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Integrative Medicine Research

journal homepage: www.imr-journal.com

Original Article

Effects of three different formulae of Gamisoyosan on lipid accumulation induced by oleic acid in HepG2 cells

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ARTICLE INFO

Article history:
Received 17 July 2017
Received in revised form
21 August 2017
Accepted 24 August 2017
Available online 1 September 2017

Keywords:
fatty liver
Gamisoyosan
menopause
phytoestrogen

ABSTRACT

Background: Gamisoyosan (GSS) is an herbal formula which has been used to treat women's diseases for several hundred years in Korea. GSS is one of the three most common prescriptions among women and is used to treat menopausal symptoms. Fatty liver disease is also common in postmenopausal women and can precede more severe diseases, such as steatohepatitis. The present study compared the effects of GSS on fatty liver using three different formulae, Dongui-Bogam (KIOM A), Korean Pharmacopeia (KIOM B) and Korean National Health Insurance (KIOM C).

Methods: In oleic acid-induced HepG2 fatty liver cells, cellular lipid accumulation, triglycerides and total cholesterol were measured after treatment with three GSS formulae and simvastatin as a positive control. To investigate the phytoestrogen activity of GSS, MCF-7 cells were treated with GSS, and hormone levels were quantified. Also, qualitative analysis was performed with UPLC.

Results: All types of GSS decreased cellular lipid accumulation. KIOM A was slightly less effective than the other two GSS formulae. KIOM B and KIOM C decreased cellular triglycerides more effective than simvastatin, but KIOM A did not affect cellular triglycerides. Cellular total cholesterol was decreased by all GSS and simvastatin. GSS showed phytoestrogen activity in MCF-7 cells. From the UPLC analysis data, geniposide, paeoniflorin and glycyrrhizin were detected form three GSS formulae.

Conclusion: These results suggest that all GSS formulae have a beneficial effect on fatty liver disease during menopause and that differences of formula have no effect on the efficacy of the prescription.

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https://doi.org/10.1016/j.imr.2017.08.004

1. Introduction

Gamisoyosan (GSS), a traditional herbal formula comprising 12 different herbal medicines, has been used in Korea to treat dysmenorrhea, insomnia, and anxiety. GSS is an herbal formula which has been used to treat women's diseases for several hundred years in Korea. GSS is one of the three major women's prescriptions and is used to treat women's menopausal symptoms. GSS has emerged as the most commonly used formula for treating menopausal symptoms in Korea, Japan, and China.[1]

Hormone balance in menopause is important for each individual. During menopause, the function of the ovaries ceases, causing hormone imbalances such as estrogen deficiency and follicle stimulation hormone (FSH) increase. Because of these hormone imbalances, menopausal symptoms including hot flushes, vaginal and urinary symptoms, and sweating can be induced.

According to oriental medicine, menopause symptoms may be caused by energy deficiency in the kidney or liver.[2–4] Especially for women, the liver's role is crucial to maintain regular blood flow and good menstrual condition, because the liver makes blood and emotion flow smoothly. In clinical application, GSS is one of the Chinese medicine formulations frequently used for management of menopausal symptoms.[3] Because GSS has the ability to promote liver qi (気) and modulate vital energy flow and blood flow, it has been frequently prescribed to patients who are easily fatigued and are inclined to have psychoneurotic symptoms including irritability and anxiety.[2] For example, according to a visual analogue scale score-based investigation, GSS relieved both vasomotor and psychological symptoms in patients with psychological symptoms.[5] GSS reduced sleep disturbance, headache and dizziness in peri- and postmenopausal women.[6]

Dysfunctional lipid metabolism can lead to several metabolic diseases, including visceral obesity, hypertension, hyperlipidemia and type 2 diabetes. Due to estrogen actions that positively regulate lipid metabolism and lead to the accumulation of subcutaneous fat rather than central fat, premenopausal women are protected from these metabolic diseases.[7] Loss of estrogen following menopause worsens lipid metabolism and is associated with an increased risk for these metabolic diseases, which can promote the development of other serious diseases, including atherosclerosis, cardiac infarction, apoplexy and fatty liver.[8,9] Nonalcoholic fatty liver disease (NAFLD) is a type of fatty liver disease that is caused without significant alcohol consumption. NAFLD occurs when fat builds up excessively in the liver; a higher prevalence of NAFLD is observed following menopause. Several studies have shown an association between menopause and NAFLD.[10,11] Furthermore, NAFLD is twice as common in postmenopausal women compared to premenopausal women.[12] The protective effect of estrogen against the development and progression of NAFLD has been suggested by studies using hormonal replacement therapy (HRT) on postmenopausal women.[13] In addition, NAFLD can progress from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocarcinoma, which are associated with cardiovascular and liver-related mortality. NASH was worsened by estrogen deficiency, and this effect was ameliorated after estrogen therapy in ovariectomized (OVX) mice.[14] Although GSS has been used for the treatment of menopausal symptoms, there are no reports about its effects on fatty liver disease during menopause. Therefore, we investigated the effects of GSS on fatty liver induced by oleic acid (OA) in HepG2 cells.

