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Original Article

Alleviative effects of Yukmijihwang-tang on cholesterol related disease in a postmenopausal rat model and lipid accumulation in HepG2 cells

Hiroe Go[1], Jin Ah Ryuk[1], Hye Won Lee[1], Ki-Jung Kil[2], Byoung Seob Ko[1]

[1]*Korean Medicine Convergence Research Division, Korea Institute of Oriental Medicine, Republic of Korea;* [2]*College of Oriental Medicine, Joongbu University, Republic of Korea*

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Abstract: Yukmijihwang-tang (YMJT), known as Liu-wei-di-huang-tang in Chinese and Lokumijio-to in Japanese, has been used as a traditional herbal formula for the treatment of various diseases. In Korean traditional medicine, YMJT is a well-known herbal prescription used to relieve Yin-Deficiency. In this study, we examined the effects of YMJT on Nonalcoholic steatohepatitis (NASH) and on diseases associated with cholesterol in a rat model of postmenopausal hyperlipidemia and the methyl-β-cyclodextrin (MβCD)-induced hepatic steatosis model in HepG2 cells. Twenty-five rats were ovariectomized (OVX), and five rats were sham-operated (Sham). Then, the OVX rats were randomly assigned to three groups (n = 5): OVX-Con, OVX with simvastatin, and OVX with YMJT (50, 150, 450 mg/kg) for 8 weeks. NASH and a number of targets associated with cholesterol were examined to confirm the effects of YMJT. Oil Red O staining and intracellular cholesterol analyses were used to quantify cellular cholesterol levels. The levels of phosphorylated AMP-activated protein kinases (AMPK) and the products of genes involved in cholesterol synthesis were measured via Western blotting. In OVX rats, YMJT reduced retroperitoneal and peri-renal fat accumulation, serum lipids, the atherogenic index, cardiac risk factors, intima-media thickness, and NASH. YMJT decreased lipid accumulation, total cholesterol, and low-density/very-low-density lipoprotein levels in HepG2 cells. Moreover, YMJT reversed the effects of MβCD on cholesterol synthesis regulators. Phosphorylation of AMPK was stimulated by YMJT. These results indicated that YMJT has cholesterol-lowering effects both *in vivo* and *in vitro*. Therefore, YMJT may have potential as a therapeutic agent for the treatment of hyperlipidemia in postmenopausal females.

Keywords: Yukmijihwang-tang (YMJT), cholesterol, hyperlipidemia, menopause, lipid, ovariectomized

Introduction

In Donguibogam, aging is said to result from the decline of blood [1]. Yukmijihwang-tang (YMJT) is used to improve health and has been used for aging care. Aging negatively affects the health of women. Hyperlipidemia is known as high lipid levels in the blood. Cholesterol is carried in the blood as lipoproteins, such as high-density lipoproteins (HDL) and low-density lipoproteins (LDL). In our body, an excess of cholesterol formation leads to an increased risk of developing cardiovascular disease (CVD) [2]. Hyperlipidemia leads to the development of fatty liver diseases, including nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) [3].

Postmenopausal females are at particular risk, with the accelerated progression of NASH and the accumulation of visceral fat being more common in these patients [4-6]. In a report about patients with NASH, premenopausal women are shown to be at a decreased risk of severe fibrosis compared with similarly aged men, but the risk is equivalent in postmenopausal women and similarly aged men [6]. NASH can be ameliorated by hormone replacement therapy (HRT) or estrogen administration [5]. HRT and estrogen replacement therapy (ERT) cause a slight increase in the risk of developing heavy diseases such as breast cancer [7]. Therefore, the development of a safe, effective method of treating or preventing NASH is needed [8].

Recent studies indicate that increased hepatic free cholesterol is an important risk factor in NASH patients [9]. In addition, hypercholesterolemia is often observed in postmenopausal females [5]. Management of increased cholesterol may be associated with a reduced risk for NASH in postmenopausal females.

The synthesis and utilization of cholesterol is a tightly regulated system mediated by the sterol regulatory element binding proteins (SREBPs), which prevent its over-accumulation and abnormal deposition within the body [10, 11]. The SREBPs are a family of transcription factors consisting of three members, namely SREBP-1a, SREBP-1c, and SREBP2. SREBP2 regulates cholesterol metabolism primarily through its regulation of genes related to cholesterol stimulation and synthesis, such as low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR) [12-14]. When cells are deprived of cholesterol, the SREBP2-NH_2-terminal fragment is translocated from the endoplasmic reticulum (ER) membrane to the nucleus and binds to sterol regulatory elements in the promoter regions of the LDLR and HMGCR genes, resulting in an increase in cholesterol uptake and synthesis [11, 15]. When cholesterol levels are high, SREBP2 is retained in the ER, resulting in the downregulation of intracellular cholesterol uptake and synthesis [11].

LDLR is a transmembrane glycoprotein that plays a critical role in the homeostatic control of blood cholesterol by mediating the turnover of LDL particles in circulation [16]. In humans, the liver contains approximately 70% of the total LDLR. The abundance of LDLR has been shown to be closely associated with NASH [17, 18].

HMGCR catalyzes a rate-limiting step of the mevalonate pathway, the cholesterol biosynthesis pathway, and is responsible for the synthesis of mevalonate from hydroxyl-methylglutaryl-coenzyme A (HMG-CoA) [19]. As the expression of HMGCR is increased in patients with NASH, HMGCR inhibitors, such as lovastatin, have been proposed as a potential treatment for NASH, although their efficacy has yet to be established.

AMP-activated protein kinase (AMPK) is seen as a mediator of the cellular adaptation to the environment, a metabolic master switch or a factor of nutritional stress [20]. The activated AMPK leads to simultaneous inhibition of anabolic pathways, such as cholesterol, fatty acids, and triglyceride synthesis, as well as to the stimulation of fatty acid oxidation and ketogenesis [17, 21]. Because AMPK plays a central role in lipid metabolism, it was considered to be an important therapeutic target for the treatment of fatty liver disease [22].

There is increasing interest in the use of traditional herbal medicines to prevent and treat various diseases [23]. YMJT, a traditional Korean remedy, is composed of six oriental herbs (*Rehmannia glutinosa* Liboschitz ex Steudel, *Dioscorea japonica* Thunberg, *Cornus officinalis* Siebold et Zuccarini, *Alisma orientale* Juzepzuk, *Poria cocos* Wolf, *Paeonia suffruticosa* Andrews) and has been used to treat diseases associated with a decline in kidney. According to Traditional Chinese Medicine, kidney energy levels are low at the onset of menopause. It has been reported that YMJT is able to prevent and cure osteoporosis induced by ovariectomy in rat models of osteoporosis [24]. In addition, in rat models of diabetes, YMJT and *Discoreae* Radix decreased total serum cholesterol and triglyceride [25]. Thus, we thought that YMJT might have effects on the symptoms of menopause, especially on cholesterol related diseases.

