Lipid-lowering effects of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) in hyperlipidemic rats and lipolytic effects in 3T3-L1 adipocytes

Kyoung Kon Kim¹, Tae Woo Kim², Yun Hwan Kang³, Dae Jung Kim², and Myeon Choe^{1,2,*}

¹Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon, Gangwon 24341, Korea

²Well-Being Bioproducts R&D Regional Innovation Center, Kangwon National University, Chuncheon, Gangwon 24341, Korea

³Applied Product Development Team, National Development Institute of Korean Medicine, Gyeongsan, Gyeongbuk 38540, Korea

Received February 12, 2016
Revised May 31, 2016
Accepted June 26, 2016
Published online October 31, 2016

*Corresponding Author
Tel: +82-33-250-8645
Fax: +82-33-250-7451
E-mail: mchoe@kangwon.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract On the basis of the antiatherosclerotic effect of *Zanthoxylum schinifolium*, the therapeutic potential of *Zanthoxylum schinifolium* seed oil (ZSO) was tested in terms of the blood lipid profile and obesity in rats. The lipolytic effects of ZSO were determined in adipocytes and the total body and liver weight were decreased in rats. Compared with the high-cholesterol high-fat (HCHF) group, the rats in the HCHF+ZSO group showed improved levels of hyperlipidemia indicators. Furthermore, western blot analysis confirmed that the improvement of hyperlipidemia indicators was induced by stimulation of lipoprotein lipase expression. Additional results indicated that the reduction in body weight was likely caused by phosphorylation of hormone-sensitive lipase (HSL) via the protein kinase A pathway, ultimately leading to lipolysis. In conclusion, the results of the *in vivo* experiment showed that ZSO improved the lipid profiles in the blood, lowering cardiovascular disease and arteriosclerosis and degrading cellular lipids by activating HSL.

Keywords: hormone-sensitive lipase, hyperlipidemia, lipolysis, protein kinase A, *Zanthoxylum schinifolium* Siebold & Zucc. seed oil

Introduction

Dietary lipids not only serve as an energy source but also play physiological roles in promoting satiety and the sense of taste by improving absorption of essential fatty acids and fat-soluble vitamins. They also enhance quality of flavor and texture and control lipid metabolism in the body (1,2). In this sense, oil from *Zanthoxylum schinifolium* is thought to be an important resource that not only serves as an energy source and regulates metabolism but also controls satiety as well as flavor and taste of food, with its own aroma and taste (3,4). Nevertheless, the production of *Zanthoxylum schinifolium* oil has not been industrialized, allowing for harvesting of only a limited amount through the cottage process, which requires further research and development.

It is known that the concentration of cholesterol in blood is an important factor of cardiovascular diseases (5), and hypercholesterolemia, together with smoking and hypertension, is known to be a major causal factor of arteriosclerosis (6). With recent economic growth and a Westernized diet, the uptake of animal products and lipids has risen, leading to an increase in the prevalence of cardiovascular diseases (7). Thus, studies on foods that can control

the blood level of cholesterol and the composition of lipoproteins have been intensively pursued to prevent and cure cardiovascular diseases.

Lipoprotein lipase (LPL) is an enzyme that belongs to the lipase family expressed in various organs and cell types such as those of the liver, heart, adipose tissue, and brain. LPL is transported to cellular membranes, the extracellular space, or endothelial cells and degrades triglycerides contained in chylomicrons or very low-density lipoproteins, converting them to a fatty acid and glycerol and facilitating cellular absorption of fatty acids (8,9). In addition, degradation of lipids and their absorption via active expression of LPL can improve the blood lipid profile (10,11). Nonetheless, this process may promote metabolic diseases via accumulation of cellular lipids (12).

Recently, numerous studies have been devoted to understanding how to prevent the accumulation of cellular lipids or to increase their degradation. It is known that lipid droplets in adipocytes perform an important function in the regulation of lipid metabolism. Degradation of triglycerides, a major component of lipid droplets, and the release of glycerol as a triglyceride degradation product are also thought to be key mechanisms that reduce cellular lipid accumulation and serve

as biomarkers (13,14). In particular, lipases are crucial for degradation of triglycerides (14).

Activation of β -adrenergic receptor (β -AR) by catecholamines increases the cellular cyclic AMP (cAMP) level, which activates PKA, eventually promoting hormone-sensitive lipase (HSL) activity. Activated HSL degrades triglycerides to glycerol and fatty acids in the adipocyte (15,16). HSL is known as one of the lipases with major involvement in the degradation of fats in lipid droplets and is regulated by hormones such as insulin and epinephrine (15). It is known that phosphorylation by PKA is an important mechanism of control over HSL. Phosphorylation of Ser660 in HSL induces translocation of HSL to lipid droplets, whereas phosphorylation of Ser563 and Ser600 activates lipase activity (15,17). In this study, we explored the beneficial effects of oil from *Zanthoxylum schinifolium* seeds on the blood lipid profile *in vivo* as well as the anti-obesity effect and its mechanism mediated by lipid degradation in adipocytes.

