's test at $P < 0.05$

Table 3 Food intake and energy expenditure

	Control (n = 12)	TTK (n = 12)	JGT (n = 12)	JGTT (n = 12)	Positive control (n = 12)
Food intake (g/day)	13.3 ± 2.1	14.8 ± 4.4	12.8 ± 2.8	14.1 ± 2.2	13.9 ± 2.3
Food efficiency (%)	12.5 ± 1.9^{a}	9.4 ± 1.2^{c}	11.8 ± 1.9^{a}	10.5 ± 1.4^{b}	8.4 ± 1.7^{c}
Energy expenditure (kcal/kg$^{0.75}$/day)	90.2 ± 9.3^{c}	114 ± 13^{a}	98.6 ± 10.4^{bc}	102 ± 11^{b}	119 ± 13^{a}
Carbohydrate oxidation (mg/kg$^{0.75}$/min)	6.5 ± 0.7^{a}	4.2 ± 0.6^{c}	5.4 ± 0.7^{b}	5.4 ± 0.7^{b}	4.5 ± 0.6^{c}
Fat oxidation (mg/kg$^{0.75}$/min)	3.1 ± 0.4^{c}	9.2 ± 1.3^{a}	6.2 ± 0.8^{b}	6.5 ± 0.9^{b}	9.4 ± 1.2^{a}

The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), 3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1%TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. At the end of the experimental period, food intake and energy expenditure were measured. Each value represents mean ± SD.
^{a,b,c}Means on the same row with different superscripts were significantly different at $P < 0.05$.

Table 4 Overnight-fasted serum glucose and insulin levels and the islet morphometry

	Control (n = 12)	TTK (n = 12)	JGT (n = 12)	JGTT (n = 12)	Positive control (n = 12)
Glucose levels (mg/dL)	123 ± 14^{a}	108 ± 12^{b}	121 ± 15^{a}	115 ± 13^{ab}	109 ± 13^{b}
Insulin levels (ng/mL)	1.45 ± 0.25^{a}	0.88 ± 0.16^{c}	1.37 ± 0.19^{a}	1.16 ± 0.18^{b}	1.14 ± 0.19^{b}
HOMA-IR	9.9 ± 1.5^{a}	5.3 ± 0.8^{c}	9.2 ± 1.4^{a}	7.4 ± 1.2^{b}	6.8 ± 0.9^{b}
β-cell area (%)	5.9 ± 0.6^{c}	7.9 ± 1.0^{a}	6.9 ± 0.8^{b}	8.1 ± 1.0^{a}	7.8 ± 0.9^{a}
Individual β-cell size (μm^{2})	1.45 ± 0.25^{a}	0.88 ± 0.16^{c}	1.37 ± 0.19^{a}	1.16 ± 0.18^{b}	1.14 ± 0.19^{b}
Absolute β-cell mass (mg)	33.4 ± 3.5^{c}	46.8 ± 4.8^{a}	40.2 ± 4.5^{b}	48.9 ± 5.1^{a}	47.7 ± 5.2^{a}
BrdU^{+} cells (% BrdU^{+} cells of islets)	0.73 ± 0.09^{b}	0.89 ± 0.09^{a}	0.75 ± 0.09^{b}	0.86 ± 0.08^{a}	0.87 ± 0.08^{a}
Apoptosis (% apoptotic bodies of islets)	0.71 ± 0.08^{a}	0.52 ± 0.06^{b}	0.51 ± 0.07^{b}	0.51 ± 0.08^{b}	0.52 ± 0.06^{b}
TNF-α expression (AU)	1.01 ± 0.19^{a}	0.78 ± 0.15^{b}	0.82 ± 0.14^{b}	0.76 ± 0.16^{b}	0.79 ± 0.15^{b}
IL-1β expression (AU)	1.03 ± 0.17^{a}	0.68 ± 0.11^{b}	0.71 ± 0.15^{b}	0.67 ± 0.14^{b}	0.72 ± 0.17^{b}

The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1%TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. At the end of the experimental period, overnight-fasted serum glucose and insulin levels, islet morphometry, and TNF-α and IL-1β expression were measured. Values represent mean ± SD.
^{a,b,c}Means on the same row with different superscripts were significantly different at $P < 0.05$.

glucose and insulin concentrations compared with the control group. JGTT tended to decrease the serum glucose, but not significantly, but did lower insulin concentrations significantly. Insulin resistance, as assessed by HOMA-IR, was much greater in the controls than positive controls and decreased from high to low in the following order: control, JGT, JGTT, positive control, and TTK (Table 4). TTK improved insulin resistance better than the positive control.