We aimed to assess whether YMJT has cholesterol-lowering effects and is effective against NASH and diseases associated with cholesterol in a postmenopausal model. Moreover, we also investigated whether YMJT has cholesterol-lowering effects in the MβCD-induced hepatic steatosis model *in vitro*.

Materials and methods

YMJT preparation

The formula of YMJT consist of 6 herbs, including *R. glutinosa* (165 g), *C. officinalis* (82.5 g), *D. japonica* (82.5 g), *P. suffruticosa* (61.88 g), *A. orientale* (61.88 g), *P. cocos* (61.88 g). The water extract of YMJT were prepared using an S-20,000 extractor (Sak IK Medical Company). Briefly, mixture of 6 herb (515.64 g) were extracted by heating for 2 h in a 10-fold volume of water. Then extractions were freeze-dried and the resulting YMJT powder (148.3 g, yield:

28.76%) was collected. YMJT extract powder was stored at 4°C until use. The YMJT extract (KIOM PH 130003) was stored at Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea) until used in this experiment.

Chromatographic conditions of high-performance liquid chromatography (HPLC)-DAD

Quantitative analysis of the reference compounds solutions; 5-HMF, morroniside, loganin, paeoniflorin, and paeonol (1,000 μg/mL) were prepared in 100% methanol and stored at 4°C. The standard solutions were prepared by six concentrations of diluted solutions (methanol). All calibration curves were attained by evaluating the peak areas at six concentrations in the range of 10-500 μg/mL for all reference compounds. The linearity of the peak area (y) vs. concentration (x, μg/mL) curve for each component was used to calculate the contents of the main YMJT. 104.0 mg of YMJT extract powder was subsequently resuspended in 10 mL distilled water for HPLC analysis. The contents of 5-HMF, morroniside, loganin, paeoniflorin, and paeonol in the YMJT water extract were analyzed using an 1100 series HPLC instrument (Agilent Technologies, USA) with a Gemini C18 column (4.6 × 250 mm, 5 μm; Phenomenex, USA). The mobile phase consisted of the solvents, distilled water (A) and acetonitrile (B). The following gradient was used: 0 min, A:B 95:5 (v/v); 30 min, A:B 60:40; and 40 min, A:B 0:100. The mobile phase flow rate was 1.0 mL/min, the column temperature was 25°C, the injection volume was 10 uL, and UV detection was at 240 nm (morroniside, loganin, and paeoniflorin) and 280 nm (5-HMF, paeonol).

Cell line and culture conditions

HepG2 human hepatocellular carcinoma cells were purchased from the Korean cell line bank (Seoul, Korea). HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were incubated at 37°C in humidified atmosphere of 5% CO_2 in air (v/v). To induce cholesterol accumulation, HepG2 cells were exposed to MβCD mixed with palmitic acid. When cells reached 70% confluence, they were incubated in 0.2% BSA-DMEM containing 20 μg/mL MβCD mixed with palmitic acid (MβCD). The cells were exposed to 30 μM simvastatin or various concentrations (250, 500, 750 μg/mL) of YMJT for 8 h. In this study, we used same data and pictures that were obtained from three groups (control, MβCD, and simvastatin) that were included in previous study [26].

Animals and experimental design

All experimental protocols and animal maintenance procedures used in this study were treated in accordance with the Guide for Care and Use of Laboratory Animals by the Institutional Animal Care and Use Committee (IACUC) of KIOM. Six week-old female Sprague-Dawley rats (weight 225±25 g) were obtained from Samtako (Osan-si, Korea). Animals were maintained at a regular 12 h light/dark cycle, at a controlled temperature (24±2°C) with a relative humidity of 50±5 % in the Laboratory Animals Center at KIOM (Approval No. 13-024). After 1 week of acclimation, the rats were performed ovariectomy (n = 25) or sham operation (n = 5) under general anesthesia by intramuscular injection. One week after the surgery, twenty-five OVX rats were randomly divided into three group: (1) high-fat (45%), high-cholesterol (1%) diet (OVX-Con) (n = 5), (2) high-fat (45%), high-cholesterol (1%) diet with simvastatin (OVX-SV; 20 mg/kg) (n = 5), (3) high-fat (45%), high-cholesterol (1%) diet with YMJT (OVX-YMJT; 50, 150, 450 mg/kg) (n = 5 per each dose). Five sham-operated rats were not assigned to a high-fat (45%), high-cholesterol (1%) diet (Sham). Simvastatin and YMJT were dissolved in phosphate-buffered saline (PBS) for daily oral administration. After eight weeks, rats were sacrificed under anesthesia and blood was collected for the estimation of total-cholesterol (TC), triglycerides (TG), HDL, LDL, atherogenic index, and cardiac risk factor. Liver tissue, retroperitoneal fat, peri-renal fat, and arterial tissue were also collected. In this study, we used same data and pictures that were obtained from three groups (Sham-operated, OVX-Con, and OVX-SV) that were included in previous study [26].

Lipid parameters

Measurements of serum TC, TG, and HDL levels were performed using a BS220 instrument (Mindary, Shenxhen, China). LDL levels were determined using the following equation: LDL = TC-HDL-(TG/5). TC and LDL/very low- density lipoproteins (VLDL) contents in HepG2 cells

were determined using an HDL and LDL/VLDL cholesterol assay kit according to the manufacturer's instructions. After treatment with 30 μM simvastatin or various concentrations of YMJT (250, 500, 750 μg/mL) with MβCD in 0.2% BSA-DMEM for 8 h, cells were washed with PBS and cellular lipids were extracted with chloroform:isopropanol: NP-40 (7:11:0.1). Then extracts were centrifuged for 10 min at 15,000 *g* and supernatants were transfer to a new tube. To remove chloroform, supernatants were dried at 50°C and 200 μL cholesterol assay buffer were added to resuspend dried lipids. TC and LDL/VLDL levels were assayed by measuring absorbance at 570 nm using a spectrophotometer.

Atherogenic index and cardiac risk factor

Atherogenic index and cardiac risk factor were determined using the following equations: atherogenic index = (TC-HDL)/HDL, cardiac risk factor = TC/HDL.

Histology

Rat livers were fixed with 4% neutral buffered formalin (NBF) and embedded in paraffin. Four-micron sections were then cut and stained with hematoxylin and eosin (H&E) for histological analysis.

Cytotoxicity

The cell viability was examined by (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) (WST) assay. Briefly, HepG2 cells were plated at a density of 2×10^4 cells/well in 96-well plates and treated with 30 μM simvastatin or various concentrations of YMJT (250, 500, 750 μg/mL) with or without MβCD in 0.2% BSA-DMEM for 8 h. Then WST solution was added to each well. After 2 h of exposure at 37°C, the absorbance was measured at 490 nm.

