Synergistic lipid-lowering effects of *Zingiber mioga* and *Hippophae rhamnoides* extracts

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Abstract. The effects of a mixture of *Hippophae rhamnoides* (HR) and *Zingiber mioga* (ZM) extract (ZH) on intracellular lipid accumulation were investigated *in vitro* and the anti-obesity effects of ZH evaluated in mice with high-fat diet-induced obesity. The results revealed that ZH inhibited lipid accumulation in 3T3-L1 adipocytes and Huh-7 cells by suppressing adipogenic and lipogenic gene and protein expression. To evaluate the anti-obesity effects of ZH, mice fed a high-fat diet were orally administered low and high doses of ZH (low, ZM 400 mg/kg + HR 100 mg/kg; high, ZM 800 mg/kg + HR 200 mg/kg) for 9 weeks. ZH significantly reduced body weight gain and adipose tissue accumulation with no reduction in food intake when compared to control treatment. Furthermore, ZH reduced hepatic triglyceride and total cholesterol levels, as well as adipose cell size, in the liver and epididymal fat pads, respectively, through inhibition of adipogenesis and lipogenesis-related gene expression. These results suggested that ZH inhibits lipid accumulation, thereby indicating its potential for use as a new therapeutic strategy for obesity.

Introduction

The prevalence of overweight and obesity increasing gradually worldwide, with ~39% of the population affected (1). Obesity is caused by a chronic energy imbalance due to excessive energy intake and reduced energy expenditure (2). Excessive energy intake due to a high-sugar and high-fat diet (HFD) leads to abnormal lipid accumulation in adipose tissues and in the liver (3,4). The accumulated lipids not only play a role in the development of metabolic syndrome, but also increase the risk of development of diabetes or cardiovascular diseases (5-8). Many methods have been proposed to overcome this problem, including inducing weight loss through treatment with nutritional supplements with anti-obesity effects (9-12); however, because nutritional supplements are generally less effective than drugs, there is a growing interest in developing combinations of different nutritional supplements that may exhibit synergistic effects, while having low numbers of side effects and increased efficacy (13-15).

Zingiber mioga (of the Zingiberaceae family) is grown in Korea and Japan, where its flower buds are eaten as pickles, in salads and brochettes. It has been suggested to exhibit diverse biological functions, including antihyperglycemic and antioxidant activity, and to improve allergic asthma and memory (16-18). A previous study by our research group demonstrated that *Zingiber mioga* extract (ZM) exerted an anti-obesity effect in HFD-induced obese mice, and revealed the effects of ZM on insulin resistance and liver gluconeogenesis (19); however, only a high concentration of ZM showed a significant effect in these previous experiments. In order to reach an effective dose, an excessive intake of the extract was required; therefore, combining multiple nutritional supplements could be a useful method for achieving effects at lower concentrations.

Hippophae rhamnoides, also known as sea buckthorn, is grown in cold-temperature climates across Europe and Asia, where its leaves and fruits have been consumed as a traditional medicine to alleviate the symptoms of a variety of diseases, including cough and indigestion (20,21). Several studies report that *Hippophae rhamnoides* leaf extract (HR) exhibits anti-inflammatory, antioxidative and cytoprotective effects (22-24). Additionally, studies into the effects of HR in treating obesity report that it inhibits the adipogenic differentiation of 3T3-L1 cells and prevents HFD-induced obesity (25,26). In the present study, a combination of HR and ZM (ZH) was investigated to establish whether HR and ZM could display synergistic inhibitory effects on adipogenic differentiation and lipid accumulation in 3T3-L1 and Huh-7 cells. Additionally, the anti-obesity effect of ZH in mice with HFD-induced obesity was explored.

Materials and methods

Extract preparation. ZM was harvested in September 2017 at Jeongeup-si, Jeollabuk-do (Korea) and lyophilized using

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an LP100 freeze-dryer (IlShin BioBase Co., Ltd.). The dried plants were pulverized and extracted at 80˚C for 2 h using a 10-fold greater volume of water. The extracts were filtered using Advantec filter paper (no. 2; pore size, 5 μm; Advantec MFS, Inc.), freeze-dried and stored at -20˚C until use. HR extract was obtained from Frombio Co., Ltd.

Cell culture. 3T3-L1 and Huh-7 cells were procured from the American Type Culture Collection. 3T3-L1 cells were cultured in DMEM with 10% calf serum and penicillin/streptomycin/glutamine, and Huh7 cells were cultured in DMEM with 10% FBS and penicillin-streptomycin (HyClone; GE Healthcare Life Sciences); thereafter, cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2 for further experiments.

Adipocyte differentiation and lipid accumulation. 3T3-L1 pre-adipocytes were seeded in 6-well plates, and the induction of adipocyte differentiation was initiated after cells reached confluence. Confluent cells were incubated in DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (cat. no. I7018; Sigma-Aldrich; Merck KGaA), 1 μM dexamethasone (cat. no. D4902; Sigma-Aldrich; Merck KGaA) and 1 μg/ml insulin (cat. no. I0908; Sigma-Aldrich; Merck KGaA) with different concentration of extracts. The treatment concentrations of ZM and HR were set at 400 μg/ml and 100 μg/ml, respectively, based on the results of previous studies (27,28). After 2 days, the medium was replaced with DMEM containing 10% FBS, 1 μg/ml insulin, and cells were incubated for a further 2 days. The medium was again replaced with DMEM containing 10% FBS and the cells were incubated for a further 2 days, after which the medium was replaced with fresh DMEM containing 10% FBS every 2 days until day 8. The extracts were renewed upon each cell medium replacement.

