Chung Shan Medical Journal 2018; 29:113-122

Original Article

Inhibitory Effect of Methanolic Extract of Black Garlic on Adipogenesis in 3T3-L1 Adipocytes

Wei-Tang Chang[1], Jiun Ling Lim[1], Chin-Lin Hsu[1,2,*]

[1] *Department of Nutrition, Chung Shan Medical University, Taichung 40201, Taiwan*
[2] *Department of Nutrition, Chung Shan Medical University Hospital, Taichung 40201, Taiwan*

Black garlic (also called aged garlic) is produced from fresh garlic and used as a food ingredient and functional food. The aim of this work was to study the effects of different solvent (water, methanol, and ethanol) extracts of black garlic (WEBG, MEBG, and EEBG) on inhibition of adipogenesis in 3T3-L1 adipocytes. The results demonstrated that MEBG has the most prominent inhibitory effects on cell numbers, intracellular triglycerides, and glycerol-3-phosphate dehydrogenase activity in 3T3-L1 adipocytes. MEBG significantly up-regulated the protein expression of adiponectin, as well as down-regulated the protein expression of fatty acid synthase in 3T3-L1 adipocytes. Real-time RT-PCR analyses of 3T3-L1 adipocytes exposed to MEBG showed that MEBG decreases the expressions of lipogenic genes (*PPARγ, C/EBPα, FAS, aP2, and ACC*) and adipokines (*leptin* and *resistin*), and increases the expressions of *PGC-1α* and *adiponectin*. These results demonstrated that MEBG efficiently inhibits adipogenesis in 3T3-L1 adipocytes.

Keywords: *Black garlic*, *Adipogenesis*, *3T3-L1 adipocytes*

Introduction

Obesity is a major source of morbidity and mortality worldwide and very complex diseases that involve genetic and environmental factors. Obesity is believed to be associated with lifestyle-related diseases such as hyperlipidemia, hypertension, arteriosclerosis, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis[1]. Currently, the only anti-obesity drugs that have been approved by the Food and Drug Administration (FDA) are Phentermine, Orlistat (Roche), Lorcaserin (Arena Pharmaceuticals) and Phentermine + topiramate (Qsymia, formerly Qnexa; Vivus)[2]. Based on the findings of previous reports, various methods have been proposed for reducing obesity, including decreased levels of energy intake, preadipocyte differentiation, preadipocyte proliferation, and lipogenesis, and increased levels of energy expenditure, lipolysis, and fat oxidation[3]. Adipogenesis is the process of cell differentiation by which preadipocytes become adipocytes. Recent reports have indicated that food ingredients may prevent the adverse health effects of high-fat diet in animals, such as polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), conjugated linoleic acid (CLA), phenolic compounds, soybeans, plant sterols, dietary calcium, and dietary fiber[4-5].

Garlic (*Allium sativum* L.) has anti-obesity, anti-bacterial, anti-viral, anti-hypertensive, blood glucose lowering, anti-thrombotic, anti-mutagenic, and anti-platelet actions *in vitro* and in *vivo*[6-12]. Sheen et al.[13] indicated that garlic oil and its organosulfur compounds ameliorate obesity induced by high-fat diet (HFD) in rats. Black garlic (also called aged garlic) is produced from fresh garlic that has been naturally fermented at controlled temperature and humidity

* Corresponding Author: Chin-Lin Hsu
Address: No. 110, Sec. 1, Jianguo N. Rd., Taichung City, 40201, Taiwan
Tel: +886-4-24730022
Fax: +886-4-23248175
E-mail: clhsu@csmu.edu.tw

for more than 30 days. During the process of natural fermentation, fresh garlic changes color from white to black and major compound-alliin is converted into stable compound-S-allylcysteine (SAC)[14]. Black garlic and its extracts have been reported to exhibit anti-oxidant, anti-hyperlipidemia, anti-hyperglycemic, anti-atherosclerotic, anti-obesity, and anti-hypertensive activities[15-19]. However, from a review of the literature, the effects of different solvent extracts of black garlic on inhibition of adipogenesis in 3T3-L1 adipocytes remain unclear.

In the present study, we investigated the inhibitory effects of black garlic extracts (water, methanol, and ethanol) on adipogenesis in 3T3-L1 adipocytes. Mouse embryo 3T3-L1 cell line was used for *in vitro* evaluation of anti-obesity effects[20-21]. Moreover, 3T3-L1 preadipocytes are a well-established cell line for the study of adipogenesis.