Oil Red O staining

To detect and quantify cellular lipid content, HepG2 cells were stained using the Oil Red O staining. Briefly, HepG2 cells were seeded at a density of 1×10^5 cells/well in 24-well plates and treated with 30 μM simvastatin or various concentrations of YMJT (250, 500, 750 μg/mL) with MβCD in 0.2% BSA-DMEM for 8 h. Then cells were gently washed with PBS and fixed using 10% formalin for 10 min at room temperature. Subsequently, cells were washed with PBS and 60% isopropanol, and stained for 1 h in a freshly diluted Oil Red O solution (stock solution, 3 mg/mL in isopropanol; working solution, 60% Oil Red O stock solution diluted in water). After staining, cells were washed with 60% isopropanol and PBS, and photographed. To quantify cellular lipids, Oil Red O stain was extracted using isopropanol and its absorbance was measured at 500 nm using a spectrophotometer.

Western blotting

Cells were cultured to 80% confluence at 37°C, incubated in 0.2% BSA-DMEM containing 20 μg/mL MβCD, and treated with 30 μM simvastatin or various concentrations of YMJT (250 and 500 μg/mL) for 8 h. Cell lysates were prepared using RIPA buffer containing protease inhibitors. Briefly, lysates were incubated at 4°C for 30 min and then centrifuged at 14,000 rpm for 15 min at 4°C to remove detergent-insoluble material.

Protein concentration was determined using a Bio-Rad protein assay. Protein samples (30-90 μg) were then separated on 4-15% Mini-Protean TGX Precast Gels and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 1% bovine serum albumin in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1% Tween 20). After blocking, the membrane was incubated with the following first antibodies: anti-HMGCR (C-1; 1:1,000), anti-SREBP2 (1C6; 1:1,000), anti-β-actin (C4; 1:1,000), anti-phospho-AMPKα (Thr172; 1:1,000), anti-AMPKα (1:1,000), anti-LDL Receptor (1:1,000) antibodies for overnight at 4°C with gentle shaking. Secondary antibodies included HRP-conjugated goat anti-mouse, goat anti-rabbit, and donkey anti-goat (1:10,000) antibodies, as appropriate. Protein bands were visualized by using an Immun-Star WesternC kit.

Statistical analyses

All of the experiments were performed in triplicate. Data are expressed as a mean ± standard deviation. Statistical analysis was performed using the SPSS software version 12.0 (SPSS, Inc., Chicago, IL) and data were evaluated for

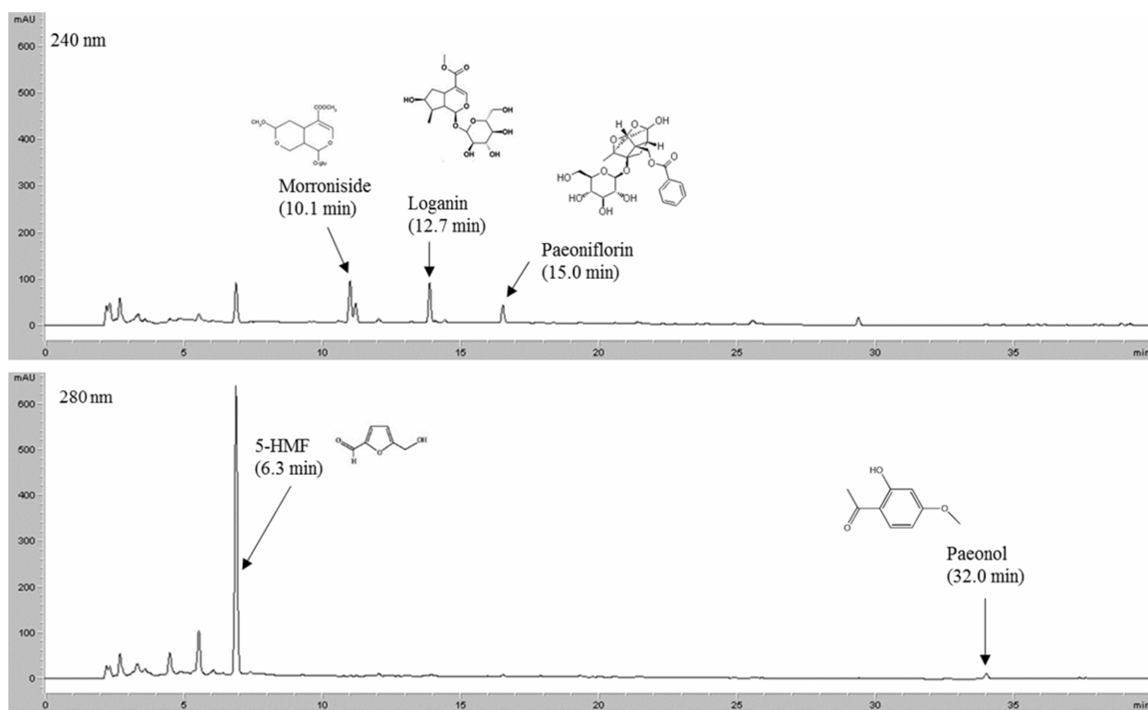

Figure 1. HPLC Chromatograms of YMJT aqueous extract and 5 reference standards of at 240 nm (morroniside, loganin, and paeoniflorin) and 280 nm (5-HMF, paeonol).

Table 1. Metabolic parameters in a rat model of menopausal hyperlipidemia

	Sham	OVX-Con	OVX-SV	OVX-YMJT (50)	OVX-YMJT (150)	OVX-YMJT (450)
Body weight gains (g)	96.44±4.8	117.34±1.9[a,*]	85.74±3.0[a,b]	70.00±1.9[b]	97.38±2.9[a,b]	102.6±2.9[b]
Liver weight (g)	7.673±0.413	17.510±1.351[a,*]	15.609±1.043[a]	16.061±0.735[a]	18.375±1.565[a]	18.540±1.554[a]
Retroperitoneal fat (mm)	119.03±8.56	217.96±18.08[a,*]	108.02±6.81[b]	84.69±6.89[b]	95.37±6.20[b]	93.57±6.46[b]
Peri-renal fat (mm)	89.4355±1.67	233.87±10.10[a,*]	100.37±11.64[b]	86.56±1.02[b]	75.76±8.08[b]	90.01±5.97[b]

Sham, sham-operated and not fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-Con, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-SV, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with simvastatin (20 mg/kg); OVX-YMJT, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with YMJT (50, 150, 450 mg/kg). After 8 weeks, body weight gains, and liver weights (g) and retroperitoneal fat and peri-renal fat sizes (mm) were measured. Values are expressed as means ± SD (n = 4-5). *Significantly different between the sham and OVX-Con at $P < 0.01$, (two-sample t-test). Values not sharing a common alphabet (a, b) as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

statistical significance using one-way ANOVA followed by a Duncan' test ($P < 0.05$). The OVX and Sham groups; control and MβCD groups were compared using two-sample *t*-tests ($P < 0.05$).