To induce lipid accumulation in Huh7 cells, the cells were seeded into 6-well plates ($2x10^5$ cells/well) and treated with 200 μM oleic acid (OA; cat. no. O3008; Sigma-Aldrich; Merck KGaA) and different concentrations of extracts (ZM, 200 μg/ml; HR, 50 μg/ml) for 24 h. Lipid droplets within Huh7 cells were then quantified following fluorescence detection of Nile Red staining.

Nile Red staining. 3T3-L1 and Huh7 cells were washed with PBS and fixed at room temperature using 4% formaldehyde for 20 min. The cells were then washed twice with PBS and permeabilized in ice-cold methanol for 20 min at -20˚C. The cells were washed again with PBS and then incubated at 37˚C with PBS-diluted Nile Red stock solution (1:10,000; cat. no. N1142; 10 mg/ml in acetone; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min. After further washing with PBS, the cells were stained at room temperature with DAPI (cat. no. 10236276001; Sigma-Aldrich; Merck KGaA; 1 μg/ml) for 5 min. Images of stained cells were obtained by capturing five different random fields of views for each well using a fluorescence microscope (model IX71; Olympus Corporation), and fluorescence intensities were measured at an excitation wavelength of 488 nm and an emission wavelength of 550 nm.

Evaluation of synergistic effects. The predicted synergistic effect was calculated using a previously described formula (29): E = (X x Y)/100; where X is the percentage inhibitory effect of ZM as compared with the control, Y is the percentage inhibitory effect of HR as compared with the control, and E is the expected inhibitory percentage of the two-extract mixture (ZH).

BODIPY staining. The OA-treated Huh-7 cells were fixed with 4% formaldehyde for 15 min and permeabilized using 0.05% saponin (cat. no. 47036, Sigma-Aldrich; Merck KGaA) in PBS for 30 min at room temperature. After blocking with 1% BSA for 1 h at room temperature, the cells were incubated with 1 μg/ml BODIPY 493/503 (cat. no. D2228; Invitrogen; Thermo Fisher Scientific, Inc.) in PBS for 20 min at 37˚C. Stained cell images were captured using an Eclipse Ti confocal fluorescent microscope (Nikon Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using the RNeasy Mini kit (cat. no. 74106; Qiagen GmbH) according to the manufacturer's instructions. cDNA was synthesized from 1 μg RNA in a volume of 20 μl using the ReverTra Ace qPCR RT kit (Toyobo Life Science). qPCR was performed using SYBR-Green real-time PCR master mix (cat. no. FSQ-101; Toyobo Life Science) and the ViiA 7 real-time PCR system (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were applied: Initial denaturation at 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Relative mRNA levels were calculated after normalizing the values against those of β-actin and the data were analyzed using the $2^{-\Delta\Delta Cq}$ method (30). The primer sequences used are shown in Table I.

Western blot analysis. Proteins were extracted using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) containing protease inhibitors and phosphatase inhibitors. After centrifugation at 16,000 x g for 10 min at 4˚C, the supernatant was removed, and protein concentration was quantified using the Pierce BCA protein assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). Proteins (10 μg/lane) were separated by SDS-PAGE (FAS, Vinculin, C/EBPα and PPARγ, 8%; AP2, 12%) and transferred onto PVDF membranes. After blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated with the following primary antibodies diluted (1:1,000) in TBS-T (0.1% Tween-20) overnight at 4˚C; Peroxisome proliferator activated receptor γ (PPARγ; cat. no. sc-7196; Santa Cruz Biotechnology, Inc.); CCAAT/enhancer binding protein-α (C/EBPα; cat. no. sc-7962; Santa Cruz Biotechnology, Inc.); sterol regulatory element-binding protein 1 (SREBP1c; cat. no. sc-13551; Santa Cruz Biotechnology, Inc.); β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc.); adipocyte protein 2 (AP2; cat. no. 2120; Cell Signaling Technology, Inc.); fatty acid synthase (FAS; cat. no. 3180; Cell Signaling Technology, Inc.); and vinculin (cat. no. V9131; Sigma-Aldrich; Merck KGaA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer (1:1,000; cat. nos. NB7539 and NB7160; Novus Biologicals, LLC) at room temperature for 1 h. Immunoreactivity was detected using an enhanced chemiluminescence western substrate (Thermo Fisher Scientific, Inc.)

Table I. Primer sequences.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
AP2	CCGCAGACGACAGGA	CTCATGCCCTTTCATAAACT
PPARγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
C/EBPα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
CD36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
SREBP1c	TGGATTGCACATTTGAAGACAT	GCCAGAGAAGCAGAAGAG
SCD1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
β-actin	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC

AP2, adipocyte protein 2; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT-enhancer-binding protein α; FAS, fatty acid synthase; SREBP1c, sterol regulatory element-binding protein 1; SCD1, acyl-CoA desaturase-1.

and the G:BOX Chemi XX6 imaging system (Syngene) and quantified using ImageJ version 1.8 (National Institutes of Health).