MATERIALS AND METHODS

Materials

Black garlic samples were provided by Professor Chin-Yin Tseng (Chung Chou University of Science and Technology, Changhua County, Taiwan). Gallic acid, Folin & Ciocalteu's phenol reagent, Trolox, 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS), sodium bicarbonate, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and insulin (INS) were obtained from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum, and antibiotic mixture (penicillin-streptomycin) were purchased from Invitrogen Co. (Carlsbad, CA, USA). Anti-β-actin and adiponectin antibodies were obtained from BioVision (Mountain View, CA). Anti-FAS antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were of the highest grade available.

Sample Extraction

A 10 g sample of black garlic was extracted with water, methanol, or ethanol (100 mL) in a rotary shaker at room temperature for 24 h. The water, methanolic, and ethanolic extracts of black garlic (WEBG, MEBG, and EEBG) were filtered through Whatman no. 1 filter paper, dried using a vacuum evaporator, and stored at -20°C until use.

Determination of Total Phenolic Content

The total phenolic contents of WEBG, MEBG, and EEBG were determined with Folin & Ciocalteu's phenol reagent and measured using gallic acid as a standard [22]. Extracts (100 µL) were added to 50% Folin & Ciocalteu's phenol reagent (100 µL). After 3 min, 2 mL of 2% Na_2CO_3 solution were added to the mixture, which was then left to stand for 30 min. The absorbance was measured at 750 nm using a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Germany). Total phenolic content is expressed as gallic acid equivalents.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

$ABTS^{\bullet+}$ was generated by the reaction of ABTS (100 µmol/L), H_2O_2 (50 µmol/L), and peroxidase (4.4 U/mL). To measure the antioxidant activity, 0.25 mL of extract (WEBG, MEBG, or EEBG) were mixed well with an equal volume (0.25 mL) of ABTS, H_2O_2, or peroxidase, and 1.5 mL of deionized water. The absorbance was measured at 734 nm after the sample was allowed to react for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. A dose-response curve was plotted for Trolox, and the antioxidant activity was expressed as TEAC value. The higher the TEAC value of a sample, the stronger the antioxidant activity. TEAC is expressed as micromoles of Trolox equivalents per gram of extract.

Cell Culture

The 3T3-L1 pre-adipocytes (BCRC 60159) used in this study were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). They were added to a 10-cm dish and maintained in DMEM supplemented with 10% bovine calf serum, 1.5 g/L sodium bicarbonate, and 100 U/mL penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator. Adipocytic

differentiation was induced by adipogenic agents (0.5 mM IBMX, 1 μM DEX, and 1 μM INS) that were added to the culture medium for eight days. After replacing with normal culture medium, fresh medium was added every 48 h. The cells were harvested 10 days after the initiation of differentiation.

Oil red O Staining of 3T3-L1 Adipocytes

The 3T3-L1 adipocytes were treated with black garlic extract (0-100 μg/mL) for 48 h. Oil red O working solution was prepared as described by Ramírez-Zacarías et al.[23]. Oil red O-stained material (OROSM) was expressed on a per cell basis using the number of cells determined from similar plates. The percentage of oil red O-stained material (OROSM, %) relative to control wells containing cell culture medium without compounds was calculated as A510 nm [antioxidant]/A510 nm [control] × 100.

Intracellular Triglyceride Content

The 3T3-L1 adipocytes were treated with black garlic extract (0-100 μg/mL) for 48 h. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS). The total triglyceride content in the cells was determined using a commercial triglyceride assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

Glycerol-3-Phosphate Dehydrogenase (GPDH) Activity Assay

The 3T3-L1 adipocytes were treated with MEBG (0-100 μg/mL) for 48 h. GPDH activity was determined with a GPDH activity assay kit (B-Bridge International, Inc., Sunnyvale, CA, USA).

Western Blot Analysis

The 3T3-L1 adipocytes were treated with 50 μg/mL MEBG for 0-6 h. The cells were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF]. The adiponectin, FAS, and β-actin proteins were assessed. The protein concentrations of the extracts were estimated on Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. Total proteins (50-60 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary antibody at 4°C overnight and then with secondary antibody for 1 h. Membranes were washed three times in PBST for 10 min between each step. The signal was detected using enhanced chemiluminescence (ECL; Perkin Elmer Life Science, Boston, MA, USA).