Materials and Methods

Preparation of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO)

To prepare ZSO, 550 g of *Zanthoxylum schinifolium* seeds was mixed with 700 mL of n-hexane and sonicated for 30 min for extraction. After 24 h of immersion, the extract was passed through a 0.45- μ m filter and then concentrated using a rotary evaporator (WB2000 and VU2000; Heidolph, Schwabach, Germany). The yield of seed oil (%) from 550 g of seeds was 20.4%. This extract was added to a high-cholesterol high-fat (HCHF) diet and presented to rats in a solid form.

Derivatization of the extract for gas chromatography (GC) analysis

For GC analysis, fatty acid methyl esterification was performed following the methods of Watkins *et al.* (18). Twenty-five milligrams of the extract was transferred to a cap tube, mixed with 1.5 mL of 0.5 N methanolic NaOH, and the mixture was heated at 100°C for 5 min using a heating block. Because plant lipids contain small amounts of triglycerides, saponification was then used to induce methylation. The heated mixture was cooled down for 1–2 min in a water bath maintaining the temperature at 30–40°C. Two milliliters of 14% BF-methanol (Supelco Inc., Bellefonte, PA, USA) was added to the cooled mixture as a catalyst. Next, the mixture was vigorously vortexed and heated for 2 min at approximately 100°C on the heating block and cooled down at 30–40°C for 1–2 min in the water bath. To separate the layers, 2 mL of iso-octane and 1 mL of saturated NaCl were added to the cooled mixture, and everything was mixed by vortexing. After incubation for 1–2 min, the supernatant was collected, filtered, and dehydrated using sodium sulfate to complete the sample preparation.

GC analysis of ZSO The fatty acid composition of the extracts was analyzed by GC following the general analytical method “1.1.5.4 fatty

acid” recommended by the Ministry of Food and Drug Safety for food processing (19). Fatty acid standards were prepared by dissolving 100 mg of fatty acid methylester mixtures (37 chemical species) FAME Mix C4-C24 (Supelco Inc.) in 1 mL of iso-octane. For quantification of fatty acids, GC (Agilent 6890N GC/FID; Agilent Technologies, Santa Clara, CA, USA) was used, and the retention time of the 37 fatty acids was detected by GC/MSD (Agilent 5975C; Agilent Technologies) using exactly the same conditions as described by Kim *et al.* (20).

Experimental animals and diets The *in vivo* experiment on animals was performed as described by Oh *et al.* (21) with modifications. Male Sprague-Dawley (SD) rats were purchased from Central Lab Animal, Inc. (Seoul, Korea) and fed a normal diet for a week. Randomized block design was utilized to include five groups with various diets, namely, a normal diet (ND), HCHF diet, HCHF and simvastatin (HCHFS), HCHF and 7% of ZSO (HCHFZ 7%), and HCHF and 15% of ZSO (HCHFZ 15%). The amount of mixed oil was determined on the basis of the studies of Kabir and Ide (22) and Ayerza and Coates (23). The composition of both the normal diet and experimental diets is listed in Table 1. The final concentration of admixed simvastatin in the diet corresponded to the dose 10 mg/[(kg body weight)·day] based on the daily uptake with food. Each group consisted of 5–6 rats, and each cage contained a single rat receiving one of the diets for 4 weeks. The growth conditions for rats consisted of 21 $\pm$ 2°C with 50 $\pm$ 10% of consistent humidity, and the light cycle was 12-h light/12-h dark. The diet was pair-fed every other day and water was provided *ad libitum*. Body weight was measured every other week. All animal experiments in the present study were conducted in accordance with the regulations of the Kangwon National University Institutional Animal Care and Use Committee (approval # KW-140306-1).

Blood plasma and tissue collection To assess the changes in lipid levels induced by the ZSO-supplemented diets, rats were anesthetized with ether, and 0.5 mL of blood was drawn from the tail artery. The blood sample was collected at 0 weeks and after 4 weeks. The collected sample was centrifuged at 4,000 $\times g$ for 20 min to separate the plasma supernatant. After the *in vivo* dietary experiment, the diet was stopped for 12 h prior to anesthesia with Avertin and killing of the rats. The abdomen was incised and blood was collected by cardiac puncture. Blood plasma was separated using the above procedure and was stored at –70°C until analysis. The liver and epididymal adipose tissues were excised and washed with normal saline, the surface moisture was blotted, and the tissues were frozen in liquid nitrogen immediately after weighing and were stored at –70°C.

Analysis of lipid composition of blood plasma Total cholesterol (TC) of the separated plasma was quantified using a cholesterol oxidase assay kit (V-cholesterol; Asanpharm, Seoul, Korea). Triglyceride

content was of plasma quantified using a kit based on free glycerol elimination (TG-s; Asanpharm). HDL was quantified using a kit that utilizes phosphotungstic acid $MgCl_2$ sedimentation (HDL-cholesterol; Asanpharm). LDL was calculated using the Friedewald equation:

$$LDL\text{-cholesterol} = \text{total cholesterol} - [\text{HDL-cholesterol} + (\text{triglycerides}/5)]$$

The atherogenic index (AI) was calculated using the method of Popa *et al.* (24):

$$\text{Atherogenic Index (AI)} = \text{TC}/\text{HDL}$$

Induction of 3T3-L1 cell differentiation and treatment 3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea) containing 10% of fetal bovine serum (FBS; Welgene) and 1% of penicillin-streptomycin (Welgene) at 37°C and 5% of CO_2 .

