Anti-Obesity Effects of Extracts from Sulfur-Grain Maggot In Obesity Model Rats

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ABSTRACT
This research tried to explain only a part of mechanisms possibly involved in anti-obesity effects of the Extracts of Sulfur-grain Maggot (ESM). Animals were classified into a normal diet group (NC, normal control), HFD (high-fat diet without ESM), HFD 15 (high-fat diet+oral administration of 15 mg of ESM extract/100 g body weight) and HFD 30 (high-fat diet+oral administration of 30 mg of ESM extract/100 g body weight). The body weight gain declined in HFD 15 and HFD 30 groups compared with the HFD group, even though the diet intake increased significantly. The weight of liver and adipose tissue decreased significantly in HFD 15 and 30 groups compared with the control HFD. Triglyceride, total cholesterol, low density lipoprotein-cholesterol (LDL-C) and Atherogenic Index (AI) decreased in HFD 15 and HFD 30 groups compared with the HFD group, but the contents of high density lipoprotein-cholesterol (HDL-C) increased significantly. Expression of sterol regulatory element binding proteins (SREBP-1α, SREBP-2)-mRNA in the liver was decreased in HFD 15 and 30 groups compared with the control HFD. Triglyceride, total cholesterol, low density lipoprotein-cholesterol (LDL-C) and Atherogenic Index (AI) decreased in HFD 15 and HFD 30 groups compared with the control HFD, but the contents of high density lipoprotein-cholesterol (HDL-C) increased significantly. Expression of sterol regulatory element binding proteins (SREBP-1α, SREBP-2)-mRNA in the liver was decreased in HFD 15 and 30 groups compared with the control HFD but the expression of lipoprotein lipase (LPL) and peroxisome proliferator-activated receptors(PPARα)-mRNA in adipose tissue increased significantly. Fat accumulation in the liver tissues and liver damage were greatly reduced in HFD 15 and 30 groups compared with the HFD group. In conclusion, this research discovered for the first time that grain maggot has anti-obesity effects, by reducing the abdominal fat of obese model animals and lowering blood lipid level through the down-regulation of SREBP-1α, SREBP-2 and the up-regulation of LPL-mRNA, not only PPARα-mRNA.

Key words: Sulfur-grain maggot, lipid profile, genes, obesity

INTRODUCTION
Today, we are witnessing a growing incidence rate of hyperlipidemia, CVD (coronary vascular disease), obesity and type 2 diabetes, all of which are metabolic diseases caused by dietary habits. Obesity is caused by an imbalance of energy metabolism, with excessive energy stored in the adipocytes and accumulated within the body. Obesity can cause hyperlipidemia, hypertension, fatty liver, diabetes, CVD and carcinogenesis by inducing insulin resistance and increasing inflammatory response (Bray et al., 2004; Adaramoye et al., 2008). The rise of blood LDL-C caused by the intake of a high-fat diet has been identified as a main cause of death from CVD (Yamaguchi et al., 2012). Gene expression of SREBP-1α, SREBP-2, PPARα and LPL regulates lipid metabolism, so it has a close relationship with obesity (Wang and Eckel, 2009; Rodriguez-Cantu et al., 2011; Rotllan and Carlos, 2012). Sulfur has been known to have antioxidation (Atmaca, 2004), anti-obesity (Ban et al., 2012) and anti-cancer effects (Wu et al., 2005; Lee et al., 2008), as well as preventive effects for rheumatoid arthritis (Hasegawa et al., 2004; Kim et al., 2006). It also has other diverse bioactive effects related to hemostasis, neural paralysis and cold limbs. To increase the efficacy of sulfur, sulfur toxicity clearance technology has been developed along with poultry production technology using sulfur.
Biotherapies began to be applied to medicine such as the treatment of patients with chronic wound infection and the treatment of burns or pressure sores by utilizing Musca domestica L. (Sherman et al., 2000; Scavee et al., 2003). In particular, Musca domestica L. treatment is being recommended for patients infected with gram positive bacteria including Staphylococcus aureus. It has been reported that Musca domestica L. extract contains strong antibacterial peptide. In Republic of Korea, Musca domestica L. has been known as grain maggot since a long time ago and has been used as a folk medicine (Jaklic et al., 2008; Park et al., 2010b). The grain maggot is non-toxic and its diverse pharmacological effects are well known. The effects of the grain maggot are described in old books as follows: Grain maggot purifies the blood and lowers fever (Chinese Dictionary) and effective for lowering fever (Ben Cao Gang Mu or Compendium of Materia Medica), reduces blood lipid and sugar and protection of alcoholic liver damage (Park et al., 2010b). Recently, in an attempt to create a synergy between the bioactive effects of sulfur and grain maggot, bioconversion technology was developed to produce high-functional sulfur-grain maggot (Park and Park, 2012). However, the bioactive effects of sulfur-grain maggot in terms of anti-obesity are not well known. This study orally administered the extract of sulfur-grain maggot to obesity model rats to identify its effect on the anti-obesity and the level of blood lipid, as well as the mechanism by which it functions.