RNA Extraction and Real-Time RT-PCR

Real-time RT-PCR was performed to determine gene expression levels in 3T3-L1 cells. Total RNA from cells was isolated using the TRIzol method (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The cDNA was synthesized from the total RNA by reverse transcription PCR using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

The following primer pairs were used: *GAPDH*, 5'-gtatgactccactcacggcaaa-3' (forward) and 5'-ggtctcgctcctggaagatg-3' (reverse); *PPARγ*, 5'-ttttcaagggtgccagtttcgatcc-3' (forward) and 5'-aatccttggccctctgagat-3' (reverse); *C/EBPα*, 5'-cgcaagagccgagataaagc-3' (forward) and 5'-cacggctcagctgttcca-3' (reverse); *FAS*, 5'-tgggttctagccagcagagt-3' (forward) and 5'-taccaccagagaccgttatgc-3' (reverse); *ACC*, 5'-gaatctcctggtgacaatgcttatt-3' (forward) and 5'-ggtcttgctgagttgggttagct-3' (reverse); *aP2*, 5'-catggccaagcccaacat-3' (forward) and 5'-cgcccagtttgaaggaaatc-3' (reverse); *PCG-1α*, 5'-tgttcccgatcaccatattcc-3' (forward) and 5'-ggtgtctgtagtggcttgattc-3' (reverse); *leptin*, 5'-aggatctgaggggtgatgtga-3' (forward) and 5'-tgaggtgaccaaggtggcatag-3' (reverse); *adiponectin*, 5'-gtctcagctgtcggtcttcccct-3' (forward) and 5'-ccctggctttatgctctttgc-3' (reverse); and *resistin*, 5'-agactgctgtgccttctggg-3' (forward) and

5'-ccctccttttccttttcttccttg-3' (reverse). Real-time RT-PCR was conducted to detect gene expression levels using StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction mixture (total volume 25 μL) contained 1× power SYBR green PCR master mix, 300 nM forward primer, 300 nM reverse primer, cDNA and DEPC-H_2O, as well as commercial reagents (Applied Biosystems, Foster City, CA, USA). The thermal profile was established according to the manufacturer's protocol: 95°C for 10 min for enzyme activation, followed by denaturing at 95°C for 15 s, and annealing and elongation at 60°C for 1 min, for a total of 40 cycles. The relative levels of gene expression were quantified using the ΔΔCt method, which results in a ratio of target gene expression to equally expressed housekeeping genes.

Statistical Analysis

Each experiment was performed in triplicate. The results are expressed as the mean ± SD. Statistical analysis was performed using SAS software. Analysis of variance was performed using ANOVA procedures. Significant differences (p<0.05) between the means were determined by Duncan's multiple range tests.

RESULTS

Extraction Yield and Antioxidant Activity of Black Garlic Extracts

Fig. 1 shows the extraction yields, total phenolic contents, and TEAC values of different solvent extracts of black garlic. The extraction yields of WEBG, MEBG, and EEBG were 58.4±1.5, 56.4±2.3, and 34.0±6.3 %, respectively (Fig. 1A). The total phenolic contents of WEBG, MEBG, and EEBG were 46.4±4.0, 13.4±0.6, and 12.2±0.7 mg/g extract, respectively (Fig. 1B). In addition, the TEAC values for WEBG, MEBG, and EEBG were 176.8±4.2, 36.1±2.8, and 15.1±1.2 μmol/g extract, respectively (Fig. 1C).

Effects of Black Garlic Extracts on OROSM and Intracellular Triglycerides in 3T3-L1 Adipocytes

Effects of black garlic extracts on OROSM and intracellular triglycerides in 3T3-L1 adipocytes are shown in Fig. 2. OROSM showed that cell numbers