Animal experimental design. Male C57BL/6J mice (age, 4 weeks; weight range, 17.39-19.61 g) were purchased from Orient Bio, Inc. (n=40). The mice housed under constant conditions (12-h light/dark cycle; temperature, 22±2˚C; humidity, 50±10%) with *ad libitum* access to food and water. After 1 week of adaptation, the mice were randomly divided into the following four groups (n=6/group): i) Normal (N); ii) HFD; iii) HFD with a low dose of ZH mixture (ZHL; ZM 400 mg/kg + HR 100 mg/kg); and iv) HFD with a high dose of ZH mixture (ZHH; ZM 800 mg/kg + HR 200 mg/kg). The N group was fed a diet of American Institute of Nutrition (AIN)-76A, including 10% fat (in kcal), and the other groups were fed a diet of AIN-76A with 45% fat (in kcal) and 0.5% cholesterol. The detailed compositions of each diet were described in a previous study (31). Body weight gain was measured weekly for 9 weeks. The design of all animal experiments was approved by the Korea Food Research Institute Animal Care and Use Committee (approval no. KFRI-M-18001). After 9 weeks, the mice were anesthetized with 2% isoflurane, sacrificed, and the liver and adipose tissues were harvested. The inguinal subcutaneous fat (hereafter referred to as subcutaneous fat) was isolated according to the method of a previous study (32). The liver and epididymal fat pads tissues were fixed in 4% formaldehyde for 36 h at 4˚C, whilst the rest were stored at -80˚C for further analysis. To obtain serum samples, blood (700-900 μl) was collected from the abdominal aorta and centrifuged at 900 x g for 20 min at 4˚C.

Hematoxylin and eosin (H&E) staining. The liver and epididymal fat pads (EPs) were fixed in 4% formaldehyde for 36 h, embedded in paraffin, cut into 5-μm sections. The sections were deparaffinized using xylene and were stained with H&E (hematoxylin; 5 min, eosin; 1 min) at room temperature. The samples were then observed under a microscope (magnification, x100; model IX71; Olympus Corporation), and adipose-tissue sizes were measured using iSolution DT 9.2 software (IMT i-Solution, Inc.)

Hepatic triglyceride (TG) and total cholesterol (TC) levels. Hepatic lipids were extracted using a method described by Folch *et al* (33). Lipid composition was measured using TG (cat. no. M-209-1), TC (cat. no. M-206-1) and high-density lipoprotein (cat. no. M-208-1) quantification kits (EMBIEL Ltd.).

Statistical analysis. Significant differences among groups were evaluated by one-way ANOVA, followed by Tukey's post hoc multiple comparison test using GraphPad Prism version 7 (GraphPad Software, Inc.). Data are presented as the mean ± SD (*in vitro*) or SEM (*in vivo*). $P<0.05$ was considered statistically significant.

Results

Synergistic inhibitory effect of ZM and HR on the differentiation of 3T3-L1 preadipocytes. To investigate the potential synergistic inhibitory effects of HR and ZM on obesity, the inhibitory effects of ZH on 3T3-L1 cell differentiation were examined and the effects was measured using Nile Red staining. ZM or HR treatment suppressed the adipogenic differentiation of 3T3-L1 cells by ~15 and ~60%, respectively, relative to untreated controls. Based on the formula proposed by Colby (29), the expected inhibition by the ZH mixture was calculated at 66%, indicating that ZH treatment was more effective than predicted, as lipid accumulation was shown to be inhibited by ~70% (Fig. 1A and B). Additionally, levels of FAS, C/EBPα, PPARγ and AP2, which are key markers of the differentiation process, were analyzed, and the results suggested that the mRNA and protein levels of these adipogenesis-related genes in 3T3-L1 adipocytes were significantly reduced in ZH-treated cells relative to cells treated with a single extract (Fig. 1C and D). These results indicated that ZM and HR exerted a synergistic effect on adipocyte differentiation by suppressing the expression of adipogenesis-related genes more effectively than single extracts.

Synergistic inhibitory effects of ZM and HR on OA-induced lipid accumulation in hepatocytes. Whether ZM and HR would also show a synergistic inhibitory effect on lipid accumulation was then investigated in hepatocytes. Although HepG2 is the most common *in vitro* model used in fatty liver studies, Huh-7