Differentiation of 3T3-L1 cells and lipid accumulation were assessed by the method of Oh *et al.* (21). To induce 3T3-L1 preadipocytes to differentiate into adipocytes, the cells were seeded in 96-well plates at 5×10^5 /well and incubated for 2 days. Differentiation was then initiated by incubating the cells with the DMEM medium containing an MDI solution (0.5 mM 3-isobutyl-1-methylxanthine [IBMX], 1 μ M dexamethasone, and 10 μ g/mL insulin) and 10% of FBS for 3 days, and differentiation was maintained in DMEM containing 10 μ g/mL insulin and 10% of FBS for 3 days. Finally, by incubating the cells in the DMEM medium supplemented with 10% of FBS only, differentiation into adipocytes (that form lipid droplets via cellular lipid accumulation) was completed. To quantify the accumulation of lipids, the size and quantity of lipid droplets were measured under a microscope after completely differentiated cells were fixed with 10% formalin and stained with an Oil Red O staining solution for 1 h. The Oil Red O dye in lipid droplets was extracted with 100% isopropanol, and absorbance at 520 nm was measured on a microplate reader to determine the relative amount of lipid droplets.

When lipid droplets were detected after cellular differentiation into adipocytes, 0.05 mg/mL ZSO was added to the medium and additional incubation was performed for 3 days to test whether the lipid droplets decreased.

In order to assess the lipolytic effect of ZSO, the expression of proteins related to lipid degradation in adipocytes was measured in adipocytes treated with ZSO for 24 h. In addition, to test whether the mechanism of the lipolytic effect of ZSO is dependent on the PKA pathway, the cells were treated with 10 μ M H89 (Sigma-Aldrich, St. Louis, MO, USA), a known PKA inhibitor, together with ZSO for 24 h.

Western blot analysis Identical amounts of protein quantified by the Bradford method were added to sample buffer containing sodium dodecyl sulfate (SDS) and β -mercaptoethanol in a ratio of 3:1, and this mixture was heated at 100°C for 3 min. The prepared protein samples were loaded onto an SDS gel for electrophoresis and then

Table 1. Composition of experimental diets (unit: g/kg diet)

	Normal	HCHF ¹⁾	HCHFS ²⁾	HCHFZ 7% ³⁾	HCHFZ 15% ⁴⁾
Casein	200	200	200	200	200
Corn Starch	223	223	223	223	223
Sucrose	250	250	250	250	250
Cellulose	50	50	50	50	50
ZSO ⁵⁾				70	150
Soybean Oil	70	150	150	80	
Lard		70	70	70	70
Mineral mix ⁶⁾	35	48	48	48	48
Vitamin mix ⁷⁾	10	21	21	21	21
Cholesterol		12.5	12.5	12.5	12.5
Cholic acid		5	5	5	5
Simvastatin			0.2		

¹⁾HCHF, high-cholesterol and high-fat diet

²⁾HCHFS, high-cholesterol and high-fat diet with simvastatin

³⁾HCHFZ 7%, high-cholesterol and high-fat diet with 7% *Zanthoxylum schinifolium* Siebold & Zucc. seed oil

⁴⁾HCHFZ 15%, high-cholesterol and high-fat diet with 15% *Zanthoxylum schinifolium* Siebold & Zucc. seed oil

⁵⁾ZSO, *Zanthoxylum schinifolium* Siebold & Zucc. seed oil

⁶⁾AIN-96M mineral mixture

⁷⁾AIN-96M vitamin mixture

transferred to a polyvinylidene fluoride membrane (0.45- μ m PVDF transfer membrane, Thermo Fisher Scientific, Waltham, MA, USA). Membrane blocking was performed for 2 h using Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% skim milk. The membrane was then incubated in a buffer containing the primary antibody cocktail against lipoprotein lipase (1:1,000), leptin (1:1,000; Abcam, Cambridge, UK), PKA (1:2,500), phospho-PKA (1:2,000), a phospho-PKA substrate (1:2,000), hormone-sensitive lipase (HSL; 1:3,000), GAPDH (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-HSL (Ser563; 1:1,000), and p-HSL (Ser660; 1:1,000; Cell Signaling Technology, Danvers, MA, USA) for 2 h and washed with TBS-T for 5 min. This step was repeated three times. The washed membrane was incubated in a buffer containing a horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology) for 1 h, and X-ray film was exposed to the membrane and developed using the enhanced chemiluminescence method. Intensity of the detected bands was analyzed in the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of released glycerol Differentiated 3T3-L1 adipocytes were incubated with 0.01 or 0.05 mg/mL ZSO for 24 h, and the medium used for incubation was collected for further experiments. A glycerol standard and the media were added to a 96-well plate and mixed with the Free Glycerol Detection Reagent (Abcam). The mixture was incubated at 37°C for 30 min, and absorbance at 570 nm was measured using the microplate reader.