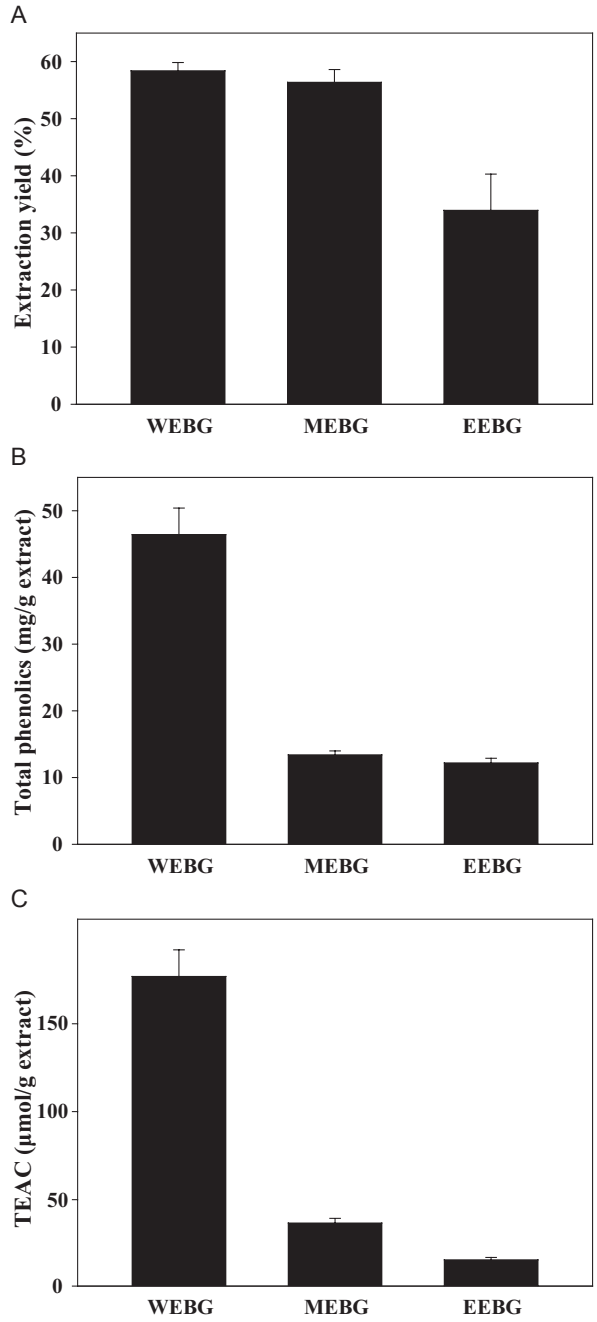

Fig.1. The extraction yields (A), total phenolic contents (B), and TEAC values (C) of different solvent extracts of black garlic. The reported values are the means ± SD (n=3). WEBG, water extract of black garlic; MEBG, methanolic extract of black garlic; EEBG, ethanolic extract of black garlic.

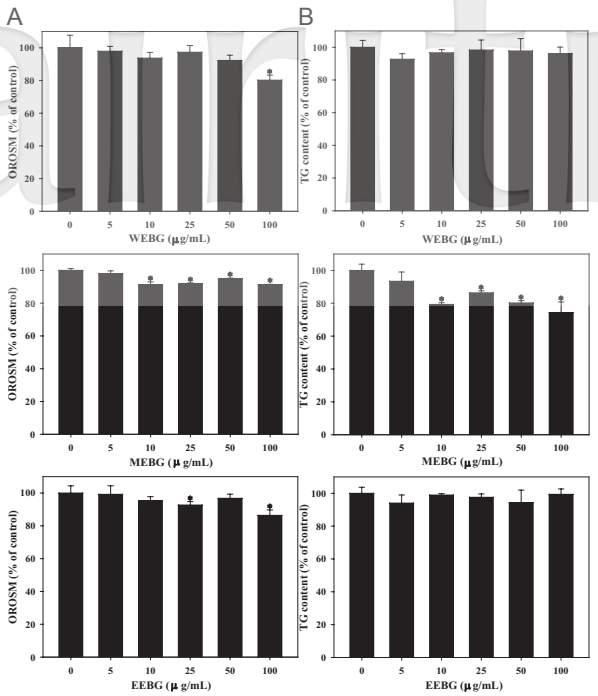

Fig.2. Effects of different solvent extracts of black garlic on oil red O stained material (OROSM) (A) and intracellular triglycerides (B) in 3T3-L1 adipocytes. The reported values are the means ± SD (*n*=3). The 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation and stained with oil red O. The 3T3-L1 cells were treated with 0-100 μg/mL of WEBG, MEBG, or EEBG for 48 h at 37°C in a humidified 5% CO_2 incubator.

are influenced by treatment with WEBG (100 μg/mL), MEBG (10-100 μg/mL), and EEBG (25 and 100 μg/mL) (Fig. 2A). However, only MEBG (10-100 μg/mL) inhibited intracellular triglycerides (Fig. 2B).

Effect of MEBG on GPDH Activity in 3T3-L1 Adipocytes

Effects of MEBG on GPDH activity in 3T3-L1 adipocytes are shown in Fig. 3A. Treatment of 3T3-L1 adipocytes with MEBG (5-100 μg/mL) caused inhibition of GPDH activity. However, GPDH activity was not influenced by the treatment of 3T3-L1 adipocytes with WEBG or EEBG. Therefore, MEBG was selected for follow-up analysis.