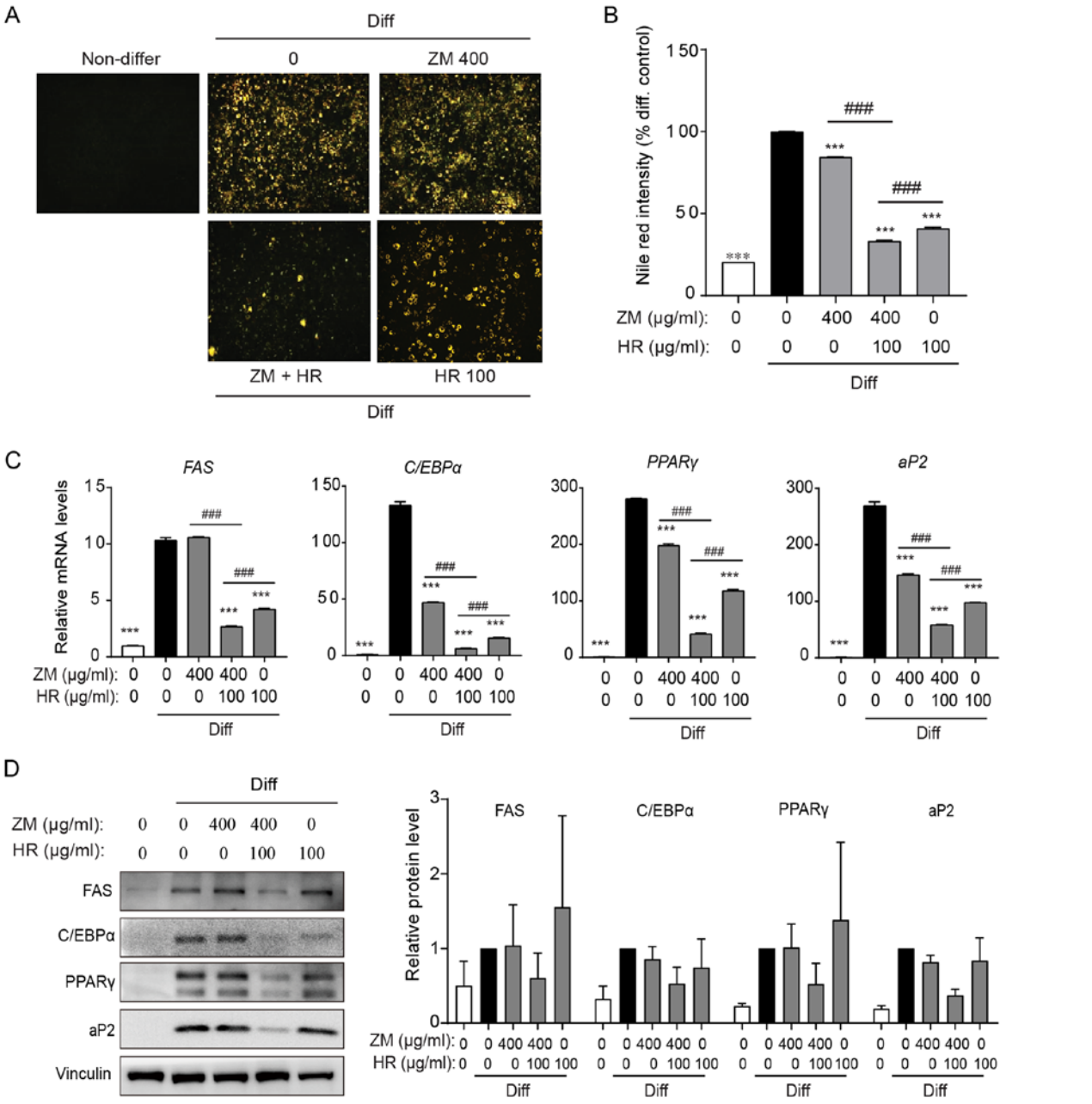

Figure 1. Effects of ZH on 3T3-L1 cell adipogenic differentiation. (A) Lipid droplets stained with Nile Red dye and (B) their fluorescence intensity (original magnification, x100). (C) Relative mRNA levels of *FAS*, *C/EBPα*, *PPARγ* and *AP2* and (D) protein levels and fold changes normalized to those in the differentiation control group. Data represent the mean ± SD of triplicate experiments. ***P<0.001 vs. differentiation control group; $^{###}$P<0.001. diff, differentiation; AP2, adipocyte protein 2; C/EBPα, CCAAT-enhancer-binding protein α; FAS, fatty acid synthase; HR, *Hippophae rhamnoides*; PPARγ, peroxisome proliferator-activated receptor γ; ZM, *Zingiber mioga*; ZH, ZM and HR extract mixture.

cells can also be used to model lipid accumulation in hepatocytes (34,35). To examine the effects, OA treated Huh-7 cell with or without extract treatment were analyzed for changes in lipid accumulation using Nile Red staining. ZM or HR treatment alone reduced lipid accumulation in Huh7 cells by ~50 and ~45%, respectively, as compared with untreated controls. The predicted value based on the Colby equation (61%) indicated that the lipid-lowering effect in the ZH-treated cells (~65%) was 4% higher than that predicted (Fig. 2A and B). To confirm the synergistic effects of ZM and HR, lipid accumulation was analyzed using BODIPY staining and the results suggested that ZH reduced lipid accumulation more effectively than either single extract (Fig. 2C). Additionally, ZM and HR treatment showed a synergistic inhibitory effect on protein levels of FAS, a factor involved in lipid synthesis (Fig. 2D). These results suggested that ZH exerted a greater inhibitory effect on lipid accumulation in hepatocytes than either ZM or HR alone.