Results

HPLC analysis of reference compounds in YMJT

The reference standard of calibration curves for the 5-HMF, morroniside, loganin, paeoniflorin, and paeonol were y = 97.8022x + 372.6578 (R^2 0.998), y = 70.2673x + 65.4569 (R^2 0.999), y = 18.9613x + 64.4805 (R^2 0.999), y = 10.4226x + 22.6496 (R^2 0.999), and y = 70.2673x + 65.4569 (R^2 0.999), respectively. HPLC analysis of YMJT and reference standard mixtures was carried out at 240 and 280 nm. The retention time of each compound was 6.37 min (5-HMF), 10.17 min (morroniside), 12.75 min (loganin), 14.94 min (paeoniflorin), and 32.14 min (paeonol), The contents of each component in the YMJT aqueous extract were 5-HMF 3.36±0.022 mg/g, morroniside 1.81±0.049 mg/g, loganin 1.84±0.011 mg/g, paeoniflorin 1.73±0.008 mg/g, and paeonol 0.04±0.006 mg/g, respectively (**Figure 1**).

Table 2. Serum lipid levels in a rat model of menopausal hyperlipidemia

	Sham	OVX-Con	OVX-SV	OVX-YMJT (50)	OVX-YMJT (150)	OVX-YMJT (450)
T-CHO (mg/dL)	113.50±2.179	221.25±6.142^{a,**}	178.50±35.624^{b}	238.00±13.934^{a}	181.00±11.712^{b}	202.50±9.152^{a,b}
TG (mg/dL)	46.6±7.80	64.06±6.06^{a,*}	48.33± 3.21^{a,b}	74.67±15.3^{a}	64.33±7.75^{a}	36.00±12.83^{b}
HDL (mg/dL)	67.2±4.03	31.8±4.5^{a,**}	41.6±3.14^{c}	37.2±4.53^{a,b}	52.8±6.3^{c}	50.4±3.33^{c}
LDL (mg/dL)	37.52±3.83	176.64± 0.43^{a,**}	127.23± 31.842^{b}	186.32±11.907^{a}	115.56±10.856^{b}	145.20±8.037^{b}

Sham, sham-operated and not fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-Con, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-SV, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with simvastatin (20 mg/kg); OVX-YMJT, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with YMJT (50, 150, 450 mg/kg). After 8 weeks, the serum levels of TC, TG, HDL, and LDL were measured. Values are expressed as means ± SD (n = 3-5). *Significantly different between the sham and OVX-Con at $P < 0.01$, $^{**}P < 0.001$ (two-sample t-test). Values not sharing a common alphabet (a, b, c) as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

Effect of YMJT on the body weight and abdominal fat accumulation

We established a rat model of menopausal hyperlipidemia using OVX rats that were fed a high-fat, high-cholesterol diet. Body weight gains of all rats after 8 weeks of treatments increased in the rats from the OVX group (OVX-Con) compared with those from the Sham group (Sham) (**Table 1**). In the YMJT treated groups (OVX-YMJT 50, 150, 450 mg/kg), YMJT inhibited the OVX-induced weight gain. Food intake remained the same in all groups (data not shown). All rats were sacrificed at week 8, and the liver weights of each group were compared (**Table 1**). In the OVX-Con group, liver weights were significantly increased compared with the Sham group; however, no significant differences were evident in the OVX-SV and OVX-YMJT groups relative to the OVX-Con group.

We also measured overall fat volumes in retroperitoneal and peri-renal fat deposits (**Table 1**). The OVX-Con group significantly increased both retroperitoneal and peri-renal fat volumes compared with the Sham group. Furthermore, retroperitoneal fat volumes and peri-renal fat volumes in all OVX-YMJT groups and the OVX-SV group decreased equally.

Effect of YMJT on serum lipids

TC, TG, and LDL levels were markedly higher in the OVX-Con group compared with the Sham group (**Table 2**). In contrast, the OVX-Con group showed decreased HDL levels compared with the Sham group (**Table 2**). In the OVX-SV group and OVX-YMJT groups (except for the group treated with 50 mg/kg), OVX-induced changes in TC and LDL levels decreased. TG levels were altered only in the OVX-SV and 450 mg/kg OVX-YMJT groups. The OVX-SV group and OVX-YMJT groups exhibited increased HDL levels compared with the OVX-Con group (**Table 2**).

Effect of YMJT on the risk of arterial sclerosis

Arterial sclerosis is a medical condition in which fatty plaques build up along the interior wall of any artery in the body as a result of fat accumulation. Increased levels of total plasma cholesterol and obesity represent marked risk factors for atherosclerosis and increased rates of cardiovascular death [27, 28]. Because levels of fat accumulation in retroperitoneal and peri-renal spaces and serum cholesterol in menopausal hyperlipidemic rats were decreased by YMJT treatment (**Tables 1** and **2**), we next examined the effect of YMJT on the risk of arterial sclerosis by measuring the atherogenic index, cardiac risk factors, lumen diameter, and intima-media thickness (**Table 3**). The OVX-Con group showed a markedly increased atherogenic index and cardiac risk factor scores compared to the Sham group. The OVX-SV and OVX-YMJT groups (except for the group treated with 50 mg/kg) exhibited a lower atherogenic index. Moreover, cardiac risk factor scores were decreased in the OVX-SV and all OVX-YMJT groups relative to the OVX-Con group (**Table 3**). Lumen diameter and intima-media thickness were also determined. The OVX-Con group showed a lower overall lumen diameter than the Sham group. In the OVX-YMJT and OVX-SV groups, lumen diameter increased but not significantly. Intima-media thickness was increased in the OVX-Con group relative to the Sham group (**Table 3**). All OVX-YMJT groups had a decreased intima-media thickness, similar to that of the OVX-SV group (**Table 3**).