Molecular Mechanisms of MEBG on Inhibition of Adipogenesis in 3T3-L1 Adipocytes

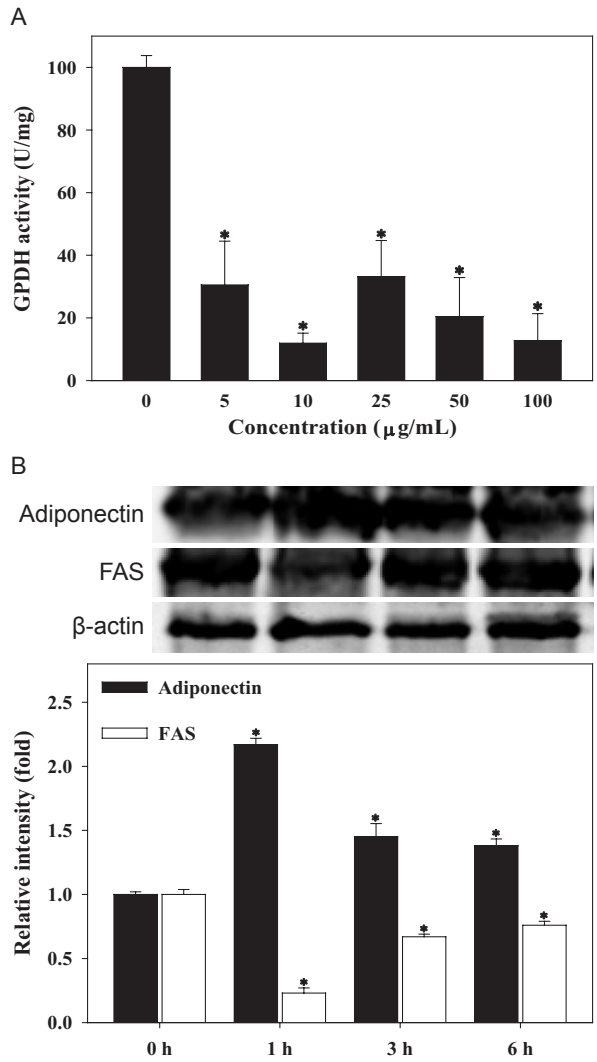

Fig.3. Effects of MEBG on GPDH activity (A) and protein expressions of FAS and adiponectin (B) in 3T3-L1 adipocytes. The reported values are the means ± SD (*n*=3). Mean values with different letters were significantly different ($p < 0.05$). The 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. (A) Cells were treated with 0-100 μg/mL of MEBG for 48 h at 37°C in a humidified 5% CO_2 incubator. (B) Cells were treated with 50 μg/mL of MEBG for 0, 1, 3, and 6 h at 37°C in a humidified 5% CO_2 incubator. The relative expressions of FAS and adiponectin in 3T3-L1 adipocytes were quantified densitometrically using the software ImageGauge 3.46, and calculated according to the reference bands of β-actin.

Effects of MEBG on protein expressions of adiponectin and FAS in 3T3-L1 adipocytes are