Following the glucose challenge, serum glucose concentrations peaked at 40–50 min by regulating both insulin secretion and insulin sensitivity (Figure 3(a)). However, from the peak, the serum glucose levels were mainly reduced by insulin sensitivity. Thus, the area under the curve of glucose (AUCG) and AUC of insulin (AUCI) for two time periods: first part (0–40 min) and second part (40–120 min) of the test. The control group had much higher AUCGs of the first and second parts than the positive-control group (Figure 3(b)). No treatments decreased the first part of the AUGG, whereas only TTK lowered the AUCG in the second part and to the same extent as the positive control. Thus, TTK improved glucose tolerance to the same extent as the positive-control group (Figure 3(b)).

Because the serum glucose is regulated by the serum insulin concentrations and sensitivity, the AUCI was calculated. Both first part and second part AUCIs were higher in control rats than in positive-control rats (Figure 3(c)). TTK and JGTT lowered the AUCIs of the first and second parts to the same extent as the positive control, whereas JGT only decreased the AUCI of the second part (Figure 3(c)).

During the IPITT, the serum glucose levels were markedly reduced until 30 min after an IP injection of insulin, after which they were further decreased and then increased from 60 to 75 min (Figure 4(a)). The decreased first part AUC for serum glucose was due to insulin resistance. During the second part of the IPITT, the concentrations of serum glucose remained steady and then slowly increased. The AUC of serum glucose concentrations in the first and second parts of the IPITT was higher for the control group