Table 3. Atherogenic index, cardiac risk factor, lumen diameter, and media thickness in a rat model of menopausal hyperlipidemia

	Sham	OVX-Con	OVX-SV	OVX-YMJT (50)	OVX-YMJT (150)	OVX-YMJT (450)
Atherogenic index	0.69±0.09	6.05±0.89[a,*]	3.31±0.80[b]	5.46±0.76[a]	2.46±0.406[b]	3.03±0.281[b]
Cardiac risk factor	1.69±0.09	7.05±0.89[a,*]	4.31±0.80[c]	6.46±0.76[b]	3.46±0.406[c]	4.03±0.281[b]
Lumen diameter (μm)	1931.25±2.86	847.78±55.50[a,**]	885.39±74.43[a]	940.72±95.35[a]	976.41±98.49[a]	994.92±121.69[a]
Intima media thickness (μm)	97.65±12.65	217.66±24.01[a,*]	118.62±11.93[b]	107.05±4.13[b]	127.48±21.83[b]	107.08±9.60[b]
Lumen diameter/Intima media thickness	20.00±2.26	3.75±0.16[a,*]	7.51±0.86[b]	8.8±0.83[b]	7.81±1.36[b]	9.34±1.15[b]

Sham, sham-operated and not fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-Con, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD); OVX-SV, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with simvastatin (20 mg/kg); OVX-YMJT, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with YMJT (50, 150, 450 mg/kg). After 8 weeks, their serum lipid levels were measured, followed by calculation of atherogenic index and cardiac risk factor scores (atherogenic index = (TC-HDL)/HDL, cardiac risk factor = TC/HDL). And lumen diameter and intima-media thickness were measured. Values are expressed as means ± SD (n = 4-5). [*]Significantly different between the sham and OVX-Con at $P < 0.01$, [**]$P < 0.001$ (two-sample t-test). Values not sharing a common alphabet (a, b, c) as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

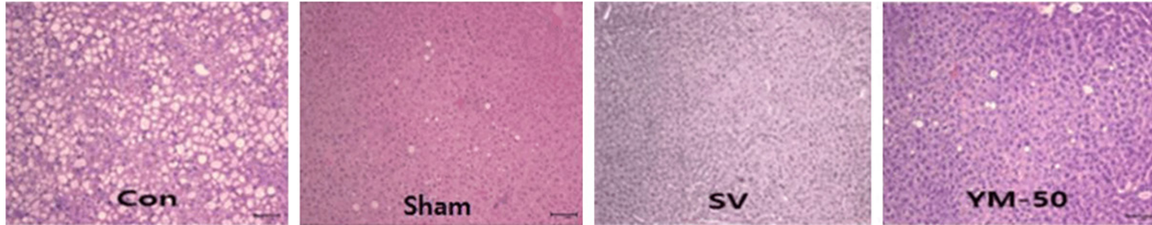

Figure 2. Histological analysis of livers in a rat model of menopausal hyperlipidemia. Sham, sham-operated and not fed a high-fat (HFD), high-cholesterol diet (HCD); Con, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD); SV, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with simvastatin (20 mg/kg); YM-50, OVX rats fed a high-fat (HFD), high-cholesterol diet (HCD) supplemented with YMJT (50 mg/kg). After 8 weeks, livers were extracted and stained with H&E (×100 magnification).

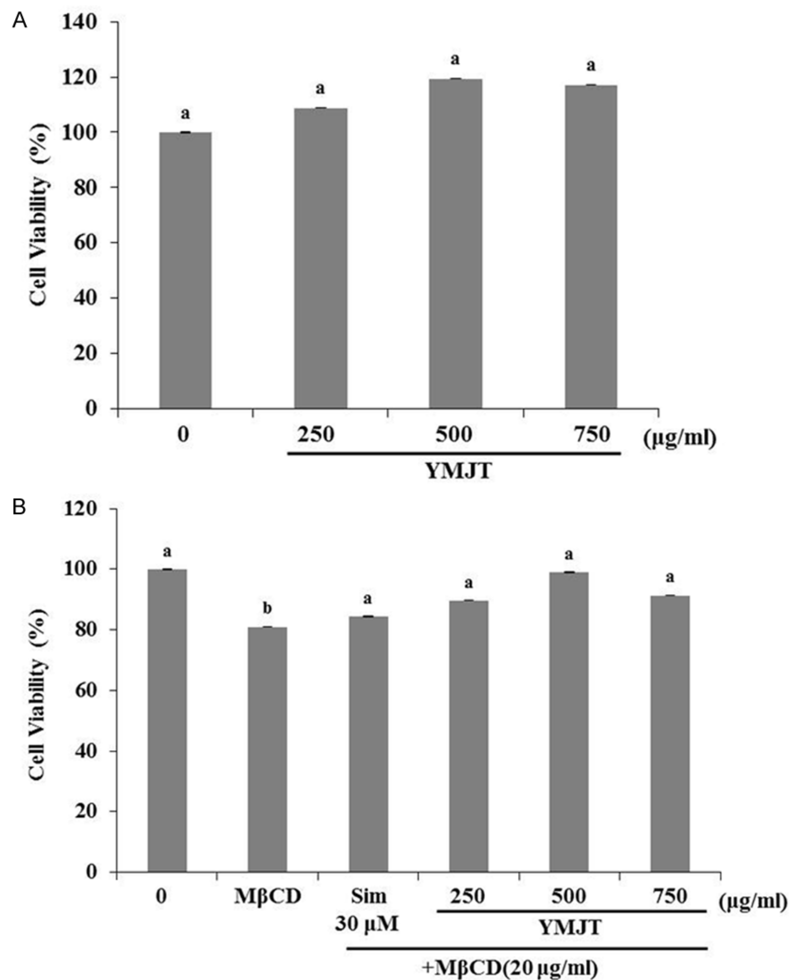

Figure 3. Cytotoxicity of MβCD and YMJT in HepG2 cells. HepG2 cells were treated with several concentrations of YMJT (250, 500, 750 μg/mL) for 8 h in 0.2% BSA-DMEM, and the cell viability was determined using WST assay (A). HepG2 cells were treated with 20 μg/mL MβCD and 30 μM simvastatin or several concentrations of YMJT (250, 500, 750 μg/mL) with 20 μg/mL MβCD for 8 h in 0.2% BSA-DMEM, and the cell viability was determined using WST assay (B). The data were mean ± SD from three samples for each group. Values not sharing a common alphabet as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

YMJT ameliorates hepatic steatosis of menopausal hyperlipidemic rats fed a high-fat, high-cholesterol diet

Obesity, diabetes, and hyperlipidemia are important risk factors for NASH; patients with fatty liver disease or an excessive accumulation of fat in liver are more likely to develop NASH [3, 8]. NASH is characterized by the presence of steatosis, lobular inflammation, and hepatocellular ballooning [29]. We examined the effect of YMJT on NASH development by histological analysis of the liver. In the OVX-Con group, a greater number of fat vacuoles were observed compared to the Sham group. However, both the OVX-SV and OVX-YMJT groups had fat accumulation levels similar to those of the Sham group (**Figure 2**).