MATERIALS AND METHODS

Preparation of extracts: Insect bio (Chuncheon, Gangwon Province, Republic of Korea) provided sulfur-maggot (grain maggot produced with 2.5% of sulfur powder in medium) that was grown in an artificial environment using corn, sugar and milk powdered formula as medium which was dried in an oven at 70°C. A defatted sample was obtained by compressing for 30 min with 1,000 PSI at a high temperature of 150°C and removing the lipid completely with hexane. After mixing a defatted sample with ethanol (w/v 1:10), reflux condensing system was used for repeated extraction three times for 3 h each at 60°C to obtain an ethanol extract sample. The extract underwent vacuum evaporation using a rotary vacuum evaporator (Eyela N-1000, Tokyo Rikakiki Co., Japan) and was stored in a refrigerator to be used as a sample. Through this method, we secured 3.50 g extract of ESM containing 0.37% of sulfur from 100 g of maggots dried.

Experimental design and feeding management: Animal testing was conducted in compliance with EEC Directive of 1986; 86/609/EEC and approval was obtained from IACUC (Institutional Animal Care and Use Committees) of Kangwon National University (No. 20100017). We purchased 40 eight-week old (average weight: 200±2.50 g) Sprague-Dawley strain male rats from Daehan Bio Link (Eumseong, Chungbuk). In order to verify the varying clinical effectiveness per gender of subject animals, we used male animals primarily and the 2nd phase with female ones is under way. Reasons for selecting males first were: Hormonal changes from estrous cycles of females may influence the results and researchers prefer males as they reduce deviations in results and improve reliability. After a week of acclimatization, they were raised for 40 days with oral administration of the extract. Treatment groups were divided into NC (normal control group with purified pellet diet), HFD (high-fat diet control group without ESM), HFD 15 (high-fat diet group with oral administration of ESM 15 mg/100 g body weight) and HFD 30 (high-fat diet group with oral administration of ESM 30 mg/100 g body weight) and rats were raised separately in individual cages with 10 repetitions. Purified pellet diet (g/100 g) was made by mixing casein 20.0, corn starch 51.15, maltodextrin 10.0, sugar 7.0, soybean oil 5.0, powdered cellulose 5.0, AIN 93G mineral mixture 1.00, AIN 93G vitamin mixture 0.30, L-cystine 0.30, Choline bitartrate 0.25 and t-Butylhydroquinone 0.0014. For obese model animals, a high-fat diet containing 40 g/100 g lipid was used (Park et al., 2013). High-fat diet (w/w, g/100 g diet) was made by mixing soybean oil 5and lard 35 g and the contents of added corn were adjusted. Temperature of the experimental animal breeding room was maintained at 20±2 with relative humidity of 60±5%. Lighting was adjusted every 12 h. Experimental diet and water were provided without limit. Average daily body weight gain and diet intake were investigated during the experimental period.

Oral administration: Purified pellet diet group (NC group) and high-fat diet groups (HFD, HFD 15 and HFD 30 groups) were fed separately and ESM was orally administered through a stomach tube 1 mm in diameter at a particular time every day. One milliliter of saline and the contents of ESM corresponding to each treatment group were orally administered to the NC group and high-fat diet groups, respectively, by dissolving in 1 mL of saline.

Collection of blood and organs and biochemical analysis: Concerning animal sacrifice, diet was withdrawn from 12 h before termination and only water was provided for the convenience of anesthesia and anatomy. The animals were anesthetized with zoletil/rompun (10 and 5 mg kg⁻¹, respectively). The blood was collected from abdominal aorta using heparinized vacuum tube (Becton Dickinson Vacutainer System, Franklin lakes, NJ 07417, USA). Centrifugation was performed for 15 min at 3,000 g to separate plasma which was quickly frozen at -196°C by liquid nitrogen and stored at -20°C until the next biochemical analysis. Liver, kidney, heart, spleen and abdominal fat were taken off immediately after blood collecting and blood was washed away with saline. Then they were absorbed onto Whatman filter paper to remove water and measure the weight of these organs. Among blood lipid fractions, triglyceride, total cholesterol, HDL-C and LDL-C were analyzed using Diagnostic Kit (Sigma chemical Co, St, Louis, MO, USA). Al
that is used to determine the risk level of CVD was calculated using the following formula: \( \frac{\text{[total cholesterol]} - \text{HDL-C}}{\text{HDL-C}} \text{[Mildner-Szkudlarz and Bajerska, 2013].} \) Liver function enzymes such as AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) were measured using a biochemical blood autoanalyzer (Fuji Dri-Chem 3500, Japan).

