Food Sci. Biotechnol. 23(1): 289-292 (2014)
DOI: 10.1007/s10068-014-0040-5

RESEARCH NOTE

Anti-obesity Effects of the Stem Bark of Japanese Horse Chestnut (*Aesculus turbinate*) in 3T3-L1 Preadipocytes

Hajin Nam, Jin-Kyu Kim, Harry Jung, and Jun Gyo Suh

Received: 3 April 2013 / Revised: 10 June 2013 / Accepted: 24 June 2013 / Published Online: 28 February 2014

Abstract The inhibitory effects of lipid accumulation on ethanol extract from stem bark of Japanese horse chestnut (JHC) were evaluated. Exposure to JHC extract (10-100 μg/mL) for a 72 h incubation period did not alter cell viability compared to the untreated control. JHC extract, with concentrations of 25, 50, and 100 μg/mL, inhibited lipid accumulation in 3T3-L1 adipocytes in a dose dependent manner. The expression of PPARγ and C/EBPα, important adpogenic key markers was significantly reduced when JHC extract was added to cells for 8 days compared with the untreated control group. These results suggest that JHC extract might be a potential therapeutic agent as a natural anti-obesity material.

Keywords: anti-obesity, *Aesculus turbinate* bark, 3T3-L1 adipocyte, lipid accumulation

Introduction

Obesity has become a worldwide epidemic and an increasingly serious public health problem (1). Obesity results from excess caloric intake being stored as triglyceride in adipose tissue and decreased energy expenditure (2). It increases of the risk of developing insulin resistance, type 2 diabetes, cardiovascular disease, hypertension, hyperlipidemia, non-alcoholic fatty liver disease, stroke, cardiovascular disease, and some cancers (3). Prevention and treatment of obesity will reduce the incidence of these diseases.

In the past, several medications were available for obesity treatment (4). However, some anti-obesity drugs have been withdrawn from the market in the last decade because of deadly side effects (5). At present, orlistat is the only approved drug for long-term use in the treatment of obesity (5). Because of an increasing number of obese patients, new anti-obesity medications with no side effects are needed.

Adipose tissue plays an essential role in maintaining energy homeostasis of body. Obesity is mostly due to the overgrowth of adipose tissue arising from an energy imbalance (6). Thus, adipocyte is one of the most important targets for obesity treatment. The expansion of adipose tissue results from increased adipocyte size (hypertrophy) and increased adipocyte numbers (hyperplasia) (6). Peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-γ (C/EBPγ) have been reported as transcriptional key factors in adipogenesis (7). The amount of adipose tissue can be reduced by inhibiting adipogenesis, which may be regulated via these transcriptional key factors (7).

Japanese horse chestnut (JHC, *Aesculus turbinate*) is a deciduous tree, and is mostly distributed in Korea and Japan. Its seeds and leaves have been used as food in Japan (8). The seeds have been reported to have several biological activities such as anti-obesity (9), anti-inflammatory (10), anti-oxidative, and anti-genotoxic effects (11). However, there are very few studies on the stem bark of JHC.

In this study the anti-obesity effects of ethanol extract of the stem bark of JHC in 3T3-L1 preadipocytes was examined to find anti-obesity effects of this natural product.

Hajin Nam, Jun Gyo Suh (✉)
Institute of Natural Medicine, Hallym University, Chuncheon, Gangwon 200-702, Korea
Tel: +82-33-248-2692; Fax: +82-33-248-2690
E-mail: jgsuh@hallym.ac.kr

Jin-Kyu Kim
Gyeonggi Institute of Science and Technology Promotion, Suwon, Gyeonggi 443-270, Korea

Harry Jung, Jun Gyo Suh
Department of Medical Genetics, College of Medicine, Hallym University, Chuncheon, Gangwon 200-702, Korea