YMJT has no toxicity against HepG2 cells

Because YMJT showed positive effects on improving hepatic steatosis, the effect and cellular mechanisms of YMJT on hepatic lipid accumulation in HepG2 he-

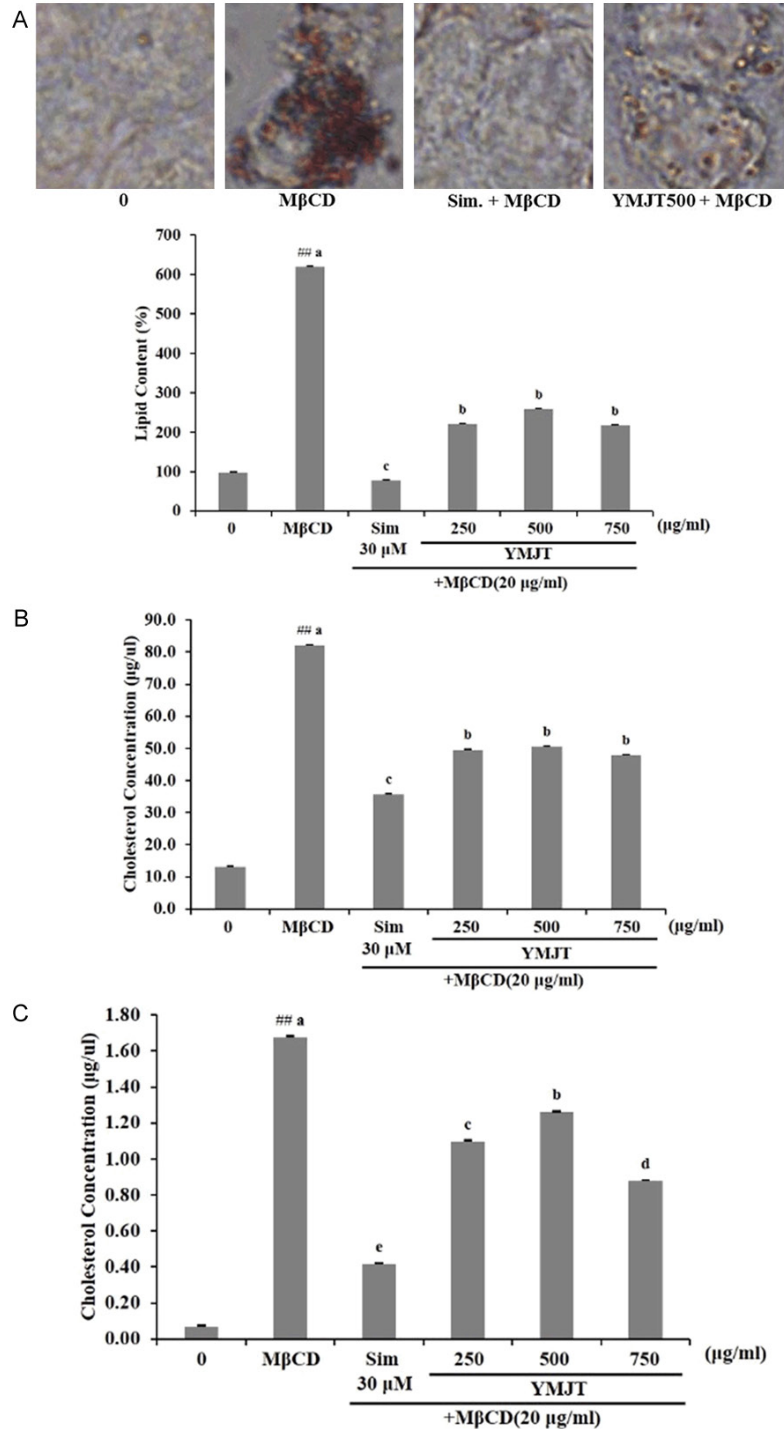

Figure 4. Effects of YMJT on lipid accumulation, total cholesterol, and LDL/VLD levels in HepG2 cells. HepG2 cells were treated with 30 μM simvastatin or several concentrations of YMJT (250, 500, 750 μg/mL) with 20 μg/mL MβCD for 8 h in 0.2% BSA-DMEM. Lipid accumulation was visualized by Oil Red O staining (×600 magnification) and quantitative analysis of lipid deposition in cells were analyzed by spectrophotometer (A). Total intracellular cholesterol (B) and LDL/VLDL (C) levels were measured by ELISA. The data were mean ± SD from three samples for each group. ##Significantly different between the control and MβCD treatment at $P < 0.01$ (two-sample t-test). Values not sharing a common alphabet as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

patocellular carcinoma were examined. We induced steatosis by MβCD. A WST assay was performed to evaluate the cytotoxic effect of MβCD, YMJT, and simvastatin on the viability of HepG2 cells. As shown in **Figure 3A**, YMJT exhibited no cytotoxic effects at any of the concentrations tested (250, 500, 750 μg/mL). Treatment of HepG2 cells with MβCD only resulted in a slight inhibition of cell growth (**Figure 3B**), but the addition of MβCD to cells cotreated with YMJT (250, 500, 750 μg/mL) or simvastatin (30 μM) was found to be non-toxic at the concentrations tested (**Figure 3B**).

YMJT inhibits cellular lipid accumulation in HepG2 cells

To evaluate the effects of YMJT on MβCD-induced lipid accumulation, HepG2 cells were treated with various concentrations of YMJT for 8 h. Simvastatin was used as the positive control. After treatment, cells were stained with Oil Red O and quantified by measuring the absorbance at 500 nm. A significant increase in lipid deposition was observed in HepG2 cells treated with MβCD; however, this effect was attenuated in YMJT- and simvastatin-treated cells (**Figure 4A**). This suggested that YMJT has inhibitory effects on MβCD-induced intracellular lipid accumulation in HepG2 cells. To confirm this result, TC and LDL/VLDL levels were quantified (**Figure 4**B and **4C**). At all concentrations of YMJT, the total