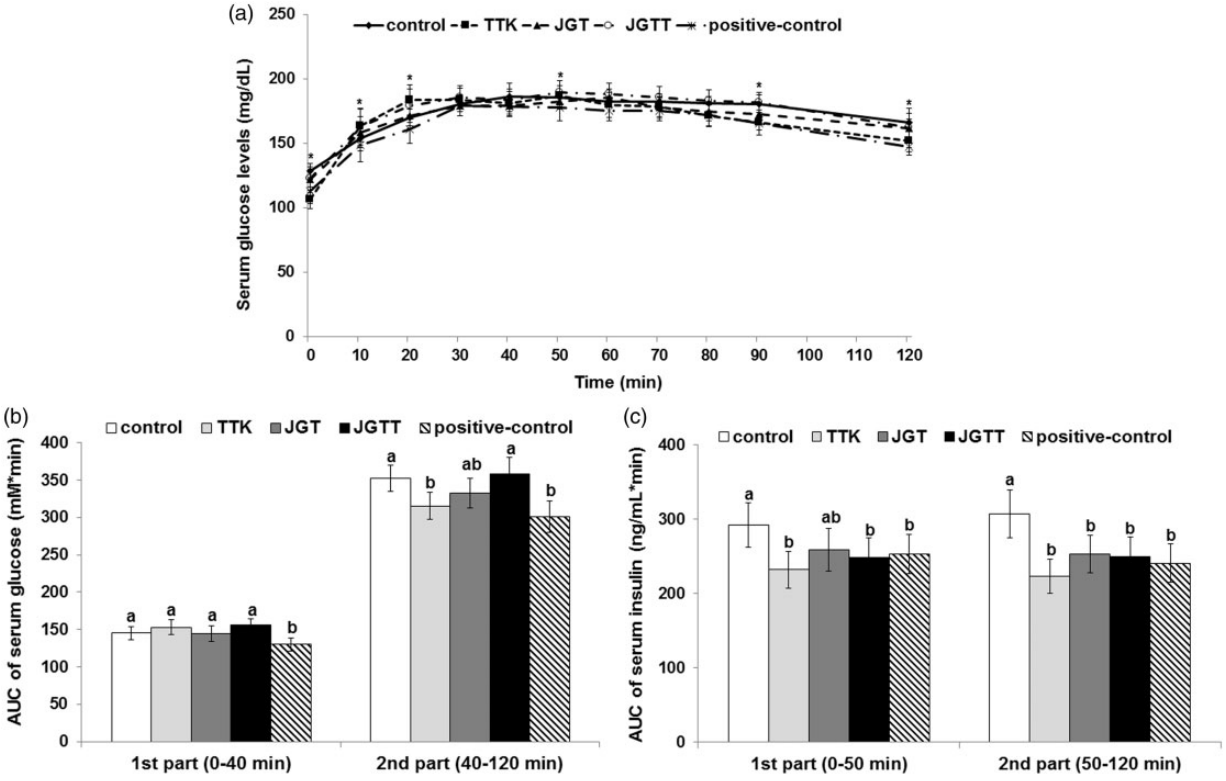

Figure 3 Serum glucose levels and areas under the curve of glucose and insulin during the oral glucose tolerance test (OGTT). The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1% TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive-control) for eight weeks. At the seventh week, 2 g of glucose/kg body weight was orally administered and the serum glucose and insulin levels were measured at the indicated times. The changes in the serum glucose levels were measured during the OGTT (a). Average area under the curve (AUC) of glucose (b) and insulin (c) for the first (0–40 min) and second phases (40–120 min) of the OGTT

Dots and bars represent the mean ± SD (n = 12). *Significantly different among all groups in one-way ANOVA at $P < 0.05$. [a, b, c] The different letters on the bars represent significant differences among the groups using Tukey's test at $P < 0.05$

than for the positive controls (Figure 4(b)). The first part of AUC was decreased in the TTK group similar to the positive controls, whereas the second part of AUC was highest in control and JGT rats and lowest in the TTK and positive-control rats (Figure 4(b)). These results indicated that TTK reduced insulin resistance to a similar extent as the positive control.

Hepatic insulin signaling

Phosphorylation of hepatic Akt and GSK-3β, a downstream modulator of Akt, suppressed more in the control rats than in the positive-control rats. This indicated that the OVX control rats exhibited impaired hepatic insulin signaling, and 17β-estradiol prevented the disturbance. The positive-control rats exhibited lower expression levels of PEPCK, which catalyzes the rate-limiting step of gluconeogenesis, than the control rats. Furthermore, AMPK phosphorylation was markedly reduced in the control rats compared with the positive-control rats (Figure 5). TTK inhibited the impaired hepatic insulin signaling (pAkt→pGSK-3β) in comparison to the control rats, and PEPCK expression was decreased to a similar extent as the positive control. In addition, TTK increased AMPK phosphorylation compared to the control rats. JGT did not improve hepatic insulin signaling as much as TTK and JGTT, the mixture of TTK and JGT did not have an additive effect (Figure 5).

Islet morphometry

Pancreatic β-cell area is a factor of both the number and size of β-cells. Larger β-cell size indicates β-cell hypertrophy due to insulin resistance. Surprisingly, the positive-control group was found to have significantly larger pancreatic β-cell area than the control group, but the sizes of individual β-cells were smaller in the positive-control group than the control group (Table 4). The β-cell area increased in the JGT group but the increase was less than the TTK and JGTT groups, and the elevated areas in the latter two groups were as high as in the positive controls (Table 4). These results indicated that the treatments increased the β-cell area through different pathways: TTK and JGTT increased the β-cell area by stimulating proliferation of the cells and not by increasing the size, but JGT only increased the area by increasing the size.