Phytoestrogens are naturally occurring plant substances that show estrogen-like activities in the body. A wide variety of food contains phytoestrogens such as coumestans, isoflavones and lignans.[15,16] Because of their similar conformation to estrogen, phytoestrogens bind to the mammalian Estrogen Receptor (ER) and exert the agonist or antagonist effects of estrogens via the ER in animals and humans. There are some reports that phytoestrogens have protective effects against several diseases, including cardiovascular disease, osteoporosis, menopausal symptoms, and hyperlipidemia .[17,18]

In this study, three different formulae of GSS, Donguibogam (KIOM A), Korean Pharmacopeia (KIOM B) and Korean National Health Insurance herbal medicine (KIOM C) were used. Among these three formulae, KIOM B and KIOM C have been mainly used for treatment of menopausal symptoms in clinical settings. KIOM A is the original recipe from Donguibogam. The three formulae have different ingredients and dosages. KIOM C has the same composition as KIOM B except for the excipient. We also wanted to know whether there were any differences in effects between these three formulae. In this report, we describe a comparative study of three different formulae of GSS regarding fatty liver improvement and phytoestrogen activity. Further, to investigate the chemical change in the compositions of GSS formulae, we analyzed the indicator components by Ultra Performance Liquid Chromatography (UPLC) qualitative analysis.

2. Methods

2.1. *General Procedures*

Geniposide was purchased from Sigma-aldrich (St. Louis, MO, USA), paeoniflorin was purchased from Wako Chemical (Osaka, Japan), nodakenin was purchased from Chemfaces (Wuhan, China) and glycyrrhizin was purchased from Ministry of Food and Drug Safety (Osong, Korea). All chemical compounds were identified with purities of ≥98%. The stock solutions of four chemicals were prepared at concentrations of 0.1 mg/mL in 80% methanol (MeOH) and 20% distilled water. The mixed standard working solutions were diluted with methanol to get a final concentration of 0.025 mg/mL. The working solutions were stored at +4°C prior to analysis. Analytical grade acetonitrile (ACN), MeOH and water were purchased from J. T. Baker (Philipsburg, NJ, USA). Extra pure grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). UPLC was performed on Agilent UPLC system equipped with a quaternary pump (G4220B), autosampler (G4228A), DAD (G4212A) and column oven (G1316A). The instrument control and data processing were carried out by an Agilent ChemStation software system (Aglient, Santa Clara, CA, USA).

2.2. Materials and Extracts of GSS formula

Among the three formulae of GSS, KIOM A and KIOM B were purchased from the Baekje medicinal herb store. KIOM C was purchased from Hankook Shinyak Corp. (Nonsan, Korea). All herbs of the three formulae were authenticated by a morphological expert, Dr. Gi Jung Kil at Joongbu University. Herbs were extracted with hot water. Then, they were mixed and extracted by heating (100°C) for 2 hours in a 10-fold volume of water. After filtration, the extracts were evaporated and lyophilized. The extracted authenticated samples were stored at KIOM (Daejeon, Korea) until use in this experiment. The composition of the three GSS formula and their chemical ingredients are shown in Table 1.

2.3. UPLC analysis condition

To assess the chemical compositions of the extracts using UPLC, a Kinetex XB-C_{18} (2.6 μm, 100 mm × 4.6 mm i.d.) column from Phenomenex (Torrance, CA, USA) was employed to compare the chromatographic patterns and chemical ingredients. The peaks of all three samples could be eluted efficiently and simultaneously by a mixture of ACN (A) and water (B). Furthermore, the addition of 0.1% formic acid to the water provided improved peak shapes. A gradient elution of A/B (v/v) = 10/90 (0 min) → 18/82 (10 min) → 21/79 (20 min) → 70/30 (30 min) → 100/0 (35 min) → 100/0 (45 min) with a flow rate of 1 mL/min at 30 °C showed optimal separation performance. Among the various wavelengths from DAD, 254 nm exhibited comprehensive absorption of all separated peaks and allowed the selection of four standards from the samples.

2.4. Sample Preparation for UPLC

The three prepared GSS samples (100 mg) were transferred into a 100 mL vial, dissolved in 10 mL of distilled water and sonicated for 10 min. After centrifugation for 10 min at 4,000 rpm, the supernatant was diluted to 0.5 mg/mL and filtered using a disposable syringe filter unit (0.2 μm, Dismic-25JP, Advantec) prior to injection into the UPLC system. The chromatographic qualitative analysis of the sample solutions was compared by their index component peak retention times and the co-injection method.

2.5. HepG2 cell culture and treatment

Human hepatocellular carcinoma HepG2 cells were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) (Hyclone, Inc., South Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Inc., South Logan, UT) and were maintained in a humidified incubator at 37 °C under an atmosphere of 5% CO_2. Prior to GSS treatment, the medium was replaced with phenol red-free DMEM supplemented with dextran-coated choral-stripped FBS for 24 hours. To induce fatty liver, HepG2 cells were exposed to 400 μM OA for 6 hours. To determine the effect of simvastatin and GSS on OA-induced HepG2 fatty liver, cells were treated with 10 μM simvastatin or 100 μg/mL GSS in 0.2% BSA-DMEM for 24 hours before treatment with 400 μM OA. Simvastatin was activated prior to use with NAOH.