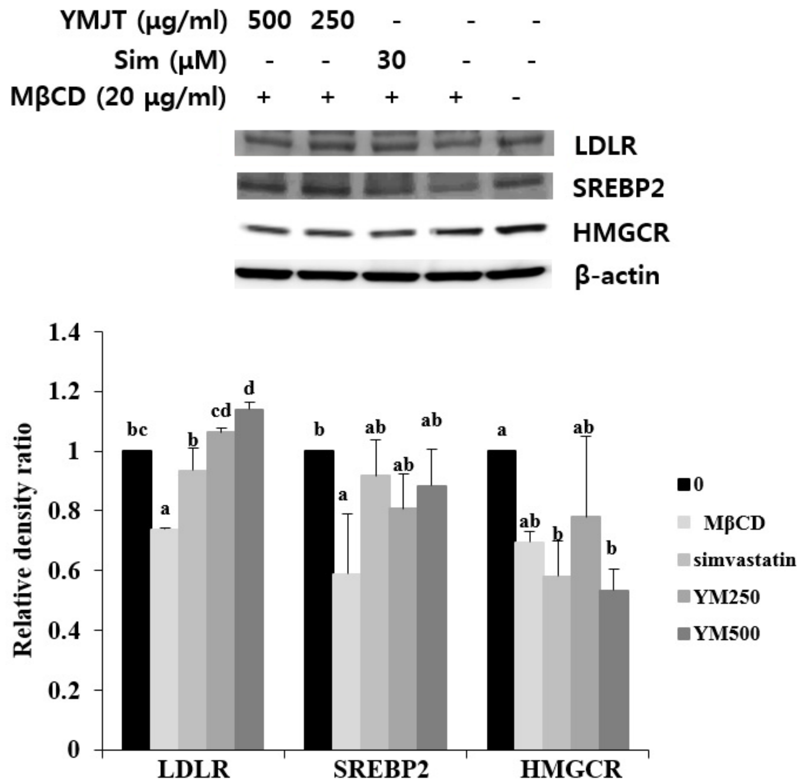

Figure 5. Effects of YMJT on cholesterol synthesis in HepG2 cells. HepG2 cells were treated with 30 μM simvastatin or YMJT (250, 500 μg/mL) with 20 μg/mL MβCD for 8 h in 0.2% BSA-DMEM. Cell lysates were then harvested by RIPA buffer and subjected to Western blotting analysis for SREBP2, HMGCR, and LDLR protein expression. Quantified data of protein levels indicates in lower panel. The values of density of proteins were all justified with β-actin. The relative density ratios of untreated cells were set at a value of 1.0. Values not sharing a common alphabet as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

cholesterol levels were significantly inhibited. In addition, the LDL/VLDL levels were lower in these cells (**Figure 4C**).

YMJT regulates cholesterol synthesis in HepG2 cells

In HepG2 cells, we identified the mechanisms and confirmed to regulate cholesterol synthesis. Cholesterol metabolism is regulated by the transcription factor SREBP2 [30], which directly regulates the transcription of important genes involved in cholesterol irritation or biosynthesis, such as HMGCR and LDLR.

We evaluated the effects of YMJT on the protein levels of the SREBP2, HMGCR, and LDLR in HepG2 cells. In MβCD-treated cells, LDLR and SREBP2 protein levels were diminished. However, they could be rescued to nearly wild-type levels following YMJT treatment (**Figure 5**). In contrast, HMGCR protein levels were decreased by treatment with MβCD or YMJT (**Figure 5**). These results show that YMJT decreased lipid accumulation through the regulation of genes involved in cholesterol synthesis.

YMJT stimulates AMPK phosphorylation in HepG2 cells

AMPK is thought to function as a metabolic master switch in response to changes in cellular energy and plays a key role in regulating fat metabolism in the liver [31, 32]. Therefore, as a marker of AMPK activity, the phosphorylation of AMPK was measured. AMPK phosphorylation was significantly increased by YMJT compared with MβCD alone (**Figure 6**). This suggests that YMJT attenuated hepatic lipid accumulation through AMPK activation.

Discussion

Menopause is well-known to an increased prevalence of a metabolic syndrome and were shown to increase the risk of cardiovascular disease and so on [33]. Progression of NASH occurs frequently in postmenopausal females [5, 6], with the accelerated accumulation of visceral fat also being common in these patients [34, 35]. Recent studies have suggested that cholesterol-lowering drugs may slow the progression of NASH. Statins have been shown to slow the progression of NASH in ovariectomized mice fed a high-fat and high-cholesterol diet [5], while atorvastatin was found to be effective in treating NASH [36, 37]. It is reported that the progression of atherosclerosis and dyslipidemia increases after menopause and estrogen administration can improve these symptoms [38, 39]. Estrogen decreased serum cholesterol levels and ameliorated steatohepatitis progression in mice [4], and protected women with

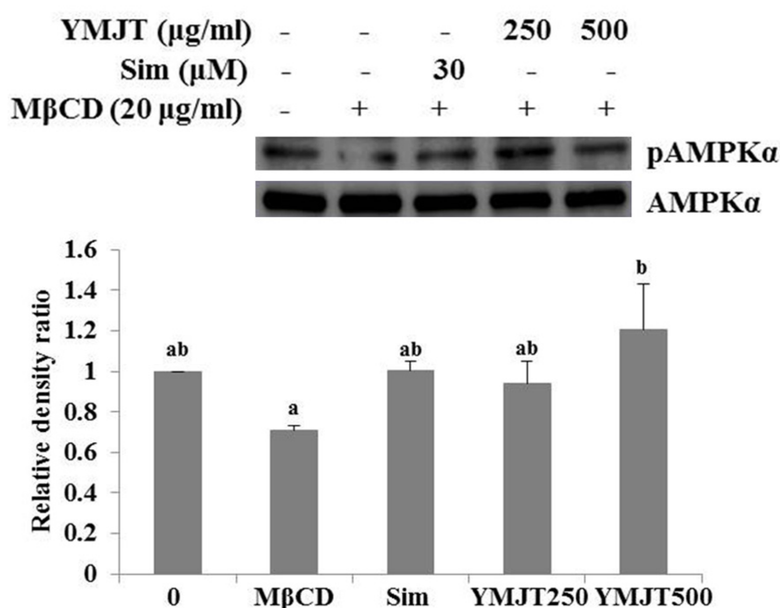

Figure 6. Effects of YMJT on AMPK phosphorylation in HepG2 cells. HepG2 cells were treated with 30 μM simvastatin or YMJT (250, 500 μg/mL) with 20 μg/mL MβCD for 8 h in 0.2% BSA-DMEM. Cell lysates were then harvested and subjected to Western blotting analysis for AMPK phosphorylation (pThr-172-AMPK). Quantified data of protein levels indicates in lower panel. The values of density of proteins were all justified with β-actin. The relative density ratios of untreated cells were set at a value of 1.0. Values not sharing a common alphabet as superscripts are significantly different from each other at the level of $P < 0.05$ (ANOVA followed by Duncan's test).

NASH from severe liver fibrosis. An aromatase knockout mouse, which cannot synthesize endogeneous estrogen, developed hepatic steatosis [40]. Thus estrogens are important regulators of lipid homeostasis and estrogen is often prescribed to treat the NASH that is associated with menopause. Because estrogens are a fundamental driving force in the development of breast cancer [41], estrogen administration could be associated with an increased risk of developing breast cancer as one of its side effects.