Pancreatic β-cell mass is calculated by multiplying the β-cell area by the pancreas weight. Pancreas weights were not different among the groups (data not shown). Nevertheless, β-cell masses were much lower in the controls group than in positive-controls group, and whereas TTK

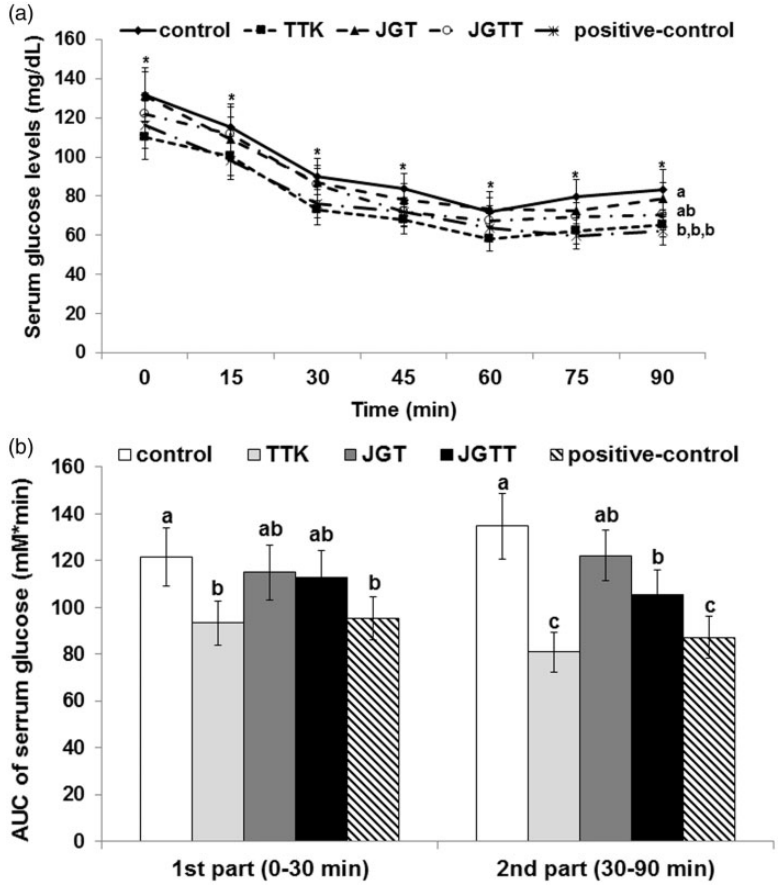

Figure 4 Changes in the serum glucose levels during an intraperitoneal insulin tolerance test (IPITT). The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1% TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. At the seventh week, an IPITT was conducted by intraperitoneally injecting insulin (1 U/kg body weight) after a 6 h fast. The serum glucose levels (a) and area under the curve of serum glucose levels (b) were measured. The dots and bars are means ± SD (n = 12). *Significantly different among all groups in one-way ANOVA at $P < 0.05$. [a, b, c] The different letters on the bars represent significant differences among the groups by Tukey's test at $P < 0.05$

and JGTT increased the mass to a similar extent as the positive control (Table 4). The number of β-cells is related to the net sum of cellular proliferation versus apoptosis. The β-cells of control rats experienced a much lower ratio of proliferation to apoptosis than the positive-control rats. TTK and JGTT increased β-cell proliferation, but JGT did not change β-cell proliferation, whereas TTK, JGT, and JGTT all decreased β-cell apoptosis (Table 4). Thus, JGT only increased the β-cell mass by decreasing apoptosis compared with the control, and the increase was less than the positive control (Table 4).

β-cell apoptosis may be linked to excessive islet expression of proinflammatory cytokines. In control rats, islet TNF-α and IL-1β expression levels were higher than those of positive-control rats. TTK, JGT, and JGTT all lowered the expression levels of these cytokines (Table 4).

Lipid metabolism

The OVX rats exhibited dyslipidemia; the serum concentrations of TC, LDL-C, and triglycerides were higher in the control rats than in the 17β-estradiol-treated rats, whereas circulating HDL-C concentrations were lower in control rats (Table 5). The TTK and JGTT treatments reduced the serum TC, LDL-C, and triglyceride concentrations and increased the serum HDL-C concentrations to the same extent as the positive-control group. However, the JGT treatment did not improve dyslipidemia (Table 5). Thus, TTK and JGTT normalized the lipid profiles to the same extent as the positive-control group.