2.6. MCF-7 cell culture and treatment

Breast cancer MCF-7 cells were purchased from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI1640 media supplemented with 10% FBS (Hyclone, Inc., South Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Inc., South Logan, UT). It was maintained in a humidified incubator at 37°C under an atmosphere of 5% CO_2.

2.7. Cell viability assay

Cell viability was examined using the EZ-CYTOX cell viability assay kit (DoGenBio Co., Ltd., Dogen, Seoul, Korea). EZ-CYTOX is based on enzyme-based methods using highly water soluble tetrazolium salts (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) (WST). WST produce water soluble formazans by mitochondrial dehydrogenases in viable cells. The amount of formazan product produced by the addition of WST correlated to the number of viable, metabolically active cells in the culture. By measuring the formazan level in the cells, the cell number can be determined. To measure cell viability, HepG2 cells were seeded at a density of 1×10^5 in 48 well plates. After treatment, the medium was removed and 200 μL PBS containing WST solution of one tenth of the total volume was added to each well and incubated for 1 h at 37°C. Then, the absorbance was measured at 450 nm.

2.8. Oil Red O staining and lipid droplets analysis by cell imaging

To measure total intracellular lipid content, HepG2 cells were stained by the Oil Red O (ORO) method. Briefly, after treatments, cells were washed three times with PBS and fixed with 10% formalin for 1 hour. After fixation, cells were washed with 60% isopropanol and the cells were stained for 1 hourh in a freshly filtered ORO solution (Sigma-Aldrich). After staining, the cells were washed with 70% ethanol once and PBS three times. The image of each group was photographed. For quantitative analysis of cellular lipids, isopropanol was added to each sample shaken at room temperature for 10 min. The extracted dye was removed by gentle pipetting and its absorbance was monitored by spectrophotometer at 500 nm.

2.9. Triglyceride and total cholesterol quantification

Triglyceride (TG) and total cholesterol (TC) contents in HepG2 cells were determined by TG and TC quantification kit (ASAN Pharm, Co., Ltd, Seoul, Korea). Briefly, after treatment, lipids of cells were extracted with chloroform: isopropanol: tween-20 (7:11:0.1) by vortex. Spin the extract 10 min at 15,000 *g* in a centrifuge. Transfer all of the liquid avoiding the pellet, to a new tube, dry at 50°C to remove chloroform. Dissolve dried lipids with 150 μL of assay buffer by vortex until homogeneous. TG and TC levels were measured spectrophotometrically at 510 and 500 nm. Results were normalized to protein concentration.

Table 1 – Composition of three different formulae of Gamisoyosan.

	Gamisoyosan (GSS)					
No.	KIOM A (Dongui-bogam/ 東醫寶鑑	Contents (%)	KIOM B (Taepyeonghyem inhwajegukbang/ 太平惠民和劑局方))	Contents (%)	KIOM C (Korean National Health Insurance)	Contents (%)
1	*Paeonia lactiflora* Pallas	12.50	*Paeonia lactiflora* Pallas	13.04	*Paeonia lactiflora* Pallas	3.39
2	*Angelica gigas* Nakai	10.00	*Angelica gigas* Nakai	13.04	*Angelica gigas* Nakai	3.39
3	*Poria cocos* Wolf	10.00	*Poria cocos* Wolf	13.04	*Poria cocos* Wolf	3.39
4	*Atractylodes macrocephala* Koidzumi	12.50	*Atractylodes macrocephala* Koidzumi	13.04	*Atractylodes macrocephala* Koidzumi	3.39
5	*Gardenia jasminoides* Ellis	5.00	*Gardenia jasminoides* Ellis	8.74	*Gardenia jasminoides* Ellis	2.27
6	*Glycyrrhiza uralensis* Fischer	2.50	*Glycyrrhiza uralensis* Fischer	8.74	*Glycyrrhiza uralensis* Fischer	2.27
7	*Anemarrhena asphodeloides* Bunge	10.00	*Bupleurum falcatum* Linne	13.04	*Bupleurum falcatum* Linne	3.39
8	*Lycium chinense* Miller	10.00	*Paeonia suffruticosa* Andrews	8.74	*Paeonia suffruticosa* Andrews	2.27
9	*Liriope platyphylla* Wang et Tang	10.00	*Zingiber officinale* Roscoe	4.30	*Zingiber officinale* Roscoe	1.12
10	*Rehmannia glutinosa* Liboschitz var. *purpurea* Makino	10.00	*Mentha arvensis* Linne var. piperascens Malinvaud ex Holmes	4.30	*Mentha arvensis* Linne var. piperascens Malinvaud ex Holmes	1.12
11	*Phellodendron amurense* Ruprecht	5.00			Excipient	74.00
12	*Platycodon grandiflorum* A. De Candolle	2.50				

2.10. *Hormone quantitation*

Estradiol concentrations of GSS were quantified using a commercial homogenous time-resolved fluorescence (HTRF) kit (CISBIO, France), and according to the instruction manual. The absorption was measured at 665 nm with a reference wavelength of 620 nm.