**Gene expression of SREBPs and PPARα:** Real-Time Polymerase Chain Reaction (RT-PCR) was used to measure the relative level of LPL-mRNA transcripts for -actin in adipose tissue and SREBP-1α, SREBP-2 and PPARα-mRNA for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in liver (Park and Park, 2014). In summary, liver and adipose tissues were collected and quickly frozen using liquid nitrogen to be stored at -80°C. Lysis buffer of Xprep Tissue RNA Mini Kit (Philekorea Technology, PKT) was used to 30 mg of liver tissues were collected and quickly frozen using liquid nitrogen to be stored at -80°C. Lysis buffer of Xprep Tissue RNA Mini Kit (Philekorea Technology, PKT) was used to 30 mg of liver (Park and Park, 2014). In summary, liver and adipose tissue to extract total RNA. The RNA concentration was measured by absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer (USA) to obtain extracted RNA 300-500 ng µL-1. cDNA synthesis kit (PKT) was used for the extracted 1 µg RNA to synthesize the 1st strand cDNA. The cDNA was amplified by being grown at 70°C for 5 min, at 42°C for 30 min and at 85°C for 5 min. Finally, the amount of 500 ng cDNA was used as template by RT-PCR and relative concentration was identified as to the mRNA transcription of each target gene for reference gene GAPDH. QuantiMix SYBR Kit (PKT) was used for RT-PCR and cDNA was diluted at a ratio of 1:5. Then, each primer (Forward and Reverse) was mixed. Gene expression was performed in accordance with the gene expression protocol manual suggested by Eco Real-Time PCR (Illumina Inc.). The GAPDH and β-actin were used to regulate the concentration of mRNA as the housekeeping gene. Specific oligonucleotide primers are as follows:

GAPDH forward 5'-TGACCACCAACTGCTTAACTG3', reverse 5'-GGATGCAGGATGTAGTTCTCCC3'; SREBP-1α forward 5'-ATGGAGAGGCTGCCCTTCCGGTTGAGGCTCCT-3', reverse 5'-CCCTGGCGATGGTGTCGCTG-3'; SREBP-2 forward 5'-TTTGTCGTTCTGATATATCAAGGGTGTCG-3', reverse 5'-GCTGCGTTCTGATATATCAAGGGTGTCG-3'; PPARα forward 5'-CCCTTTCTCTCAGCTGTTACCCG-3', reverse 5'-CCACAAGGCTTCTCTCATGAGATG-3'; β-actin forward 5'-CTCTCCAGGCCCTCTTCTCT-3', reverse 5'-AGCAC TGATTGCGATACAG-3'; LPL forward 5'-CAGCTG GCCAATCTTTTAGG-3', reverse 5'-AATGGGTTC TCCAATGTTCG-3'.

**Electron microscopic examination of liver and adipose cells:** To observe liver and adipose cells, liver and abdominal fat tissues were obtained after blood collecting and were cut into 1 mm² samples within 3 min. Then, the samples were buffered with 0.1 M cacodylate (pH 7.4) and fixed and washed with 4% glutaraldehyde. After fixing with 2% osmium tetroxide (OsO₄, 2%), they were washed with buffer solution and dried with ethanol. The samples were substituted with propylene oxide and dehydration solution within tissue was infiltrated and embedded with EPON resin. They underwent embedding and polymerization at 60°C and a 300 nm thick slice was made with ultramicrotome, which was dyed with toluidine blue. Liver and adipose cells were observed using an Energy Filtering-Transmission Electron Microscope (EF-TEM, Leo 912AB, Carl Zeiss Inc., Germany).

**Statistics analysis:** For statistical interpretation of the analyzed data, the SAS software package was used. After calculating average and standard error of each treatment group and performing analysis of variance, significance (p<0.05) was examined at a level of 95% through Duncan's multiple range test (SAS., 2004).