Effect of ZH on body weight gain and organ weight in HFD-induced obese mice. It was next determined whether ZH would show anti-obesity effects in an animal model. The ZHL and ZHH groups showed significantly reduced gains in body weight as compared with the HFD group, with no differences in food intake during the HFD feeding period (Fig. 3A, B and D). To investigate whether this difference was due to the inhibition of lipid accumulation, the weights of the liver and adipose

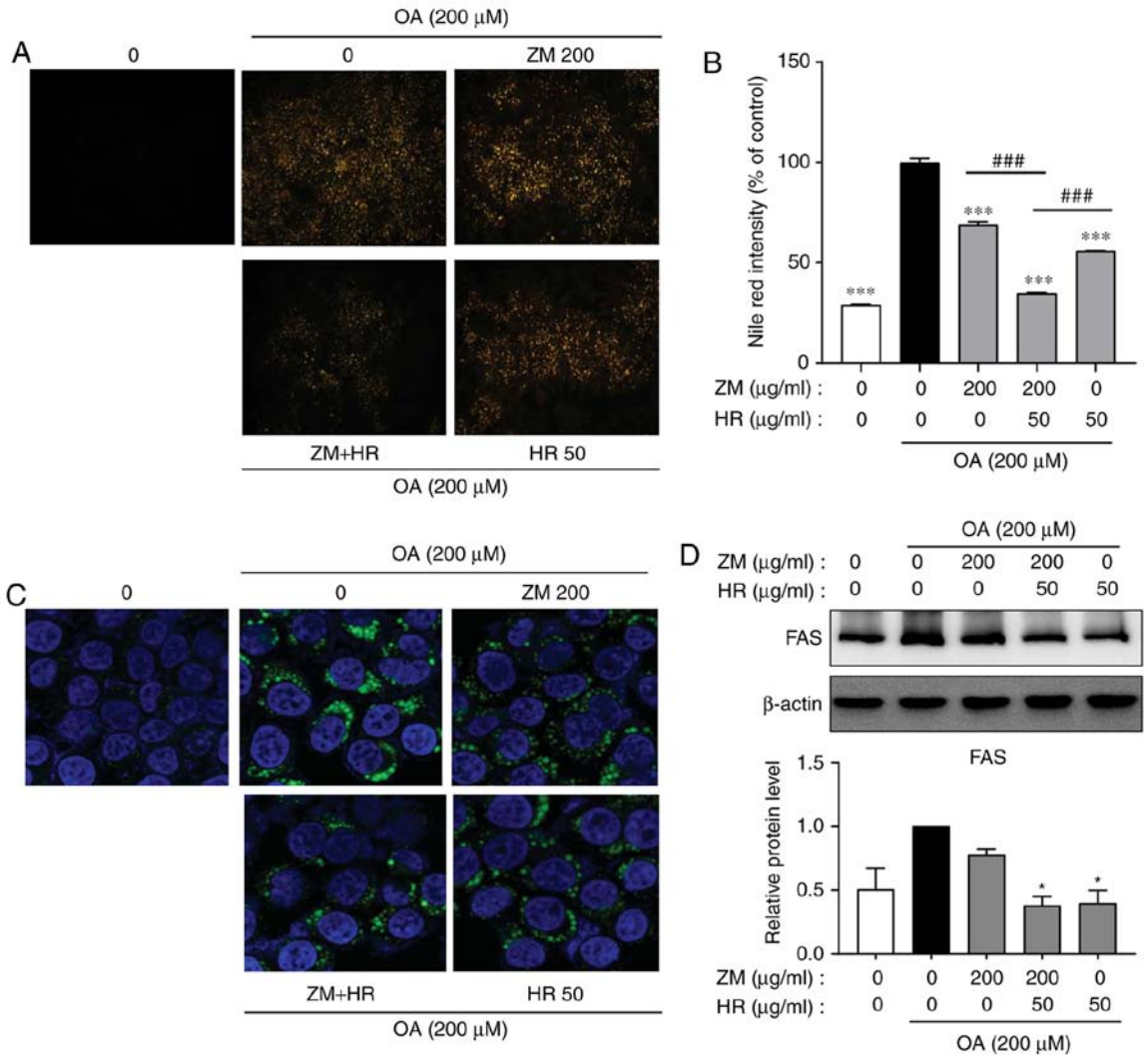

Figure 2. Effects of ZH on OA-induced lipid accumulation in Huh-7 cells. (A) Lipid droplets stained with Nile Red and (B) their fluorescence intensity (original magnification, x100). (C) Lipid droplets evaluated by BODIPY staining (original magnification, x600). (D) Protein levels of FAS and the fold change normalized to that in the OA-treated control group. Data represent the mean ± SD of triplicate experiments. FAS, fatty acid synthase; HR, *Hippophae rhamnoides*; OA, oleic acid; ZM, *Zingiber mioga*. *P<0.05, ***P<0.001 vs. OA-treated control; $^{###}$P<0.001. FAS, fatty acid synthase; HR, *Hippophae rhamnoides*; OA, oleic acid; ZM, *Zingiber mioga*; ZH, ZM and HR extract mixture.

tissues from mice in all groups were measured. Both ZHL and ZHH reduced liver weight significantly, and the subcutaneous fat weight was significantly reduced in only the ZHH group (Fig. 3C). Although the weight of epididymal fat tissues was reduced in ZHH group compared with HFD group, no significant differences were observed (Fig. 3C). H&E staining of the liver showed that both the ZHL and ZHH groups displayed reduced lipid accumulation as compared with the HFD group (Fig. 3E). Additionally, analysis of TG and TC concentrations indicated that the ZH groups showed reductions in both TG and TC concentrations as compared with those in the HFD group (Fig. 3G). Additionally, adipocytes in the EPs were smaller in mice in the ZH groups than in those from the HFD group (Fig. 3F and H), although ZH supplementation did not significantly reduce EP weight. These results suggested that ZH supplementation effectively reduced the size of adipocytes in white adipose tissue and lipid accumulation in the liver.