2.11. *Statistical analysis*

Statistical analysis was performed using Prism software version 7.0 (GraphPad software Inc., San Diego, CA, USA). Values are presented as the mean ± SD ($n \geqq 3$). Statistical significance of group differences was determined using an analysis of variance followed by Tukey's post hoc test. Control and OA groups were compared using t-tests. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. *GSS has no toxicity to cell viability*

The cytotoxicity of GSS to HepG2 cells in the presence of OA was determined using a WST assay. As shown in Fig. 1, we found that GSS showed no inhibition of HepG2 cell viability in the presence of OA.

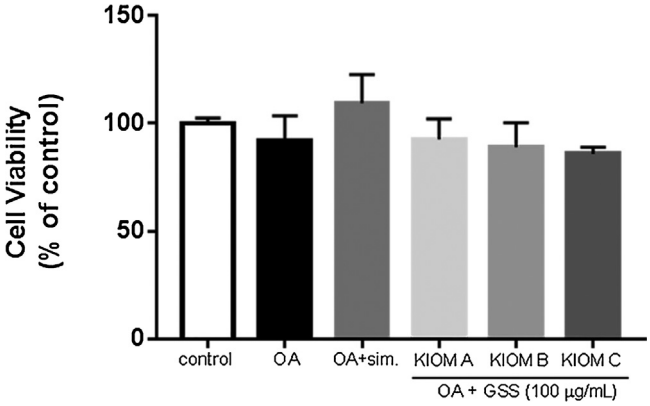

Fig. 1 – Effects of GSS on cell viability in the presence of OA in HepG2 cells. sim., simvastatin; OA, oleic acid; GSS, Gamisoyosan.

3.2. *GSS decreases lipid accumulation in OA-induced fatty liver*

To investigate the effect of GSS on fatty liver and compare the effects of three different formulae of GSS on fatty liver, we monitored intracellular lipid droplets after GSS and simvastatin treatment in OA-induced HepG2 fatty liver using ORO staining. As shown in Fig. 2, treatment with all GSS formulae decreased lipid accumulation. In comparison to simvastatin,