The use of traditional herbal medicines to prevent and treat a variety of diseases has generated considerable interest. Recently, several effects of YMJT have been demonstrated scientifically. For example, YMJT prevents and treats the diseases related to the kidney [42]. YMJT has regenerative effects on injured peripheral nerve fibers [43]. Antioxidative effects of YMJT in the liver of a senescence-accelerated mouse have been reported [44]. According to Traditional Korean Medicine (TKM), menopausal symptoms are associated with a decline in kidney energy [1], and YMJT has been used to improve the function of the kidney. Moreover, YMJT decreased serum cholesterol and triglyceride in rat models of diabetes [25]. YMJT can regulate glucose and lipid metabolism in rat models of diabetes [45]. In obese Zucker rats, Yukmijihwangtang-Jahage improved the lipid profile [46]. Therefore, we evaluated the therapeutic effects of YMJT on lipid related diseases of menopausal hyperlipidemia by utilizing at in vitro and in vivo model.

We performed a quantitative analysis of 5 reference standards (morroniside, loganin, paeoniflorin, 5-HMF, paeonol) in YMJT using a HPLC. Identifying the major components is of great importance because their origin is relevant to the effects of the herbal medicine.

In Korea, some traditional prescription or herbal products are used to manage metabolic syndrome during menopause. In our previous report, Palmiwon showed therapeutic effects against metabolic syndrome as measured by weight gain and a decrease in the lipid profile in OVX rats [26]. As shown in this report, YMJT will be therapeutic and protective effects against the metabolic syndrome during menopause. We think YMJT has effects not only on one symptom but also on multiple targets involved in lipid related diseases.

This study was done to measure the effects of YMJT on targets associated with lipid factors, including retroperitoneal fat, peri-renal fat, and serum lipid levels. YMJT reduced retroperitoneal and serum TC, TG, and LDL levels, the atherogenic index, cardiac risk factors, and the intima-media thickness. YMJT was resulted to improve NASH and increase the lumen diameter and HDL levels to those similar to the positive controls. These results show that YMJT can improve symptoms caused by menopausal hyperlipidemia.

In previous studies, some natural products were identified to manage metabolic syndrome during menopause [47]. YMJT has a therapeutic and protective effect against metabolic syndrome related to cholesterol during menopause. Dietary cholesterol in NASH is an important factor both in rodents and humans [5]. In the present study, YMJT ameliorated NASH by reducing serum cholesterol levels.

Some reported studies, menopausal symptoms were reported a relationship between the intake of natural products. Linum Usitatissimum (Linseed) Seed Oil, a prescribed dose from 40 to 50 mg/day, was resulted to have cholesterol-lowering properties in postmenopausal women [48, 49]. An isoflavone(genistein, daidzein, formononetin, and biochanin) from red clover increased HDL and decreased apolipoprotein B levels when ingested the prescribed doses of 28.5, 57, or 85.5 mg/day in postmenopausal women [50]. The intake of 90 mg/day of Isoflavone (1:1:0.2 genistein: daidzein: glycitein) improved in postmenopausal women with hypercholesterolemia [51]. Dehydroepiandrosterone at a dose of 25 mg/day showed improved lipid patterns [52]. Determining whether YMJT could improve cholesterol related disease in postmenopausal females will be a subject addressed by our future research.

In addition, we performed an experiment using the MβCD-induced hepatic steatosis model in HepG2 cells in order to examine the cholesterol-decreasing effect of YMJT. We determined that MβCD treatment induced a significant increase in lipid accumulation, TC and LDL/VLDL levels, all of which were reduced following treatment with YMJT (**Figure 4**). These results show that YMJT exerts cholesterol-lowering effects on HepG2 cells.

The SREBP-2 regulates the expression of many genes involved in cholesterol synthesis and uptake. Activation of SREBP2 is dependent on the cholesterol status of the cell [14, 53]. MβCD increased cholesterol levels and caused of the down-regulation of the effector proteins SREBP2, LDLR, and HMG-CoA. Co-treatment with YMJT and MβCD decreased cholesterol levels, bring in increased expression of SREBP2 and LDLR (**Figure 5**). There are some reports proposing that the up-regulation of either SREBP2 or LDLR is responsible for appreciated cholesterol levels. The green alga *Haematococcus pluvialis* shows a hypocholesterolemic result through the upregulation of LDLR expression [54]. Also, Resveratrol has anti-atherogenic effects and increases the activity of LDLR in hepatocytes through the activation of SREBPs [55]. Our result, in contrast with the normal regulatory response, the expression of HMGCR was decreased (**Figure 5**).

The pattern of protein expression of hepatic LDLR and SREBP2 was very similar to our previous study [26]. This study demonstrated that YMJT could reduce body weight and improve the blood lipid profile. In HepG2 cells, YMJT regulated the expression of SREBP2, suggesting that the effects of YMJT on the lipid profile might also be linked to the SREBP2 pathway. We used simvastatin as a positive control. This drug is known to suppress HMGCR activity [56]. The data shown here suggest that the response to YMJT was very similar to that of simvastatin in terms of decreasing cholesterol synthesis in HepG2 cells exposed to MβCD.

Increased AMPK phosphorylation was also observed following YMJT treatment (**Figure 6**). AMPK has become the focus of many recent studies as a therapeutic target of metabolic disease and as a central regulator of lipid metabolism pathways [57-59]. Based on its role, AMPK has appeared as a good target for the treatment of fatty liver disease. In this study, YMJT might also regulate cholesterol synthesis via AMPK phosphorylation [32, 58, 60, 61]. Overall, the present in vitro data suggest that inhibition of lipid synthesis on MβCD-induced HepG2 cells by YMJT blocks the progression of hepatocyte steatosis. Therefore, YMJT might negatively regulate the lipid metabolism by activating AMPK phosphorylation.

YMJT is a TKM formula that is utilized when a patient has been determined as having inadequate kidney and liver yin. Patients exhibiting kidney and liver Yin insufficiency have been used to supply energy. YMJT has been supported from: flushed or red face, headaches, hot flashes, night sweats, nocturnal emission, uneasiness or mental unrest. This is the aspect of the symptoms of menopause which we must consider. In this viewpoint, we examined the effects of cholesterol related disease. YMJT was showed almost the same effect as Palmiwon without relation on yin or yang [26].

We think that YMJT is a novel prescription medicine that can improve multiple lipid-associated factors in menopause. Further studies are required to determine the significance of the up-regulation of AMPK phosphorylation by YMJT.

Conclusions

In conclusion, we have shown that YMJT may be used as a novel agent to treat or prevent high-cholesterol and high-fat-induced cholesterol related disease during menopause. Our results also support the view that the anti-steatosis effects of YMJT may be attributed to the regulation of the cholesterol synthesis pathway and activation of AMPK.

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Disclosure of conflict of interest

None.

Address correspondence to: Byoung Seob Ko. Korean Medicine Convergence Research Division, Korea Institute of Oriental Medicine, Republic of Korea. Tel: +82-42-868-9542; Fax: +82-42-868-9293; E-mail: bsko@kiom.re.kr

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