Statistical analysis The data were expressed as mean $\pm$ standard error (SE), and comparisons of data involved Student's unpaired *t*

test or one-way analysis of variance (ANOVA), where appropriate. Mean values were considered significantly different at $p<0.05$.

Results and Discussion

The fatty acid profile of ZSO The composition of ZSO was analyzed on the basis of the retention time of a standard fatty acid mix (FAME Mix C4-C24; Supelco Inc.). The results revealed that unsaturated fatty acids including oleic acid, linoleic acid, and linolenic acid were the major components, and palmitic acid and stearic acid were detected as minor components in ZSO, consistent with the report by Oh and Chung (25). Based on the amount of undecanoic acid used as an internal standard, it was confirmed that unsaturated fatty acids constituted more than 70% of ZSO, with oleic acid at 29.23% (w/w), linoleic acid at 23.97%, and linolenic acid at 18.19%, whereas saturated fatty acids constituted approximately 20% with palmitic acid at 16.22%, stearic acid at 2.15%, and palmitoleic acid at 2.07%; trace components were responsible for 4.56% of ZSO contents (Table 2). Although these data showed an approximately 1-5% discrepancy with the results of Oh and Chung (25), who reported oleic acid at 35.36%, linoleic acid at 22.60%, linolenic acid at 15.63%, and palmitic acid at 18.51% (which is 2% greater than the level we observed), the discrepancy was marginal and did not affect the total amount of saturated fatty acids. Although ZSO showed a lower concentration of unsaturated fatty acids in our study compared to a report by Yoon and Choi (3), it is likely that the discrepancy is due to differences in the experimental procedures such as a different extraction method and harvest timing.

The effects of ZSO on body and tissue weights SD rats were fed the HCHF diet to induce hyperlipidemia. There were no significant differences in dietary intake among the groups during the experiment

Table 2. Fatty acid profiles of *Zanthoxylum schinifolium* Siebold&Zucc seed oil (ZSO)

No.	Name (acid methyl esters)	Ret time (min)	Area	Area %
1	Undecanoic acid (ISTD) ¹⁾	29.12	26.22	3.61
2	Oleic acid	47.34	212.04	29.23
3	Linoleic acid	49.35	173.85	23.97
4	Linolenic acid	51.63	131.94	18.19
5	Palmitic acid	41.61	117.67	16.22
6	Stearic acid	45.90	15.57	2.15
7	Palmitoleic acid	43.28	15.03	2.07
8	others	-	-	4.56
Total				100

¹⁾Undecanoic acid was used as an internal standard.

(data not shown). Their body weights and lipid weight in the liver and the epididymal fat were then compared with those of groups fed a diet containing simvastatin at the dose 20 mg/kg for the purpose of treatment of hyperlipidemia, or with groups fed a diet containing 7% or 15% of ZSO. The results are summarized in Fig. 1. This figure shows that the weight increase (53.20±10.61 g) induced by the HCHF diet was significantly attenuated (to 37.05±6.33 g) in the group fed 15% of ZSO after the 2-week treatment. After 4 weeks, the group fed only HCHF showed a body weight of 88.36±14.34 g, whereas the groups fed simvastatin and ZSO at 15% experienced significant losses of body weight (resulting body weight 71.08±10.97 and 67.88±7.36 g, respectively). Figure 1B shows the changes in the lipid weight in the liver and in the epididymal fat after the experiment was completed. The liver weight increase was significantly attenuated in the groups treated with simvastatin or ZSO as compared to HCHF alone, with the group fed 15% of ZSO showing the smallest weight increase. Furthermore, the liver weight increase was suppressed in the two groups treated with simvastatin or ZSO. Although the effect was not

Fig. 1. Effect of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on body, liver and epididymal adipose tissue weight in SD rats fed a HCHF diet. (A) Change of body weight during the experiment. (B) Weight of liver and epididymal adipose tissue after the end of experiment. The values shown are mean±SE (n=5-6). # $p<0.05$, ## $p<0.01$ compared to ND (white bar), * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to HCHF (black bar). ND, normal diet; HCHF, high-cholesterol and high-fat diet; HCHF5, high-cholesterol and high-fat diet with simvastatin; HCHFZ 7%, high-cholesterol and high-fat diet with ZSO 7%; HCHFZ 15%, high-cholesterol and high-fat diet with ZSO 15%.

Fig. 2. Effect of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on blood lipids of SD rats fed a HCHF diet. (A) Change of total cholesterol contents during the experiment. (B) Change of triglyceride contents during the experiment. (C) Change of HDL-cholesterol contents during the experiment. (D) Change of LDL-cholesterol contents during the experiment. (E) Atherogenic index (AI) evaluation. The values shown are mean $\pm$ SE ($n=5-6$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to HCHF (black bar). ND, normal diet; HCHF, high-cholesterol and high-fat diet; HCHFS, high-cholesterol and high-fat diet with simvastatin; HCHFZ 7%, high-cholesterol and high-fat diet with ZSO 7%; HCHFZ 15%, high-cholesterol and high-fat diet with ZSO 15%; HDL, high density lipoprotein; LDL, low-density lipoprotein.

significant, epididymal adipose tissue in the rats tended to be reduced. These results pointed to a suppressive effect of simvastatin and ZSO on the weight increase and lipid accumulation.