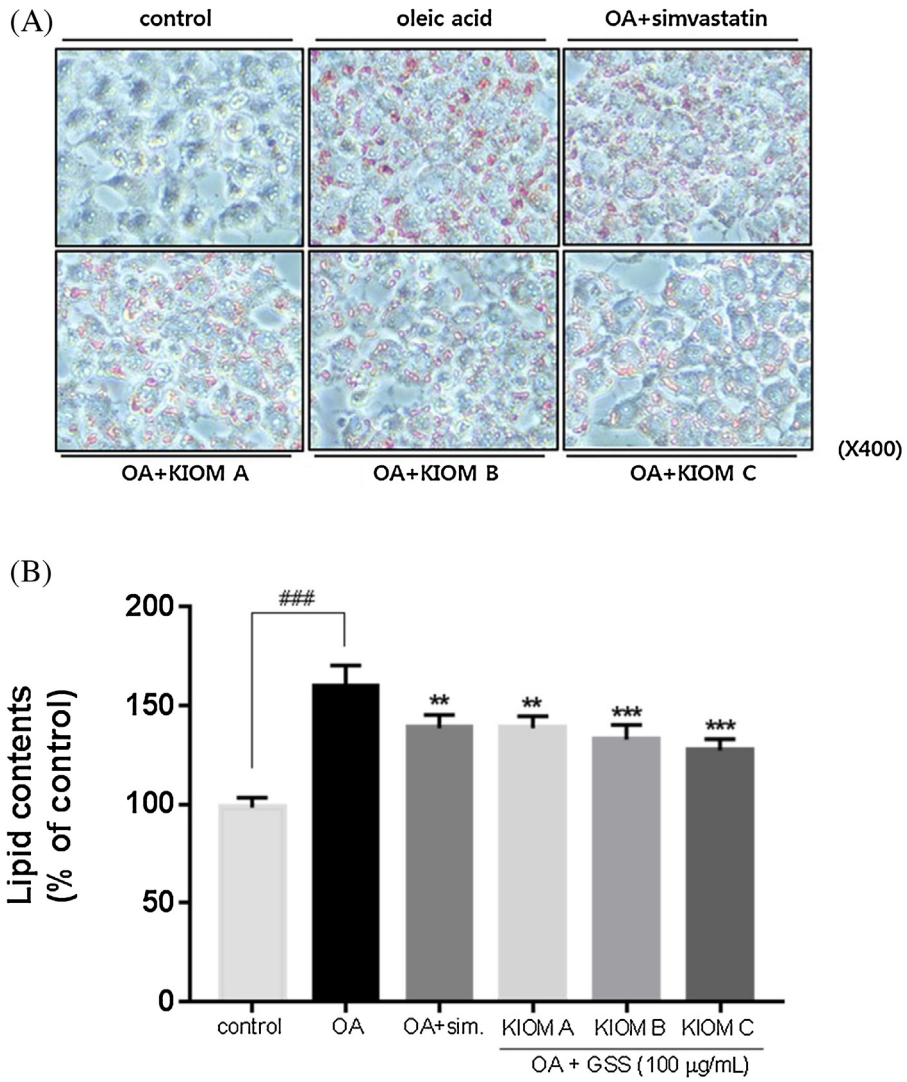

Fig. 2 – Effects of GSS on lipid accumulation induced by OA in HepG2 cells. (A) ORO staining of HepG2 cells after treatment with three GSS and simvastatin. (B) ORO-based quantitative assay. ###$p < 0.01$ versus control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus OA. sim., simvastatin; OA, oleic acid; GSS, Gamisoyosan.

KIOM A was less effective than the other two GSS formulae (Fig. 2).

3.3. *GSS decreases cellular TG and TC in OA-induced fatty liver*

Following OA stimulation, intracellular TG and TC levels were significantly increased (Fig. 3). Significant decreases of TG levels were seen in the KIOM B and KIOM C treatment groups but not in the KIOM A treatment group. In contrast, TC levels were significantly decreased after treatment by all GSS formulae and simvastatin.

3.4. *GSS enhances phytoestrogen activity in MCF-7 cells*

The estradiol concentrations of GSS in the supernatant of the MCF-7 cell culture medium were significantly increased in KIOM A and KIOM B (0.068 ng/mL, 0.055 ng/mL vs. 0.009 ng/mL in control, respectively) (Fig. 4). However, KIOM C was not surveyed for estradiol concentration using the standard curve (Fig. 4). Since KIOM C contains an excipient, it is possible that the excipient may interfere with the analysis of the contents.

3.5. *UPLC quantitative analysis of GSS*

The standard compound (STD) was used as an indicator to examine changes in the components of the three GSS formulae. Under the UPLC analysis conditions, each of the three indicator components of GSS was verified to contain geniposide, paeoniflorin and glycyrrhizin. However, nodakenin was not detected in KIOM C. The retention times of each compound were observed at 10.80 min of geniposide, 13.69 of paeoniflorin, 19.79 of nodakenin and 29.60 of glycyrrhizin. Moreover, to reconfirm the components in the samples, the co-injection method was used. As the result, all spiked standard peaks were increased at the same retention time in all three samples (Fig. 5).

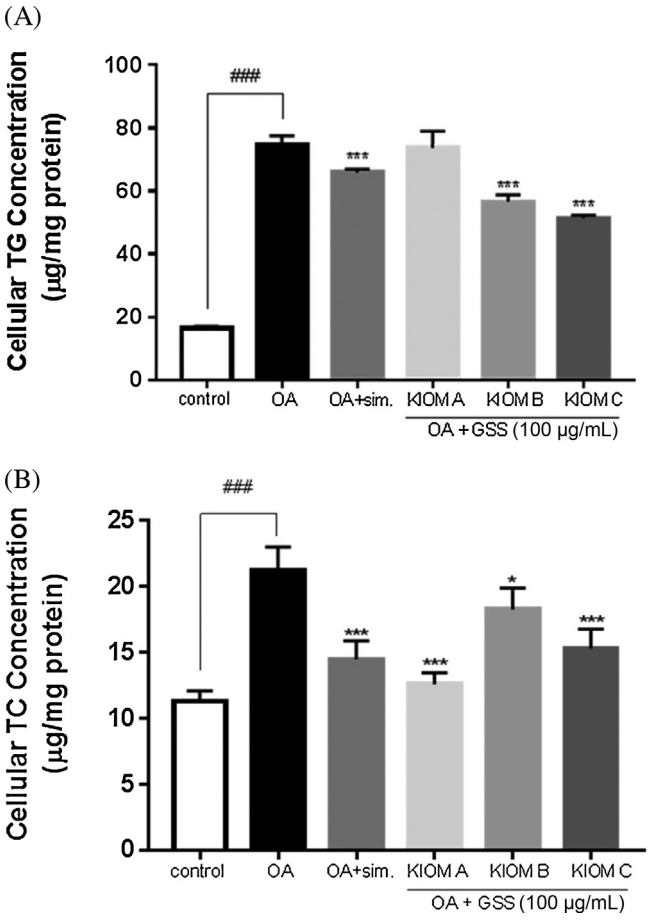

Fig. 3 – Effects of GSS on intracellular TG and TC induced by OA in HepG2 cells. (A) Inhibition of intracellular TG by GSS. (B) Inhibition of intracellular TC by GSS. ###$p < 0.0001$ versus control. **$p < 0.01$, ***$p < 0.001$ versus OA. TG, triglyceride; TC, total cholesterol; sim., simvastatin; OA, oleic acid; GSS, Gamisoyosan.