**RESULTS**

**Growth performance:** When ESM was administered orally to obesity animals that ingested a high-fat diet, diet intake, the body weight gain and dieting efficiency are as summarized in Table 1. Diet intake was significantly higher in HFD 30, HFD 15 and NC groups compared with HFD group. The HFD 15 and HFD 30 groups recorded a lower value than NC group but there was not a difference between HFD 15 and HFD 30 groups. Body weight gain was significantly lower in HFD 15, HFD 30 and NC groups compared with the HFD group but there was no difference among HFD 15, HFD 30 and NC groups. The HFD 15 and HFD 30 groups showed body weight decrease by 25.55% compared with the HFD group which was similar to the NC group. Diet efficiency was lower among the HFD 15, HFD 30 and NC groups compared with the HFD group and there was a significant difference among the treatment groups.

**Weight of organs:** Weight change of liver, kidney, heart, spleen and adipose tissue after ESM administration is described in Table 2. Except for kidney, heart and spleen, which showed no difference among the treatment groups, the weights of liver and adipose tissues were significantly lower among HFD 15, HFD 30 and NC groups compared with HFD group but there was no significant difference between HFD 30 and NC groups. Compared with HFD group, liver weight...
Blood lipid profiles, AST and ALT: The change of blood lipid profiles, AST and ALT in the event of ESM administration to high-fat diet induced obesity animals is described in Fig. 1. Triglyceride was significantly lower in the NC group than in the HFD group (p<0.05) in the high-fat diet induced obesity animals. Similarly, the weight of adipose tissue was significantly lower in the NC group than in the HFD group (p<0.05) in the high-fat diet induced obesity animals.
Fig. 2(a-d): Effect of oral administration of ESM on gene expressions of (a) SREBP-1α and (b) SREBP-2 mRNA in liver or (c) PPAR-α and (d) LPL mRNA in adipose tissue in obesity rats. Bars are Mean±Standard errors (n = 10). a,b,c,dValues are significantly different at p<0.05.

HFD 15, HFD 30 and NC groups compared to the HFD group, by 27.36, 32.40 and 35.12%, respectively and HFD 15 and HFD 30 group registered higher values than NC group. Total cholesterol was significantly lower in HFD 30, HFD 15 and NC groups compared to HFD group, by 26.33, 26.70 and 27.53%, respectively but there was no difference among these 3 groups. LDL-C was significantly lower in HFD 15, HFD 30 and NC groups compared to HFD group, by 17.06, 38.70 and 39.27%, respectively. HFD 30 and NC groups showed lower value than HFD 15 group, but there was no difference between the two groups. The HDL-C was significantly higher in HFD 15, HFD 30, compared to HFD group by 142.27, 106.17 and 109.09%, respectively. The value of HFD 15 and HFD 30 groups was significantly lower than that of NC group, by 5.81 and 8.33%, respectively. The AI was significantly lower in HFD 30, HFD 15 and NC groups, by 11.12, 59.72 and 66.91%, respectively, compared to HFD group. AI and AST was significantly lower in HFD 30, HFD 15 and NC groups, by 27.30, 38.97 and 39.22%, respectively, compared to HFD group. The HFD 15 and HFD 30 groups registered a significantly higher value than that of NC group, by 119.39% but there was no difference between NC and HFD 30 groups. The HFD 15 group showed a significantly higher value than NC group, by 119.39% but there was no difference between NC and HFD 30 groups. The PPAR-α mRNA increased significantly in NC, HFD 15 and HFD 30 groups compared to HFD group, by 286.67, 193.33 and 188.89%, respectively. The HFD 15 and HFD 30 groups showed a significantly lower value than that of NC group by 32.56 and 34.11%, respectively but there was no difference between the two groups. The SREBP-2 mRNA decreased significantly in the HFD 15, NC and HFD 30 groups, by 24.02 and 46.75%, respectively. The HFD 15 group showed a significantly higher value than NC group, by 119.39% but there was no difference between NC and HFD 30 groups. The PPAR-α mRNA increased significantly in NC, HFD 15 and HFD 30 groups compared to HFD group, by 286.67, 193.33 and 188.89%, respectively. The HFD 15 and HFD 30 groups showed a significantly lower value than that of NC group by 32.56 and 34.11%, respectively but there was no difference between the two groups. The SREBP-1α mRNA decreased significantly in the HFD 15, HFD 30 and NC groups compared to HFD group, by 37.68, 39.86 and 46.38%, respectively. The HFD 15 and HFD 30 groups registered significantly higher values compared to NC group, by 166.92 and 112.12%, respectively, but there was no difference between the two groups. The LPL mRNA expression of adipose tissue increased in the NC, HFD 30 and HFD 15 groups compared to HFD group, by 120.90, 176.12 and 201.49%, respectively and there was a significant difference among the three groups.