Discussion

SERMs act as estrogen agonists and antagonists in different tissues by influencing conformational changes in the ERs.[26] TTK and JGT were chosen because they affected growth of MCF-7 human breast adenocarcinoma cells and might modulate menopausal symptoms by compensating for estrogen depletion in estrogen-sensitive tissues. We determined that the long-term consumption of these herbal extracts alleviates the progression of postmenopausal symptoms and metabolic disturbances in OVX rats fed a high fat diet, and their mechanism of action was explored. Although JGT and TTK acted in a similar manner as an ER agonist and antagonist, respectively, in breast cancer cells, neither caused uterine proliferation in the OVX rats. TTK attenuated the menopausal symptoms better than JGT and

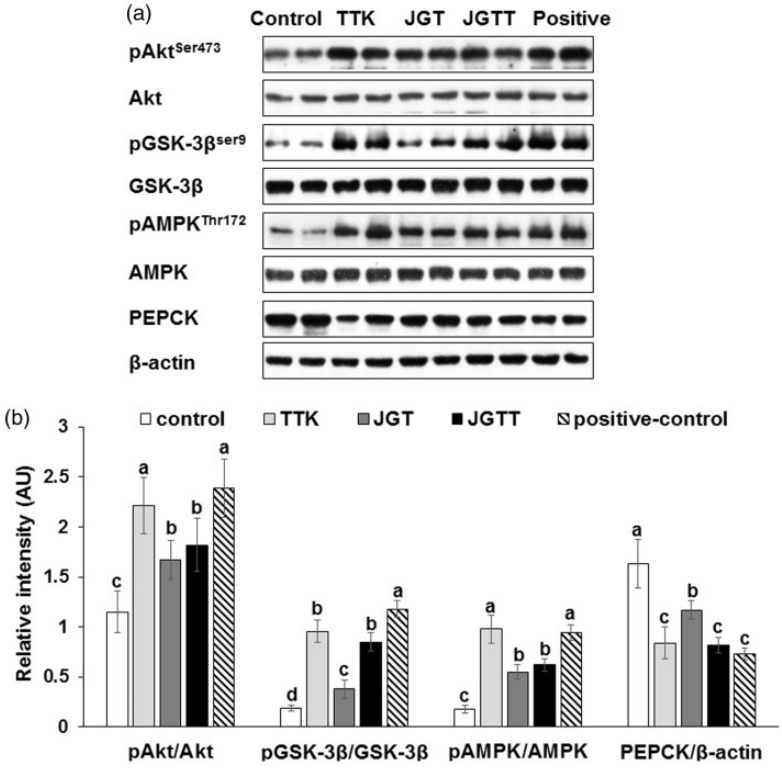

Figure 5 Hepatic insulin signaling at the end of experiment. The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1% TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. After completing the IPITT, the animals were provided food for 6 h, after which they were deprived of food for 16 h. Regular human insulin (5 U/kg body weight) was injected through their inferior vena cava. The hepatic insulin signaling was measured with immunoblotting. The band intensity was measured with image analyzer. The values are means ± SD (n = 6). [a, b, c] The different letters on the bars represent significant differences among the groups by Tukey's test at $P < 0.05$

Table 5 Lipid profiles in overnight-fasted rats

	Control (n = 12)	TTK (n = 12)	JGT (n = 12)	JGTT (n = 12)	Positive control (n = 12)
Total cholesterol (mg/dL)	104.5 ± 8.4^{a}	95.3 ± 7.8^{b}	98.6 ± 7.9^{ab}	96.9 ± 7.2^{b}	94.6 ± 7.6^{b}
HDL cholesterol (mg/dL)	23.3 ± 1.7^{b}	26.5 ± 2.1^{a}	24.9 ± 2.2^{ab}	27.4 ± 2.4^{a}	27.8 ± 1.9^{a}
LDL cholesterol (mg/dL)	66.1 ± 5.2^{a}	56.2 ± 5.1^{b}	58.1 ± 5.3^{ab}	56.4 ± 5.9^{b}	53.5 ± 5.6^{b}
Triglyceride levels (mg/dL)	74.1 ± 6.8^{a}	63.1 ± 5.3^{b}	77.8 ± 6.1^{a}	65.9 ± 5.8^{b}	66.2 ± 5.8^{b}

The ovariectomized (OVX) rats were provided with a 45% fat diet containing (1) 2% dextrin (placebo; OVX control), (2) 2% *Tetragonia tetragonioides* (Pall.) Kuntze water extract (TTK), (3) 2% JakYakGamCho-Tang (JGT), (4) 1% JGT+1%TTK (JGTT), or (5) 30 μg/kg body weight 17β-estradiol+2% dextrin (positive control) for eight weeks. At the end of the experimental period, lipid profiles in the circulation were measured. Each value represents mean ± SD.
[a,b]Means on the same row with different superscripts were significantly different at $P < 0.05$.