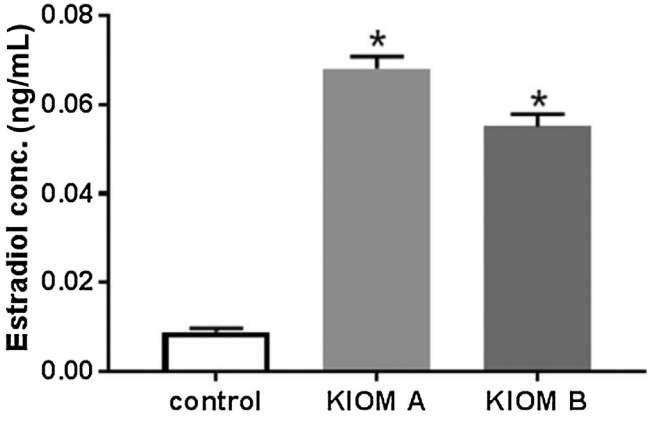

Fig. 4 – Estradiol concentration of GSS. KIOM A (100 ug/mL); KIOM B (100 ug/mL). *$p < 0.05$ versus control.

Table 2 – Peaks area and area (%) of three different formulae of Gamisoyosan from UPLC qualitative analysis.

Compound	Rt[a](min)	Peak Area/Area (%)[b]		
		KIOM A	KIOM B	KIOM C
Geniposide	10.80	34.91/53.14	100.01/67.19	19.49/59.80
Paeoniflorin	13.69	7.03/10.71	8.84/5.94	4.22/12.96
Nodakenin	19.79	6.83/10.40	9.26/6.22	N.D[c]
Glycyrrhizin	29.60	16.92/25.76	30.73/20.64	8.88/27.24

[a] Peak retention time.
[b] Calculated based on the peak area% of the detected STD compound in the UPLC chromatograms.
[c] Not Detected.

From the identified compound peaks, we compared the peak area of the three different GSS formulae. The peak areas were integrated in KIOM A as 34.91 (geniposide), 7.03 (paeoniflorin), 6.83 (nodakenin) and 16.92 (glycyrrhizin). In KIOM B, the peak areas were integrated as 100.01 (geniposide), 8.84 (paeoniflorin), 9.26 (nodakenin) and 30.73 (glycyrrhizin). The peaks were integrated as 19.49 (geniposide), 4.22 (paeoniflorin), and 8.88 (glycyrrhizin) in KIOM C. In addition, the calculated peak area % based on the STD compounds in the chromatograms showed similar patterns of integrated peak values: 53.14 (geniposide), 10.17 (paeoniflorin), 10.40 (nodakenin) and 25.76% (glycyrrhizin) for KIOM A, 67.19 (geniposide), 5.94 (paeoniflorin), 6.22 (nodakenin) and 20.64% (glycyrrhizin) for KIOM B and 59.80 (geniposide), 12.96 (paeoniflorin), and 27.24% (glycyrrhizin) for KIOM C (Table 2).

4. Discussion

In women's diseases, GSS, which is known to have favorable influences on psychiatric disorders and blood flow, is a useful herbal prescription for dysmenorrhea, infertility and insomnia induced by stress. Therefore, persons who have neuropsychiatric symptoms including vertigo, irritability, anxiety, insomnia, depression and suffer from hot flushes, shoulder stiffness, or premenstrual syndrome are often prescribed GSS.[19] GSS has also been used for menopausal woman with unidentified complaints.

In clinical settings, KIOM B or KIOM C (but not KIOM A) is often used for treatment, and the composition and dosage of herbal medicines between these three formulae are different from each other. KIOM C is a drug supplied by Korean National Health Insurance, consisting of medicinal herbs as in KIOM B combined with excipient. KIOM B is based on the prescription of Taepyeonghyeminhwajegukbang. There are no reports of comparative studies of the effects of these three GSS formulae on menopausal disorders. Therefore, we investigated whether there are any differences between these three GSS formulae on menopausal disorders, especially fatty liver *in vitro*.

Estrogen plays an important role in lipid metabolism, and reduction of estrogen induces several changes in lipid profiles, including increased VLDL, LDL and triglycerides and decreased HDL.[20] Fatty liver is caused by the accumulation of lipids, and menopausal women have a high prevalence of fatty