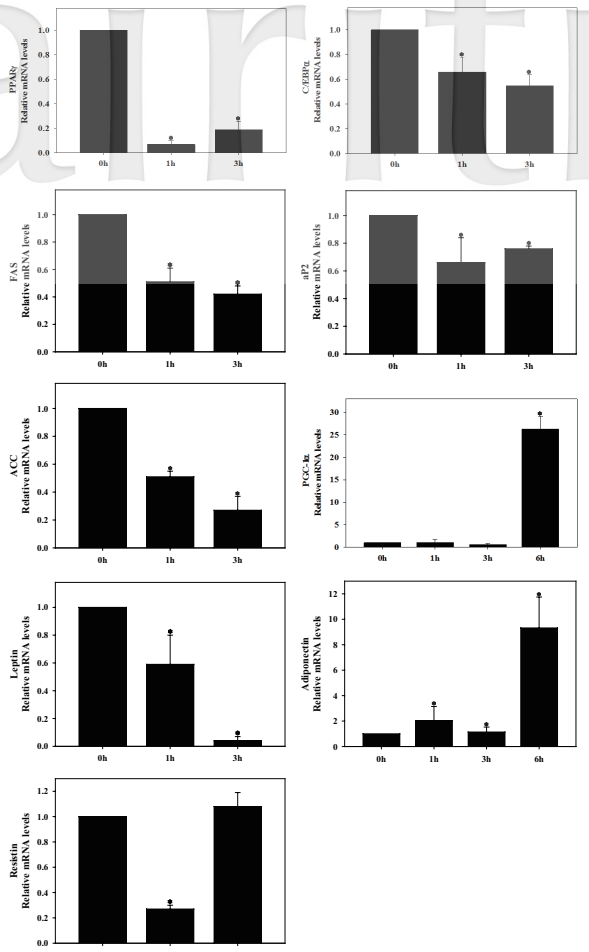

Fig.4. Effect of MEBG on gene expressions of *PPARγ*, *C/EBPα*, *FAS*, *aP2*, *ACC*, *PGC-1α*, *leptin*, *adiponectin*, and *resistin* in 3T3-L1 adipocytes. Mean ± SD *(n=3)*. Mean values with different letters were significantly different ($p < 0.05$). The 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The 3T3-L1 adipocytes were treated with 50 μg/mL of MEBG for 1-6 h at 37°C in a humidified 5% CO_2 incubator. The values of *PPARγ*, *C/EBPα*, *FAS*, *aP2*, *ACC*, *PGC-1α*, *leptin*, *adiponectin*, and *resistin* mRNA were normalized to the value of *GAPDH*.

shown in Fig. 3B. Exposure of 3T3-L1 adipocytes to MEBG caused the up-regulation of adiponectin, to a maximum of 2.17-fold relatively intensity after treatment with 50 μg/mL MEBG for 1 h. Moreover, MEBG inhibited the protein expression of FAS in 3T3-L1 adipocytes, to a maximum of 0.23-fold relative intensity after treatment with 50 μg/mL MEBG for 1 h.

Effect of MEBG on gene expressions of *PPARγ*, *C/EBPα*, *FAS*, *aP2*, *ACC*, *PGC-1α*, *leptin*, *adiponectin*, and *resistin* in 3T3-L1 adipocytes are shown in Fig. 4. Real-time RT-PCR analyses of 3T3-L1 adipocytes exposed to MEBG showed that MEBG decreases lipogenic gene expressions (*PPARγ*, *C/EBPα*, *FAS*, *aP2*, and *ACC*) (50 μg/mL MEBG for 1-3 h) and increases lipolytic gene expression (*PGC-1α*) (50 μg/mL MEBG for 6 h). The treatment of 3T3-L1 adipocytes with MEBG caused the down-regulation of gene expressions of *leptin* (50 μg/mL MEBG for 1-3 h) and *resistin* (50 μg/mL MEBG for 1 h), as well as the up-regulation of gene expression of *adiponectin* (50 μg/mL MEBG for 1-6 h).

DISCUSSION

Black garlic is a fermented food ingredient and functional food in Asian countries. Sato et al.[24] indicated that the superoxide dismutase (SOD)-like activity, free radical scavenging activity, and polyphenol content of black garlic were significantly higher compared to fresh garlic. Black garlic and its extracts have been reported to possess important roles and functions, including anti-oxidant, anti-hyperlipidemia, anti-hyperglycemic, anti-atherosclerotic, anti-obesity, and anti-hypertensive activities[15-19]. We studied the inhibitory effects of water, methanolic, and ethanolic extracts of black garlic (WEBG, MEBG, and EEBG) on adipogenesis in 3T3-L1 adipocytes and its bioactive compounds. Prior et al. [25] provides a basis of rationale for antioxidant capacity methods, including total phenolic contents, TEAC, and ORAC. Our data indicated that the extraction yield, total phenolic content, and TEAC value for WEBG were significantly higher than for MEBG and EEBG (Fig. 1). Lee et al. [16] indicated that the TEAC values of aged black garlic were higher than those of fresh garlic.

During adipocyte differentiation, enhanced levels of transcriptional factors such as PPARγ and C/EBPs lead to the expression of many adipocyte specific proteins [26]. Tang et al. [27-28] indicated that the levels of PPARγ and aP2 play an important role in adipogenesis. Our previous report indicated that flavonoids and

phenolic acids (such as *o*-coumaric acid and rutin) are involved in the control of adipogenesis [29]. The levels of OROSM and intracellular triglyceride accumulation are often used as markers of adipogenesis [29-30]. Our data indicated that MEBG has the most prominent inhibitory effect on OROSM and intracellular triglycerides in 3T3-L1 adipocytes (Fig. 2). Wise and Green[31] indicated that the cytosolic enzyme GPDH plays an important role in the conversion of glycerol into triglyceride. Adiponectin is an adipocytokine that has been shown to have anti-atherogenic, anti-inflammatory, and anti-diabetic roles and might influence glucose homeostasis and insulin sensitivity[32]. MEBG significantly decreased the GPDH activity and regulated the protein expressions of adiponectin and FAS in 3T3-L1 adipocytes (Fig. 3).