Effect of ZH on mRNA expression of liver lipogenic and WAT adipogenic genes. Whether ZH reduces the mRNA expression of lipogenic and adipogenic genes in the liver and EPs was next examined. *SREBP1c*, *SCD1* and *FAS* in the liver and *PPARγ*, *AP2* and *C/EBPα* in adipose tissue are associated with lipogenesis and adipogenesis, respectively (36,37). In the liver, *SREBP1c* and *SCD1* levels decreased significantly in the ZHH group as compared with those in the HFD group, whereas *FAS* expression decreased slightly but not significantly (Fig. 4A). All other genes showed no significant difference in expression in the ZHL group as compared with the HFD group, although their expression levels were slightly reduced. Additionally, *PPARγ* expression was only significantly reduced by ZHH supplementation in EPs (Fig. 4B). These results suggested that ZHH administration to HFD-induced obese mice partially suppressed the expression of adipogenic genes in the liver and EPs.

Discussion

Studies are currently underway to demonstrate the synergistic effects of two or more substances to improve the physiological

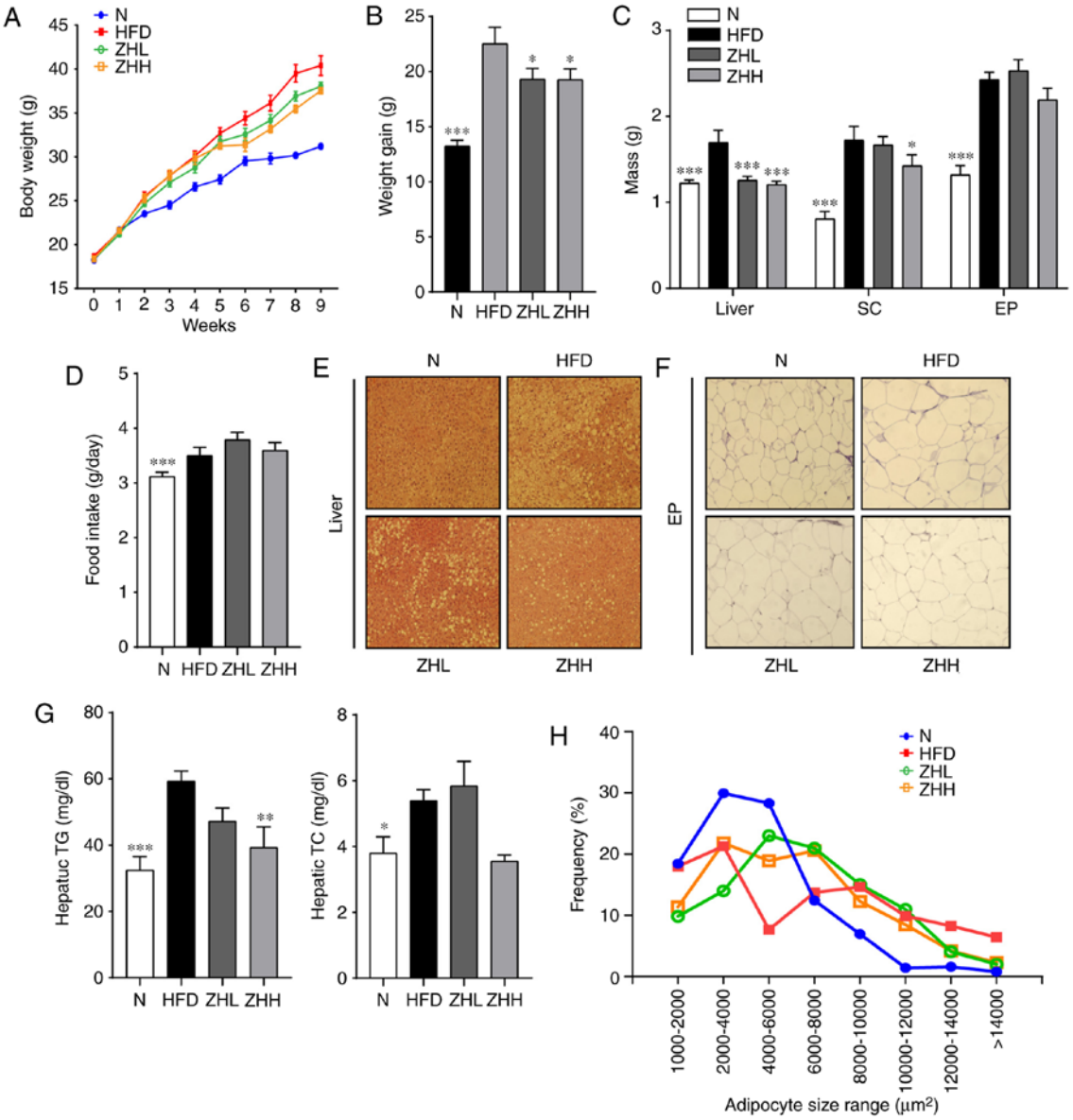

Figure 3. Anti-obesity effects of ZH in HFD-fed mice. (A) Body weight change and (B) body weight gain during 9 weeks of ZH supplementation in HFD-fed mice. (C) Liver and adipose tissue weights. (D) Food intake. (E) Hematoxylin and eosin staining of the liver and (F) EPs (original magnification x200). (G) Hepatic TG and TC levels in each group. (H) Frequency of adipocyte cell size in EPs. Data represent the mean ± SEM. n=6 mice/group. *P<0.05, **P<0.01, and ***P<0.001 vs. HFD group. EP, epidymal fat pad; H, high-dose; HFD, high fat diet; HR, *Hippophae rhamnoides*; L, low-dose; N; Normal fat diet; TC, total cholesterol; TG, triglyceride; ZM, *Zingiber mioga*; ZH, ZM and HR extract mixture.

activity of well-known materials, with the aim of developing nutritional supplements or dietary supplements (15,38,39). Until recently, most studies have focused on identifying the efficacy of single materials rather than mixtures. Studies of the synergistic effects of a mixture comprised of two or more materials to promote human health could provide more favorable alternative treatment options. In the present study, the inhibitory effects of the ZH mixture on lipid accumulation *in vitro* were examined and its anti-obesity effects in HFD-induced obese mice evaluated. The results indicated that treatment with individual extracts inhibited lipid accumulation in 3T3-L1 adipocytes, as shown in previous studies (19,25). This effect was found to be higher than predicted (using the equation proposed by Colby (29) following treatment with a mixture containing both materials. Subsequent evaluation of the efficiency of the mixture in reducing lipid accumulation revealed that ZM and HR displayed a synergistic effect in reducing lipid accumulation in 3T3-L1 adipocytes and hepatocytes.

Various intracellular proteins participate in adipocyte differentiation in 3T3-L1 cells. Specifically, PPARγ and C/EBPα are transcription factors that induce the expression of several genes involved in adipogenic differentiation (40). Previous studies have shown that ZM and HR inhibit fat accumulation by suppressing the expression of these genes (19,26). The results of the present study indicated that ZH treatment inhibited the expression of genes related to adipocyte differentiation in 3T3-L1 cells to a degree greater than that observed following treatment with either single extract. Moreover, ZH suppressed lipid accumulation more effectively than the single extracts in hepatocytes exhibiting OA-induced lipid accumulation. However, this study did not identify a mechanism controlling