JGTT, and the mixture of TTK and JGT did not have a synergistic activity in reducing the menopausal symptoms. Thus, TTK might have potential as a therapeutic agent for menopausal symptoms in postmenopausal women.

Estrogens exert potent regulatory actions on the reproductive, cardiovascular, skeletal, and central nervous systems.[1,3] Estrogen is important for regulating energy, glucose, lipid, and bone metabolism in both animal models and humans.[2,17] Estrogen is involved in insulin sensitivity; lipid accumulation; and inflammation in cells of the skeletal muscle, liver, adipose, and immune systems, and these activities are closely related.[27] In addition, estrogen is involved in regulating insulin secretion and pancreatic islet β-cell mass.[28] Estrogen deficiency promotes metabolic disease such as obesity, type 2 diabetes, and dyslipidemia, in addition to menopausal symptoms such as hot flashes, insomnia, and night sweats.[2,5] The dysregulation of energy metabolism leads to body weight and fat gain in humans and animal models, mainly due to decreased energy expenditure.[2] Increased visceral fat gain is characterized by increased inflammatory adipokine levels and macrophage infiltration, which promote ectopic fat storage in the liver and muscles.[27] The increased inflammation induces insulin resistance in the periphery to impair

glucose metabolism.[29] The OVX rats used in this study exhibited impairments of energy, glucose, and lipid metabolisms that increased body weight, abdominal fat accumulation, dyslipidemia, and glucose intolerance. Therefore, this study evaluated the potential of JGT and TTK to reverse those disturbances.

Menopausal symptoms, including metabolic impairments, are partially prevented by HRT with estrogen and progestin.[4] However, the Women's Health Initiative[30] has reported that HRT increases the risk of serious diseases such as breast cancer, heart disease, and stroke. HRT prescriptions have declined substantially from a high of 37% of all women treated for symptoms of menopause in 2002 to 14 and 4% in 2003 and 2004.[31] Menopausal women need alternative treatments to relieve their symptoms. Menopausal symptoms are associated with increased inflammation in both estrogen-deficient women and experimental animals.[27] Both TTK and JGT have demonstrated anti-inflammatory and antioxidant properties that inhibit NF-κB signaling.[32] Surprisingly, the present study showed that TTK prevented the decrease in energy expenditure which lowered visceral fat mass, without changing the serum levels of 17β-estradiol or uterine weight in the OVX rats. Increases in adipose tissues induce proinflammatory adipokine and cytokine expression which are correlated with their levels in the circulation.[33] Thus, the levels of cytokines in the circulation can be used as an indicator of the inflammatory state. The release of adipokines and cytokines, by either adipocytes and/or adipose tissue-associated macrophages, causes chronic low-level inflammation that increases the chance to develop insulin resistance and type 2 diabetes.[34] The present study also showed that the control rats exhibited increased serum TNF-α and MCP-1 levels, which are indicators of inflammation, and that TTK and estrogen treatments resulted in lower levels. Kim et al.[35] also showed that the OVX mice exhibited increased fat mass, serum MCP-1 levels, and reactive oxygen species levels, but MCP-1 knockout mice prevented the increase. These results indicated that the increased inflammation mediated by MCP-1 may be an important factor in the metabolic perturbations in the OVX rats. Therefore, the suppression of the inflammatory status during estrogen treatment may attenuate the metabolic impairments. TTK might act as an anti-inflammatory agent that ameliorates the metabolic disturbances in OVX rats.