The beneficial effect of ZSO on the lipid profile Because abnormal upregulation of lipids in blood can cause hyperlipidemia and arteriosclerosis, it is necessary to improve the blood lipid profile. To assess the influence of the ZSO-supplemented diet on the lipid profile in blood, plasma (collected after 0 and 4 weeks) was analyzed, and the results are shown in Fig. 2. These data revealed that the HCHF diet alone successfully induced hyperlipidemia, with significantly altered levels of all the parameters analyzed as compared to the ND group. During the treatment with ZSO or simvastatin, all treatment groups showed reduced levels of TC, triglycerides, and LDL-cholesterol in comparison with the HCHF group (Fig. 2A, 2B, and 2D). When the dietary experiment was completed, group HCHF showed the highest level of TC (209.72 ± 18.52 mg/dL) followed by HCHFS (157.30 ± 50.07 mg/dL), HCHFZ 7% (148.38 ± 24.26 mg/dL), and HCHFZ 15% (138.92 ± 17.51 mg/dL), all of which showed significant differences with the

HCHF group.

A similar pattern was observed for triglycerides, with the HCHF group having the highest levels (111.20 ± 23.82 mg/dL), followed by HCHFS (71.66 ± 12.39 mg/dL), HCHFZ 7% (61.03 ± 13.73 mg/dL), and HCHFZ 15% (60.79 ± 21.93 mg/dL). Compared to group HCHF, all treatment groups had significantly lower levels of triglycerides. LDL levels were the highest in the HCHF group (169.34 ± 1.57 mg/dL), whereas groups HCHFZ 7%, HCHFZ 15%, and HCHFS had 104.89 ± 1.24 , 97.80 ± 1.21 , and 95.28 ± 2.03 mg/dL, respectively, thus each showed significant differences with the HCHF group. All treatment groups showed significantly increased HDL levels as compared to the HCHF group (18.15 ± 5.56 mg/dL), with HCHFZ 15% (28.95 ± 4.91 mg/dL), HCHFZ 7% (31.27 ± 11.27 mg/dL), and HCHFS (47.68 ± 4.11 mg/dL) showing p values <0.05 , <0.01 , and <0.001 , respectively (Fig. 2C). Based on all the parameters measured, the atherogenic index (AI) was computed, and the results revealed that the HCHF group had the highest value, at 10.55 ± 0.57 , whereas the HCHFS group the lowest level: 2.29 ± 0.09 . Groups fed ZSO at 7 and 15% showed levels similar to those of the HCHFS group: 3.74 ± 0.09 and 3.79 ± 0.5 , respectively

Fig. 3. Effect of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on lipoprotein lipase (LPL) and leptin expression in epididymal adipose tissue of SD rats fed a HCHF diet and lipoprotein lipase (LPL) expression and lipid accumulation in 3T3-L1 adipocytes. (A) The expression of LPL protein in epididymal adipose tissue of SD rats fed a HCHF diet. (B) The expression of leptin protein in epididymal adipose tissue of SD rats fed a HCHF diet. The values shown are mean $\pm$ SE ($n=5-6$). (C) The expression of LPL protein in 3T3-L1 adipocytes. (D) The lipid accumulation in 3T3-L1 adipocytes. (A) and (B): *** $p<0.001$ compared to HCHF (black bar). ND, normal diet; HCHF, high-cholesterol and high-fat diet; HCHFS, high-cholesterol and high-fat diet with simvastatin; HCHFZ, high-cholesterol and high-fat diet with ZSO 15%. (C) and (D): * $p<0.05$, *** $p<0.001$ compared to adipocyte (black bar). MDI indicates 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μ M dexamethasone, and 10 μ g/mL insulin mixture and this mixture induces adipocyte differentiation.

(Fig. 2E).

These results revealed that ZSO and simvastatin (separately) could reduce the risk of cardiovascular diseases and arteriosclerosis (26,27). Furthermore, significant upregulation of HSL, which reduces cholesterol and protects blood vessels (28), was observed in the groups treated with simvastatin or ZSO, suggesting that ZSO ingestion improves the lipid profile in blood and lowers the risk of arteriosclerosis. The AI, which is based on the formula of Popa *et al.* (24), also revealed a significant beneficial effect of ZSO, although it was weaker than that of simvastatin.