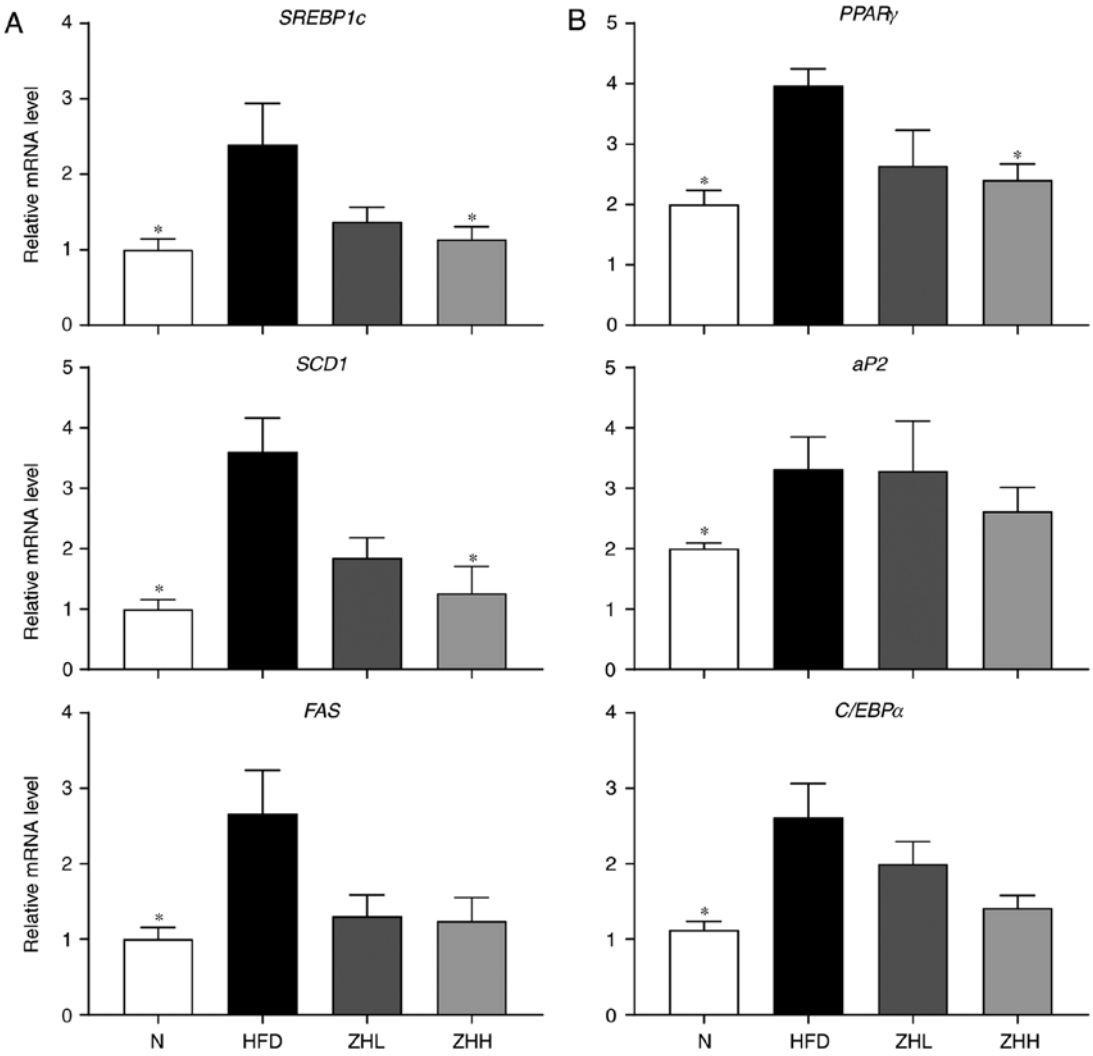

Figure 4. Effects of ZH on lipogenesis- and adipogenesis-related mRNA levels. (A) mRNA levels of *SREBP1c*, *SCD1* and *FAS* in the liver (n=6). (B) mRNA levels of *PPARγ*, *AP2* and *C/EBPα* in EPs (n=6). Data represent the mean ± SEM. *P<0.05 vs. HFD group. AP2, adipocyte protein 2; C/EBPα, CCAAT-enhancer-binding protein α; FAS, fatty acid synthase; HR, *Hippophae rhamnoides*; PPARγ, peroxisome proliferator-activated receptor γ; SCD1, acyl-CoA desaturase-1; SREBP1c, sterol regulatory element-binding protein 1; ZM, *Zingiber mioga*; ZH, ZM and HR extract mixture.

the lipid-lowering effect of ZH. To elucidate such a mechanism, it will be necessary to identify the functional components in each extract, followed by confirmation of additional effects between the components. ZM is a perennial herb whose flower buds have a pungent aroma and flavor caused, at least in part, by the presence of mioganal and miogadial (41,42). A previous study reported that miogadial stimulates the transient receptor potential (TRP) cation channel V1 and TRPA1 receptors (43); however, to the best of our knowledge, there have been no studies focused on their roles in relation to obesity. The leaves of HR are reportedly rich in flavonols, such as kaempferol, isorhamnetin and quercetin glycosides (44), with many studies demonstrating that these flavonol glycosides exert an inhibitory effect on lipid accumulation (45,46). Furthermore, recent studies have been conducted to improve their specific functions by mixing different compounds (47,48). Further research is required to determine which components of HR and ZM display the synergistic effects observed here.

To investigate whether ZH reduces lipid accumulation *in vivo*, its anti-obesity effect in HFD-induced obese mice was investigated. ZH treatment significantly reduced body weight gain in obese mice as compared to that in the control group; however, no concentration-dependent effects were observed, and its activity was effective even at a lower concentration. Obesity is characterized by an increase in the size of adipocytes (49), a major component of adipose tissue. Here, the results suggested that ZH treatment significantly decreased adipocyte size. Obesity can be