Glucose metabolism is regulated by the combined effects of insulin sensitivity and secretion.[28] Estrogen deficiency impairs glucose metabolism not only by increasing insulin resistance but also by impairing insulin secretion. Insulin resistance is induced in insulin-responsive tissues, especially skeletal muscle, adipose tissue, and liver.[28] It is associated with increased inflammation due to increased adipose tissue mass.[29] Increased insulin resistance decreases glucose uptake by skeletal muscles and adipose tissue in response to insulin, elevates fat storage in these tissues, and increases hepatic glucose output in a hyperinsulinemic state.[23] The increased insulin resistance elevates insulin secretion to normalize the serum glucose levels and it can exhaust β-cell function and reduce β-cell mass.[23,28] This vicious cycle eventually increases the susceptibility to develop type 2 diabetes in estrogen-deficient animals and humans. Menopause exacerbates insulin resistance.[27,29] In the present study, control rats exhibited increased insulin resistance, as determined by the HOMA-IR and the IPITT, as well as increased fat storage in the skeletal muscles and liver. These observations might be related to the increased serum TNF-α and IL-1β levels. In addition, hepatic insulin signaling was attenuated which increased glucose output in the hyperinsulinemic state, producing hyperglycemia in the OVX rats. TTK and JGT reduced the cytokine levels in the circulation and decreased insulin resistance in comparison to the control rats, but TTK produced a greater reduction. It has been reported that JGT and its component herbs have anti-inflammatory activities that reduce cytokine production and inhibit NF-κB signaling in various cell types.[32,36,37] A few studies have been conducted about TTK as a functional food. TTK is known to possess antiulcerogenic and anti-inflammatory activities[38] and TTK has also demonstrated to have antioxidant activities higher than α-tocopherol.[39] The present study exhibited TTK had a better anti-inflammatory activity than JGT and consequently improved insulin sensitivity better than JGT. Thus, TTK may improve insulin sensitivity by enhancing antioxidant activities.

In addition to insulin resistance, the insulin secretion capacity is indirectly modulated by inflammation.[29] Insulin secretion capacity is regulated by the serum glucose levels, which affects the intracellular glucose levels in the islets to change cAMP and ATP levels in an acute phase, whereas the insulin secretion capacity is modified by β-cell mass in a chronic phase.[40] In particular, maintaining the β-cell mass is important for delaying or preventing type 2 diabetes, which develops when the β-cells are exhausted.[23] In the OVX rats, the β-cell mass was reduced, which was prevented by the estrogen treatment.[28] TTK also prevented the reduction of β-cell mass, which in turn led to increased insulin secretion and decreased inflammatory cytokine expression in the OVX rats. Thus, in OVX rats, insulin secretion is impaired and when they are unable to compensate for insulin resistance, they in turn results in type 2 diabetes due to the loss of β-cell mass. TTK supplemented rats maintained β-cell mass equally as well as estrogen-treated rats by increasing proliferation and suppressing apoptosis in β-cells. In particular, TTK prevented the decrease in β-cell proliferation more than JGT, but TTK and JGT reduced β-cell apoptosis as effectively as estrogen treatment. Thus, the decreased expression of the inflammatory cytokines including TNF-α and IL-1β, in the islets prevented β-cell apoptosis in the TTK, JGT, and JGTT groups, but TTK might use another pathway to improve β-cell proliferation.

In conclusion, estrogen-deficient rats exhibited disturbances in energy, glucose, and lipid metabolism, which were associated with increased proinflammatory cytokine levels in the circulation and islets. TTK itself prevented the OVX-induced impairments in energy, lipid, and glucose metabolism to the same extent as the positive control without changing either serum 17β-estradiol levels or uterine weights. TTK and JGT had no synergistic activity on the menopausal symptoms. Although the lack of effect of

TTK on uterine weights suggests that it does not possess estrogenic activity at the dosages used in this study, TTK may be a useful therapeutic agent for menopausal symptoms with effects that overlap with many of those of SERMs. Additional research is necessary to confirm the safety and efficacy of TTK in human studies.

Authors' contributions: JAR, BSK, and SP participated in the experimental design; HWL analyzed indicative compounds of the herbs; DSK and SK conducted the animal studies; YHL performed biochemical studies. JAR, BSK, and SP wrote the manuscript. All authors participated in analysis of the data and review of the manuscript.

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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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