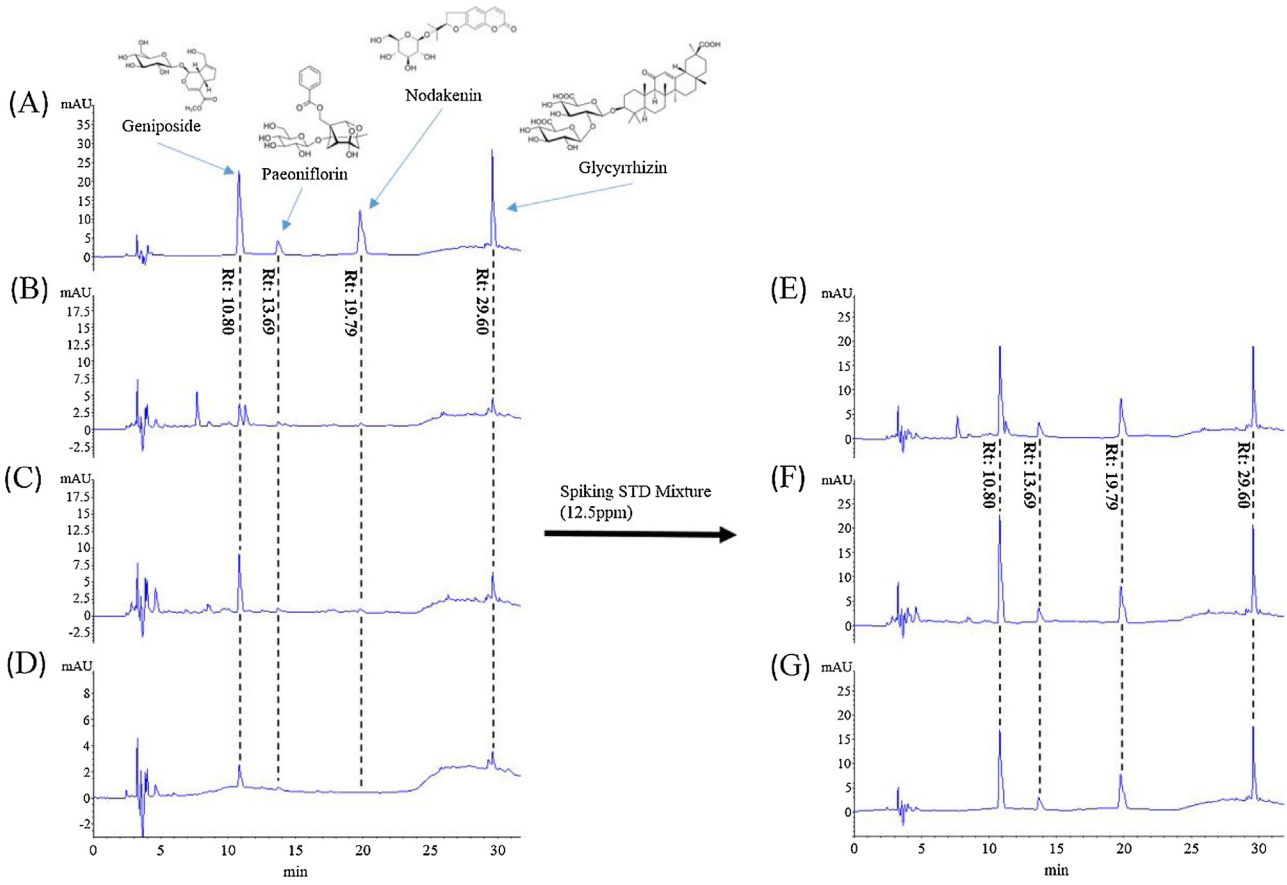

Fig. 5 – UPLC chromatograms of standard mixture (0.25 mg/mL) (A), KIOM A sample (0.5 mg/mL) (B), KIOM B sample (0.5 mg/mL) (C), KIOM C sample (0.5 mg/mL) (D), KIOM A (0.5 mg/mL) + standard mixture (E), KIOM B (0.5 mg/mL) + standard mixture (F), KIOM C (0.5 mg/mL) + standard mixture (G) at 254 nm detection.

liver. Because GSS is a well-known menopause prescription, we thought it might also have improving effects on fatty liver. In our results, all GSS formulae exhibited significant inhibition of lipid accumulation, and little difference existed between the three formulae of GSS (Fig. 2). There have been some reports that components of GSS such as Lycii Cortex Radicis, Platycodi Radix, Bupleuri Radix, Moutan Cortex Radicis, *Zingiber officinale*, Angelicae gigantis Radix, and Paeoniae Radix have known to improve hyperlipidemia or inhibit lipid accumulation.[21–27] For example, Lycii Cortex Radicis moderated triglycerides, fatty acids, HDL cholesterol, and LDL cholesterol in hyperlipidemic rats.[21] A methanol extract of Bupleuri Radix reduced total cholesterol in hyperlipidemia.[23] *Zingiber officinale* lowered the contents of total cholesterol, triglycerides, free cholesterol and cholesteryl ester in the liver of hyperlipidemic rats.[25] Therefore, we thought that the improvements in fatty liver induced by GSS in this paper might be attributed to these herbal medicines.

According to oriental medicine, blood stasis and liver qi (気) stagnation are known to pathogenic factors for fatty liver disease.[28] The liver is an important organ as it stores blood and maintains a smooth flow of qi (気) throughout the body. Since qi (気) moves blood, when there is insufficient qi (気) the blood stops. If liver qi (気) stagnates, it will fail to store the blood properly, causing the blood to congeal and liver blood stagnation. We thought the main action of GSS, improvement of liver qi (気) depression, would also be positively related to amelioration of fatty liver in HepG2 cells.