Real-time RT-PCR analyses indicated that MEBG decreases the expressions of lipogenic genes (*PPARγ*, *C/EBPα*, *FAS*, *aP2*, and *ACC*) and gene expressions of adipokines (*leptin* and *resistin*), as well as increases the gene expressions of *PGC-1α* and *adiponectin* (Fig. 4). Lipogenesis-related factors such as FAS and ACC are also regarded as important markers for adipogenesis[33]. Phytochemicals (such as capsaicin, *o*-coumaric acid, and rutin) block adipogenesis by suppression of the expression of adipogenic transcription factors, including *PPARγ* and *C/EBPα*[29, 34]. Regulation of adipocyte-specific genes, such as *aP2*, *leptin*, *adiponectin*, *FAS*, and *PGC-1α*, leads to morphological changes and lipid accumulation within the adipocytes[35-36].

In conclusion, the effect of MEBG on inhibition of adipogenesis in 3T3-L1 adipocytes, as indicated by decreases in OROSM, intracellular triglycerides, and GPDH activity, have been elucidated. MEBG inhibits adipogenesis through the down-regulation of lipogenesis and expressions of adipokines, as well as the up-regulation of the expressions of *PGC-1α* and *adiponectin*. These results demonstrated that MEBG efficiently suppresses adipogenesis in 3T3-L1 adipocytes.

ABBREVIATIONS USED

ABTS, 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate); ACC, acetyl-CoA carboxylase; alliin, S-Allyl-L-cysteine sulfoxide; aP2, adipocyte fatty acid-binding protein 2; C/EBPα, CCAAT enhancer binding protein alpha; CLA, conjugated linoleic acid; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EEBG, ethanolic extract of black garlic; FAS, fatty acid synthase; GPDH, glycerol-3-phosphate dehydrogenase; HFD, high-fat diet; IBMX, 3-isobutyl-1-methylxanthine; MEBG, methanolic extract of black garlic; MUFAs, monounsaturated fatty acids; OROSM, oil red O-stained material; OSC, organo-sulfur compounds; PGC-1α, peroxisome proliferator-activated receptor-coactivator 1α; PPARγ, peroxisome proliferator-activated receptor-gamma; PUFAs, polyunsaturated fatty acids; PVDF, polyvinyldifluoride; SAC, S-allylcysteine; TEAC, trolox equivalent antioxidant capacity; WEBG, water extract of black garlic.

ACKNOWLEDGEMENT

This research work was partially supported by grant NSC98-2622-B-235-001-CC1 from the National Science Council, Taiwan, Republic of China.

References

1. Kopelman PG: Obesity as a medical problem. Nature 2000; 404: 635-43.
2. Dietrich MO, Horvath TL: Limitations in anti-obesity drug development: the critical role of hunger-promoting neurons. Nature Reviews, Drug Discovery 2012; 11: 675-91.
3. Wang YW, Jones PJ: Conjugated linolic acid and obesity control: efficacy and mechanisms. International Journal of Obesity and Related Metabolic Disorders 2004; 28: 941-55.
4. Hsu CL, Yen GC: Phenolic compounds: Evidence for inhibitory effects against obesity and their underlying molecular signaling mechanisms. Molecular Nutrition & Food Research 2008; 52: 53-61.
5. Trigueros L, Peña S, Ugidos AV, Sayas-Barberá E, Pérez-Álvarez JA, Sendra E: Food ingredients as anti-obesity agents: a review. Critical Reviews in Food Science and Nutrition 2013; 53: 929-42.