Morphological change of liver and fat cells: The morphological change of liver and fat cells as a result of ESM administration is shown in Fig. 3 and 4. In the liver cells of HFD group, porous-coated fats and very large fats are accumulated compared to normal diet fed NC group. As the
entire liver cell was covered with fat globule, it was impossible to observe the form of the cell and the cytoplasm looked white due to the accumulation of excessive fat inside. It induced fatty liver, with an unclear membrane and form of cell organelle and the size of adipocyte within adipose tissue increased (The number and size of adipocytes were not measured). In the liver cells of HFD 15 and HFD 30 groups, fat accumulation was reduced compared with the HFD group and Kupffer’s cell was activated. Normal liver mesenchymal cell is seen clearly surrounding the central vein and liver damage resulting from a high-fat diet was greatly alleviated. Concerning adipose tissue of HFD 15 and HFD 30 groups, the size of adipocytes within adipose tissue became diminished compared with the HFD group and the size of adipocytes displayed a tendency of decreasing, to close to the NC group.

**DISCUSSION**

The result of this study has found that ESM administration to obesity model animals can reduce body weight significantly. These findings are supported by the anti-obesity effects of sulfur-containing thiacremonone separated from garlic for obese model animals through body weight loss and the reduction of blood triglyceride and glucose (Ban et al., 2012). These are also supported by the paper by Park and Park (2012), which observed body weight decrease in obesity model animals fed with grain maggot extract. The result was affected by high energy intake through the high-fat diet during testing period. Furthermore, body weight increased despite a relatively low diet intake because of the increase of body fat accumulated along with hepatomegaly instead of muscle.
Since antibacterial peptide regulates blood lipid metabolism by inhibiting SREBP-1α and SREBP-2 mRNA in liver while, facilitating the expression of PPArRα mRNA in adipose tissue (Park and Park, 2014). Of the target genes relating to lipid metabolism, Sterol regulatory element binding proteins (SREBPs: SREBP-1α, SREBP-1c, SREBP-2) is an important transcription activating gene that activates the expression of more than 30 genes involved with biosynthesis of neutral fat and cholesterol (Ji et al., 2011; Rodriguez-Cantu et al., 2011). SREBP-1α facilitates and regulates the expression of LDL receptor transcription gene and biosynthesis enzyme of cholesterol and fatty acid, while SREBP-2 controls a gene that is essential for the homeostasis of cholesterol by facilitating and regulating the expression of LDL receptor transcription gene (Frazier-Wood et al., 2013; Rotllan and Carlos, 2012). Peroxisome proliferator-activated receptors (PPARs: alpha, gamma, delta) are nuclear receptor proteins that have a close relationship with obesity as a major regulator of lipid metabolism in liver. The activation of PPAR-α enhances the transport of fatty acids and the securing, use and decomposition of fatty acids by facilitating and modulating the gene involved with -oxidation of fatty acid in peroxisome and mitochondria (Kelley and Azhar, 2005; Frazier-Wood et al., 2013). Gene expression of SREBPs mRNA is inhibited among hyperlipidemia model animals compared to chow diet rats and this may be a reason behind the reduction of blood lipid (Rogi et al., 2011). The LPL lowers blood neutral fat by hydrolyzing neutral fat and providing free fatty acids to adipocyte (Yamaguchi et al., 2012). The LPL, as a rate-limiting enzyme in charge of lipolysis, is involved with weight control by increasing lipid partitioning in obese tissues (Wang and Eckel, 2009). In conclusion, ESM administration down-regulates gene expression of cholesterol biosynthesis by inhibiting SREBP-1α and SREBP-2 mRNA in animal liver and up-regulates the lipolysis gene by activating PPArRα mRNA and LPL mRNA. This will ultimately helping prevents obesity by regulating gene expression.
The testing newly discovered that ESM administration can be a great help in protecting animal liver from being damaged and reducing the number of adipocytes. It is considered that fatty liver occurred in the HFD group, because excess fat was accumulated in the liver with animals ingesting a high-fat diet. As excess fat continues to be accumulated in adipocytes, the size of adipocytes continues to grow until the fat is consumed as energy. It is known that obesity is caused by an increase in the size of adipocytes rather than in their number (Greenberg and Obin, 2006). Meanwhile, the size of visceral adipocytes becomes huge with the increase in insulin resistance due to the accumulation of visceral fat. Obesity is also known to be a powerful risk factor for metabolic syndrome and CVD (Hanauer, 2005).

CONCLUSION

In conclusion, results of this study suggest that sulfur-grain maggot extracts has anti-obesity effects, by reducing the abdominal fat of obesity model animals and lowering blood lipid level through the down-regulation of SREBP-1α and SREBP-2 mRNA and the up-regulation of PPAR-α mRNA.

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