We used simvastatin as a positive control for anti-lipid accumulation. Simvastatin is known to be a cholesterol-lowering drug that inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme for cholesterol synthesis,[29] and GSS showed inhibitory effects of lipid accumulation that were comparable to simvastatin (Fig. 2). These observations may explain the beneficial effects of GSS on chronic diseases such as fatty liver. Simvastatin is known to lower not only cholesterol but also triglycerides. In clinical study, simvastatin was effective in decreasing serum triglyceride levels.[30] In vivo high-fat/cholesterol-fed rabbits, simvastatin also showed triglyceride-lowering effects.[31] In contrast, there is a report that simvastatin increased triglycerides in HepG2 cells.[32] In our results, simvastatin significantly decreased cellular total cholesterol but had little effect on triglycerides, suggesting that simvastatin has less efficacy on cellular triglyceride levels in our *in vitro* systems. The statins, including simvastatin, are known to be the most effective drugs at lowering LDL-cholesterol but are less effective than other lipid-regulating drugs, such as fibrates, at reducing triglyceride concentrations.[33] KIOM A effectively decreased cellular total cholesterol but not cellular triglycerides (Fig. 3).

These results indicate that KIOM A is more effective at reducing total cholesterol than triglycerides, similar to simvastatin. In terms of lipid lowering efficacy, KIOM A has effects similar to simvastatin compared to KIOM B or KIOM C.

In this study, we conducted a chromatographic qualitative analysis of GSS using four index compounds found in GSS, including geniposide, paeoniflorin, nodakenin and glycyrrhizin (Fig. 5). From the UPLC analysis, geniposide, paeoniflorin and glycyrrhizin were detected in all three different GSS formula at 254 nm. The integrated peak areas of geniposide and glycyrrhizin were higher than paeoniflorin and nodakenin in our analysis conditions. However, the paeoniflorin peak was higher at another wavelength (240 nm). Nodakenin was not detected or observed at other wavelengths (data not shown). Therefore, we cautiously anticipate that the three compounds (geniposide, paeoniflorin and glycyrrhizin) have a role as major activators in GSS. However, to verify the relationship between their activities and components more clearly, quantitative analysis of the quality index components of GSS will likely be required. Therefore, more studies are necessary to investigate the active compounds of our GSS formulate and to develop appropriate validation methods to compare various GSS formulae. Three of these compounds, geniposide, paeoniflorin, and glycyrrhizin, which were contained in all three GSS formulae, have been reported to have positive effects on lipid metabolic abnormalities. For example, geniposide ameliorates abnormal lipid metabolism in free fatty acid-treated HepG2 fatty liver cells by increasing the expression of fatty acid oxidation related genes and peroxisomal proliferator-activated receptor (PPARα).[34] Paeoniflorin inhibited de novo lipid synthesis and prevented lipid accumulation in palmitate-induced HepG2 cells.[35] Elevated PPAR-gamma and glucose transporter 4 proteins contributed to the positive effects of glycyrrhizin on several metabolic diseases, including dyslipidemia, insulin resistance and hyperglycemia.[36] Based on these reports, we suggest that the mechanism of action of the three GSS formulae on lipid accumulation might be related to enhanced fatty acid oxidation or inhibited expression of lipid synthesis related genes, such as sterol response element binding proteins, fatty acid synthase and HMGCR. Moreover, phytoestrogen-like actions of geniposide have been reported.[37] Phytoestrogens have been used for the treatment of estrogen imbalances during menopause as an alternative to HRT, which is generally used to treat menopausal symptoms. However, several complications, such as the risk of breast cancer, are increased.[38] In terms of fatty liver, there are some reports on improvements due to phytoestrogens.[39,40] In agreement with a previous report using an estrogen-chimeric receptor/Gal4-response element regulated/luciferase reporter gene assay in HeLa cells,[41] GSS indicated the presence of phytoestrogens (Fig. 4). These results suggest that the phytoestrogen-like actions of their inherent components could be involved in the actions of the three GSS formulae on lipid accumulation. Further studies are required to quantitatively analyze the three GSS and determine the active relationship between their ingredients.

In conclusion, the results of this study suggest that GSS inhibits fatty liver induced by oleic acid in HepG2 cells, and all three formulae of GSS (KIOM A, KIOM B and KIOM C) show promise for therapeutic applications in terms of fatty liver improvement. Any of these formulae could be useful to treat fatty liver in clinical settings. In addition, our results led us to a hypothesis that all three formulae of GSS shows phytoestrogen activity that is useful for menopausal fatty liver. Interestingly, the dosage of drugs and excipient did not affect the effectiveness of the prescription. The Korean National Health Insurance medicine, KIOM C, appears to be effective, and testing should continue. We will consider including this set of *in vivo* experiments in future studies.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by the Korea Institute of Oriental Medicine (grant K17291) and the Korea Research Fellowship Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (grant 2015H1D3A1066241).

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