prevented by decreasing adipogenesis and inhibiting lipogenesis (50). Adipocyte formation is regulated by a cascade of transcription factors, including PPARγ and C/EBP, which are master regulators of adipocyte differentiation and regulate the expression of downstream target genes, such as *AP2*, *FAS* and *SCD1*, leading to lipid accumulation in cells (40,51). In the present study, ZH treatment resulted in a slight decrease in *C/EBPα* and *AP2* levels, with only *PPARγ* expression showing a significant decline. Increasing the number of experimental animals will potentially provide more meaningful results.

The effects of ZH treatment were more prominent in the liver, as shown by the significant reduction in liver weight and lipid accumulation in the present study. SREBP1c is a transcription factor that induces the expression of lipogenesis-related

genes, such as *FAS* and *SCD1* (52). An HFD stimulates fat production through activation of these genes in the liver and causes hepatic steatosis (53-55). ZH effectively reduced the mRNA levels of *SREBP1c*, *FAS* and *SCD1*, which might have triggered a reduction in lipid and TG accumulation in the liver. In particular, these mRNA levels were significantly reduced in the ZHH group as compared with the HFD group.

The results of the present study suggested that ZH inhibited adipocyte differentiation and hepatic lipid accumulation, and that this effect was significantly more pronounced than that of treatment with each extract alone. Furthermore, the results suggested that the ZH mixture suppressed lipid accumulation in the liver and adipose tissue by regulating gene expression *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

CHJ contributed to the study concept and design; CHJ and SHP wrote the manuscript; SHP, HIC and DHL performed the *in vivo* experiments; SHP and DHL performed the *in vitro* experiments; and JA supported *in vivo* experiment design and related result analyses. YJJ and TYH contributed in the revisions of manuscript and performed statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Korea Food Research Institute Ethnics Committee (approval no. KFRI-M-18001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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