The impact of ZSO on the expression of LPL and leptin in epididymal adipose tissue, LPL expression in 3T3-L1 adipocytes, and on lipid accumulation LPL is known to be a lipolytic enzyme expressed in various tissues, including tissues of the liver, heart, and brain and adipose tissue, whereas leptin is known as one of the adipokines released by adipose tissue into the bloodstream. Using the protein extracted from epididymis fat of the rats used in this experiment, LPL and leptin expression was assessed. The results revealed that LPL expression was significantly increased in groups HCHFS (2.08-fold) and HCHFZ 15% (1.48-fold) compared to the HCHF

Fig. 4. Effect of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on protein kinase A (PKA) activity and PKA dependence of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on HSL phosphorylation in 3T3-L1 adipocytes. (A) The expression of PKA and phosphorylated PKA (p-PKA) protein in 3T3-L1 adipocytes. (B) The expression of p-PKA substrate proteins in 3T3-L1 adipocytes. (C) The expression of PKA and phosphorylated PKA (p-PKA) protein in 3T3-L1 adipocytes. (D) The expression of p-PKA substrate proteins in 3T3-L1 adipocytes. (E) The expression of p-HSL^{ser563} and p-HSL^{ser660} proteins in 3T3-L1 adipocytes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to adipocyte (black bar), ## $p < 0.01$ compared to ZSO (gray bar). ZSO, *Zanthoxylum schinifolium* Siebold & Zucc. seed oil 0.05 mg/mL.

group (Fig. 3A). Leptin expression was significantly decreased in groups HCHF5 (0.65-fold) and HCHFZ 15% (0.57-fold) compared to the HCHF group (Fig. 3B).

According to the increased LPL and decreased leptin levels

observed in the *in vivo* experiment described above, we hypothesized that 3T3-L1 adipocytes would show a similar response to ZSO treatment. To test this hypothesis, Oil Red O staining was performed to evaluate LPL expression and suppression of lipid accumulation.

Fig. 4. Continued

The results revealed that LPL expression increased up to 2.61- and 4.38-fold after treatment with ZSO at 0.01 or 0.05 mg/mL, respectively, indicating a dose-dependent increase (Fig. 3C). Fig. 3D shows that lipid accumulation was significantly reduced in the differentiated 3T3-L1 adipocytes after treatment with 0.05 mg/mL ZSO.

It was reported that simvastatin serves as an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (29), and *Ocimum sanctum* L. suppresses lipid synthesis (30): examples of a mechanism underlying improvement of blood lipid profiles. In this study, when the ZSO treatment was added to the HCHF diet, analysis of the blood lipid profile revealed downregulation of triglycerides to the level of the ND group and increased expression of LPL. The latter is known to play a crucial role in cellular absorption of triglycerides (8,9). By means of the protein extracted from epididymal adipose tissue of the rats, it was confirmed that ZSO treatment increased the expression of LPL protein, as did simvastatin. It is expected that the upregulation of LPL by ZSO will degrade triglycerides in chylomicrons or very low-density lipoprotein, promoting cellular absorption of triglycerides.

Leptin is a hormone that maintains storage of body lipids in adipocytes. The production of leptin increases when lipid tissue increases (31). In groups treated with simvastatin and ZSO, a significant reduction in leptin expression was detected as compared to the HCHF diet group, pointing to a suppressive effect of ZSO on the body weight increase and lipid accumulation.

Dependence of ZSO effects on the PKA pathway and the impact of ZSO on lipolysis in 3T3-L1 adipocytes We found that lipid accumulation in adipocytes decreased after ZSO treatment, and this result prompted us to test expression of PKA and its activity in order to elucidate the mechanism of cellular lipid degradation. The results

showed that there was no change in PKA expression in cells treated with ZSO at 0.01 or 0.05 mg/mL, whereas phosphorylated PKA (p-PKA) was upregulated in a dose-dependent manner, showing an increase of 2.86- and 3.49-fold, respectively, as compared to untreated cells (Fig. 4A). Phosphorylation of a PKA substrate was also measured to test PKA activity indirectly. The results revealed that the levels of phosphorylated PKA substrates (p-PKA substrates) increased up to 3.42- and 5.35-fold in cells treated with ZSO at 0.01 or 0.05 mg/mL, respectively, as compared to untreated adipocytes; thus, there was a dose-dependent increase (Fig. 4B).

HSL is the most well-known lipase that degrades lipids accumulated in adipocytes and is known to be regulated by PKA activity. We found that ZSO increased PKA activity in this study and next tested whether the effect of ZSO on HSL is dependent on the PKA pathway and whether activated PKA promotes HSL activation. The results showed that the increased p-PKA level (a 2.4-fold increase relative to untreated adipocytes) in cells treated with 0.05 mg/mL ZSO was significantly attenuated after treatment with the PKA inhibitor H89 at 10 μ M, showing a 25% reduction in comparison with the cells treated with 0.05 mg/mL ZSO without the inhibitor (Fig. 4C). The amount of phosphorylated PKA substrate protein in the cells treated with 0.05 mg/mL ZSO increased up to approximately twofold as compared with untreated adipocytes. When the ZSO-treated cells were incubated with H89, that phosphorylation level decreased to almost the level of untreated adipocytes (Fig. 4D). Analysis of HSL expression activated via phosphorylation by PKA revealed that treatment with either ZSO or H89 did not change the expression of HSL, but ZSO treatment caused phosphorylation of Ser563 in HSL, which promotes its enzymatic activity, increasing p-HSLSer563 1.5-fold (as compared to untreated adipocytes). In addition, we found that the level of p-HSLSer563 that was increased by ZSO treatment