6. Agarwal KC: Therapeutic actions of garlic constituents. Medicinal Research Reviews 1996; 16: 111-24.
7. Rahman K: Historical perspective on garlic and cardiovascular disease. The Journal of Nutrition 2001; 313: 977S-9S.
8. Gorinstein S, Jastrzebasi Z, Namiesnik J, Leontowicz H, Leontowicz M, Trakhtenberg S: The atherosclerotic heart disease and protecting properties of garlic: contemporary data. Molecular Nutrition & Food Research 2007; 51: 1365-81.
9. Morihara N, Nishihama T, Ushijima M, Ide N, Takeda H, Hayama M: Garlic as an anti-fatigue agent. Molecular Nutrition & Food Research 2007; 51: 1329-34.
10. Rahman K: Effects of garlic on platelet biochemistry and physiology. Molecular Nutrition & Food Research 2007; 51: 1335-44.
11. Joo H, Kim CT, Kim IH, Kim Y: Anti-obesity effects of hot water extract and high hydrostatic pressure extract of garlic in rats fed a high-fat diet. Food and Chemical Toxicology 2013; 55: 100-5.
12. Nasri H: Effect of garlic extract on blood glucose level and lipid profile in normal and alloxan diabetic rabbits. Advances in Clinical and Experimental Medicine 2013; 22: 449-50.
13. Sheen LY, Chen HW, Kung YL, Liu CT, Lii CK: Effects of garlic oil and its organosulfur compounds on the activities of hepatic drug-metabolizing and antioxidant enzymes in rats fed high- and low-fat diets. Nutrition and Cancer 1999; 35: 160-6.
14. Corzo-Martinez M, Corso N, Villamiel M: Biological properties of onions and garlic. Trends in Food Science & Technology 2007; 18: 609-25.
15. Kang MJ, Lee SJ, Shin JH, Kang SK, Kim JG, Sung NJ: Effect of garlic with different processing on lipid metabolism in 1% cholesterol fed rats. Journal of the Korean Society of Food Science and Nutrition 2008; 37: 162-9.
16. Lee YM, Gweon OC, Seo YJ, Im J, Kang MJ, Kim MJ, Kim JI: Antioxidant effect of garlic and aged black garlic in animal model of type 2 diabetes mellitus. Nutrition Research and Practice 2009; 3: 156-61.
17. Seo YJ, Gweon OC, Im J, Lee YM, Kang MJ, Kim JI: Effect of garlic and aged black garlic on hyperglycemia and dyslipidemia in animal model of type 2 diabetes mellitus. Journal of Food Science and Nutrition 2009; 14: 1-7.
18. Seo DY, Lee S, Figueroa A, Kwak YS, Kim N, Rhee BD, Ko KS, Bang HS, Baek YH, Han J: Aged garlic extract enhances exercise-mediated improvement of metabolic parameters in high fat diet-induced obese rats. Nutrition Research and Practice 2012; 6: 513-9.
19. Ried K, Frank OR, Stocks NP: Aged garlic extract reduces blood pressure in hypertensives: a dose–response trial. European Journal of Clinical Nutrition 2013; 67: 64-70.
20. Green H, Kehinde O: Sublines of mouse 3T3 cells that accumulate lipid. Cell 1974; 1: 113-6.
21. Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA: Inhibition of adipogenesis by Wnt signaling. Science 2000; 289: 950-3.
22. Taga MS, Miller EE, Pratt DE: Chia seed as a source of natural lipid antioxidants. Journal of the American Oil Chemists' Society 1984; 61: 928-31.
23. Ramírez-Zacarías JL, Castro-Muñozledo F, Kuri-Harcuch W: Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry 1992; 97: 493-7.
24. Sato E, Kohno M, Niwano Y: Increased level of tetrahydro-b-carboline derivatives in short-term fermented garlic. Plant Foods for Human Nutrition 2006; 61: 175-8.
25. Prior RL, Wu X, Schaich K: Standardized Methods for the Determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 2005; 53: 4290-302.
26. Gregorie FM, Smas CM, Sul HS: Understanding adipocyte differentiation. Physiological Reviews 1998; 78: 783-809.
27. Tang QQ, Otto TC, Lane MD: Mitotic clonal expansion: A synchronous process required

for adipogenesis. Proceedings of the National Academy of Sciences of the United States of America 2003a; 100: 44-9.
28. Tang QQ, Otto TC, Lane MD: CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proceedings of the National Academy of Sciences of the United States of America 2003b; 100: 850-5.
29. Hsu CL, Yen GC: Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. Journal of Agricultural and Food Chemistry 2007a; 55: 8404-10.
30. Hsu CL, Lin YJ, Ho CT, Yen GC: Inhibitory effects of garcinol and pterostilbene on cell proliferation and adipogenesis in 3T3-L1 cells. Food & Function 2012; 3: 49-57.
31. Wise LS, Green H: Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. The Journal of Biological Chemistry 1979; 254: 273-5.
32. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Enjel J, Brownlee M, Scherer PE: Structure-function studies of the adipocyte-secreted hormone acrp30/adiponectin. The Journal of Biological Chemistry 2003; 278: 9073-85.
33. Rosen ED, Spiegelman BM: Molecular regulation of adipogenesis. Annual Review of Cell and Developmental Biology 2000; 16: 145-71.
34. Hsu CL, Yen GC: Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. Journal of Agricultural and Food Chemistry 2007b; 55: 1730-6.
35. Fu Y, Luo N, Klein RL, Garvey WT: Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. The Journal of Lipid Research 2005; 46: 1369-79.
36. Cristancho AG, Lazar MA: Forming functional fat: a growing understanding of adipocyte differentiation. Nature Reviews Molecular Cell Biology 2011; 28: 722-34.