Fig. 5. Effect of *Zanthoxylum schinifolium* Siebold & Zucc. seed oil (ZSO) on glycerol release in 3T3-L1 adipocytes. After differentiation, the 3T3-L1 adipocytes were incubated with ZSO 0.01 mg/mL and 0.05 mg/mL. Glycerol release was measured using a commercial glycerol assay kit. The values shown are mean±SE. *** $p < 0.001$ compared to adipocyte (white bar).

decreased when 10 μ M H89 was added to the culture medium. Nonetheless, there was no alteration of phosphorylation of Ser660, which is known to promote HSL transfer to lipid droplets (Fig. 4E). To test the impact of ZSO on lipolysis in 3T3-L1 adipocytes, we quantified glycerol released into the medium. The results showed that the medium from cells incubated with 0.01 or 0.05 mg/mL ZSO accumulated higher levels of glycerol (102.09±3.03 and 115.04±2.83%, respectively) as compared to untreated adipocytes (100±3.17%), suggesting that the glycerol release depended on the ZSO concentration (Fig. 5).

To identify the mechanism underlying the suppression of body weight and lipid accumulation, another experiment was conducted on 3T3-L1 adipocytes. First, ZSO treatment showed induction of LPL and suppression of lipid accumulation just as in epididymal adipose tissue. We hypothesized that activation of a lipase was required to promote lipid degradation in order to prevent cellular lipid accumulation. To test this hypothesis, we assessed the activity of HSL among various lipases in adipocytes. HSL is known to be a major enzyme that together with adipose triglyceride lipase degrades triglycerides. It is also known that degradation of cellular lipids is regulated by extracellular signals, and HSL is known as a rate-limiting enzyme of diglyceride degradation (32). By quantifying the phosphorylated form of PKA and a phosphorylated PKA substrate protein that activates HSL via its phosphorylation, we showed here that ZSO treatment activated cellular PKA via phosphorylation of Ser563, which activates lipolytic function of HSL (16).

Nonetheless, it was found that ZSO treatment did not affect phosphorylation of Ser660 which induces the transfer of HSL to lipid droplets. In addition, by treating cells with H89 (PKA inhibitor) together with ZSO, we could obtain evidence that the mechanism of ZSO action involves activation of PKA. Finally, quantification of the glycerol released into the medium after incubation of cells with ZSO revealed that activation of HSL promotes degradation of cellular triglycerides and significantly increases the glycerol release into the extracellular space.

In conclusion, ZSO contains high levels of physiologically useful unsaturated fatty acids. Results of the *in vivo* experiment showed that ZSO improves the lipid profile of blood (possibly lowering the risk of cardiovascular disease and arteriosclerosis) and causes degradation of cellular lipids by activating HSL. Taken together, our findings provide evidence that ZSO may be a useful resource with health-promoting effects.

Acknowledgments This research was supported by High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs (312001-03-03-HD040), Well-being Bioproducts R&D Regional Innovation Center project (B0009702) and Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the ministry of Education (2014R1A1A2053358).

Disclosure The authors declare no conflict of interest.

References

1. Lim JK. Oil and fat industry. *Food Ind.* 181: 10-37 (2004)
2. Gurr MI, Harwood JL, Frayn KN. *Lipid biochemistry*. 5th ed. Blackwell Science Ltd., Oxford, UK. pp. 127-169 (2002)
3. Yoon DH, Choi YS. Influence of red pepper (*Capsicum annuum* L.) seed oil and sancho (*Zanthoxylum schinifolium*) seed oil on serum and liver lipids profiles in rats. *Korean J. Food Sci. Technol.* 40: 96-100 (2008)
4. Mun SI. Effects of *Zanthoxylum schinifolium* and its active principle on serum lipid levels in carbon tetrachloride-treated mice. *J. Korean Soc. Food Sci. Nutr.* 13: 249-254 (2000)
5. NIH Consensus Development Conference Statement. Lowering blood cholesterol to prevent heart disease. *Arteriosclerosis* 5: 404-412 (1985)
6. Muntner P, He J, Astor BC, Folsom AR, Coresh J. Traditional and nontraditional risk factors predict coronary heart disease in chronic kidney disease: Results from the atherosclerosis risk in communities study. *J. Am. Soc. Nephrol.* 16: 529-538 (2005)
7. Kim MJ, Jun HY, Kim JH. Antiadipogenic effect of Korean glasswort (*Salicornia herbacea* L.) water extract on 3T3-L1 adipocytes. *J. Korean Soc. Food Sci. Nutr.* 43: 814-821 (2014)
8. Wang H, Eckel RH. Lipoprotein lipase: from gene to obesity. *Am. J. Physiol. Endoc. M.* 297: E271-E288 (2009)
9. Braun JE, Severson DL. Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem. J.* 287: 337-347 (1992)
10. Amar MJ, Sakurai T, Sakurai-Ikuta A, Sviridov D, Freeman L, Ahsan L, Remaley AT. A novel apolipoprotein C-II mimetic peptide that activates lipoprotein lipase and decreases serum triglycerides in apolipoprotein E-knockout mice. *J. Pharmacol. Exp. Ther.* 352: 227-235 (2015)
11. Weinstock PH1, Bisgaier CL, Aalto-Setälä K, Radner H, Ramakrishnan R, Levak-Frank S, Essenburg AD, Zechner R, Breslow JL. Severe hypertriglyceridemia, reduced high density lipoprotein, and neonatal death in lipoprotein lipase knockout mice. Mild hypertriglyceridemia with impaired very low density lipoprotein clearance in heterozygotes. *J. Clin. Invest.* 96: 2555-2568 (1995)
12. Kopelman PG. Obesity as a medical problem. *Nature* 404: 635-643 (2000)
13. Okabe Y, Shimada T, Horikawa T, Kinoshita K, Koyama K, Ichinose K, Aburada M, Takahashi K. Suppression of adipocyte hypertrophy by polymethoxyflavonoids isolated from *Kaempferia parviflora*. *Phytomedicine* 21: 800-806 (2014)
14. Chaves VE, Frasson D, Kawashita NH. Several agents and pathways regulate lipolysis in adipocytes. *Biochimie* 93: 1631-1640 (2011)
15. Holm C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. T.* 31: 1120-1124 (2003)
16. Jaworski K, Sarkadi-Nagy E, Duncan RE, Ahmadian M, Sul HS. Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue. *Am. J. Physiol.-gastr. L.* 293: G1-G4 (2007)
17. Kim SO, Sakchaisri K, Asami Y, Ryoo IJ, Choo SJ, Yoo ID, Soung NK, Kim YS, Jang JH, Kim BY, Ahn JS. Illudins C2 and C3 stimulate lipolysis in 3T3-L1 adipocytes and suppress adipogenesis in 3T3-L1 preadipocytes. *J. Nat. Prod.* 77: 744-750 (2014)
18. Watkins BA, Shen CL, McMurtry JP, Xu H, Bain SD, Allen KG, Seifert MF. Dietary

- lipids modulate bone prostaglandin E2 production, insulin-like growth factor-I concentration and formation rate in chicks. *J. Nutr.* 127: 1084-1091 (1997)
19. Ministry of Food and Drug Safety. Available from: http://fse.foodnara.go.kr/residue/RS/jsp/menu_02_01_01.jsp. Accessed Apr. 03, 2015.
 20. Kim KK, Kang YH, Kim DJ, Kim TW, Lee JI, Choe M. Analysis of fatty acid composition and effects of pumpkin seed oil on human umbilical vein endothelial cells. *J. East Asian Soc. Diet. Life* 24: 351-358 (2014)
 21. Oh SD, Kim M, Min BJ, Choi GS, Kim SK, Bae H, Kang C, Kim DG, Park BJ, Kim CK. Effect of *Achyranthes bidentata* Blume on 3T3-L1 adipogenesis and rats fed with a high-fat diet. *Evid-Based Compl. Alt.* 2014: 158018 (2014)
 22. Kabir Y, Ide T. Activity of hepatic fatty acid oxidation enzymes in rats fed α -linolenic acid. *Biochim. Biophys. Acta* 1304: 105-119 (1996)
 23. Ayerza R, Coates W. Ground chia seed and chia oil effects on plasma lipids and fatty acids in the rat. *Nutr. Res.* 25: 995-1003 (2005)
 24. Popa CD, Arts E, Fransen J, van Riel PL. Atherogenic index and high-density lipoprotein cholesterol as cardiovascular risk determinants in rheumatoid arthritis: the impact of therapy with biologicals. *Mediat. Inflamm.* 2012: 785946 (2012)
 25. Oh M, Chung MS. Effects of oil and essential oils from seeds of *Zanthoxylum schinifolium* against foodborne viral surrogates. *Evid-Based Compl. Alt.* 2014: 135797 (2014)
 26. Ansell BJ, Watson KE, Fogelman AM. An evidence-based assessment of the NCEP adult treatment panel II guidelines. national cholesterol education program. *JAMA* 282: 2051-2057 (1999)
 27. Anthony MS, Clarkson TB, Bullock BC, Wagner JD. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscl. Throm. Vas.* 17: 2524-2531 (1997)
 28. Sirtori CR. HDL and the progression of atherosclerosis: New insight. *Eur. Heart J. Suppl.* 8(supplement F): F4-F9 (2006)
 29. Vickers S, Duncan CA, Vyas KP, Kari PH, Arison B, Prakash SR, Ramjit HG, Pitzenberger SM, Stokker G, Duggan DE. *In vitro* and *in vivo* biotransformation of simvastatin, an inhibitor of HMG CoA reductase. *Drug Metab. Dispos.* 18: 476-483 (1990)
 30. Suanarunsawat T, Boonnak T, Na Ayutthaya WD, Thirawarapan S. Anti-hyperlipidemic and cardioprotective effects of *Ocimum sanctum* L. fixed oil in rats fed a high fat diet. *J. Basic Clin. Physiol. Pharmacol.* 21: 387-400 (2010)
 31. Behme MT. Leptin: Product of the obese gene. *Nutr. Today* 31: 138-141 (1996)
 32. Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, Zechner R. Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *J. Biol. Chem.* 277: 4806-4815 (2002)