Materials and Methods

Materials 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM), β-actin antibody, insulin, 3-isobutyl-1-methylxanthine (IBMX), Oil Red O solution, Dimethyl sulfoxide (DMSO, D2650), and dexamethasone were provided by Sigma-Aldrich Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS), newborn calf serum (NCS), 25% Trypsin-EDTA, and Antibiotics-Antimycotic were obtained from Gibco (Rockville, MD, USA). A cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). A Quant-iT protein assay kit was purchased from Invitrogen (Auckland, New Zealand). Antibodies against PPARγ and C/EBPα were provided by Cell Signaling Technology (Beverly, MA, USA). Horse radish peroxidase-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Preparation of *Aesculus turbinate* extract The stem bark of *Aesculus turbinate* was provided by the Gangwon Institute of Forest Research (GIFR). A voucher specimen (G-11-09) was deposited in the GIFR herbarium. The JHC stem bark (1 kg) was ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to a particle size of 0.25 mm or less and extracted twice with 10 L of 70% ethanol at room temperature for 3 days. The extract solution was filtered through Whatman filter paper (Whatman, Maidstone, UK). The extract solution was concentrated in vacuo at 40°C, and the aqueous residue freeze dried, yielding 78.7 g of powder. In order to using the test, the powder was re-dissolved in DMSO.

Cell culture 3T3-L1 preadipocytes were maintained in DMEM containing 10% NCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C until confluence. To induce adipogenesis, the cells were cultured till two days after confluence (day 0) and then induced by a differentiation mixture containing 5 μg/mL insulin, 0.5 μM dexamethasone, and 0.5 μM IBMX in DMEM with 10% FBS. Thereafter, the medium was changed with 10% FBS/DMEM containing 5 μg/mL insulin every two days. The test compounds were treated with 0, 10, 25, 50, and 100 μg/mL of JHC extract at the initiation of differentiation and with every medium change for 8 days (day 0-8).

Oil Red O staining Oil Red O staining was performed using the procedures of Kim *et al.* (12) with minor modifications. In brief, differentiated adipocytes were washed with phosphate-buffered saline (PBS), and then fixed with 10% formalin in PBS for 60 min at room temperature (RT). The fixed cells were then washed 3 times with PBS, and stained with filtered Oil Red O solution for 30 min at RT and then the cells were rinsed 2 times with distilled water. The morphology of the cells was examined under an inverted microscope (ZEISS, Oberkochen, Germany) and the images were captured using a digital camera (Nikon, Tokyo, Japan). Stained oil droplets were dissolved with isopropanol and quantified using luminescence microplate readers (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The values were calculated as percentages of the untreated control and expressed as means±SD.

Cell viability assay Cell viability was determined using CCK-8, according to the manufacturer's instructions. Briefly, 3T3-L1 cells stimulated by the adipogenic cocktail in the absence or presence of different concentrations of JHC extract (0, 10, 25, 50, and 100 μg/mL) were employed in this experiment in 96-well plates (5,000 cells/well) for 72 h. After each indicated time period, DMEM with test compounds were replaced by DMEM with 20 μL of CCK-8 solution and incubated in the dark for 2 h. The amount of dehydrogenase activity in viable cells was detected using luminescence microplate readers (Spectramax/M2^e; Molecular Devices) at 450 nm. Each assay was carried out in triplicate.

Western blot analysis Cells were seeded in 100 mm dish plates. Adipocyte differentiation and JHC extract treatment were carried out as described above. The cell extracts were prepared by adding protein extraction solution (PRO-PREP; Intron Biotechnology, Sungnam, Korea). The protein content in cell lysates was determined according to the method described by Bradford (13). Protein (20 μg) were separated by 10% SDS-PAGE and blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20. Blots were incubated at 4 overnight with primary antibodies, including PPARγ (1:200) and C/EBPα (1:5,000), and goat anti-mouse IgG-HRP secondary antibody (1:5,000) in a blocking solution. The target protein was detected using ECL western blotting detection reagent (GE Healthcare, Seoul, Korea).

Statistical analysis Data are presented as the mean± standard deviation (SD). To determine statistical significance, data were analyzed using student's *t* test. A value of $p<0.05$ was considered to be statistically significant.

Results and Discussion

Effect of JHC extract on anti-adipogenesis during adipocyte differentiation In the course of screening for anti-adipogenic activity in dozens of natural products using

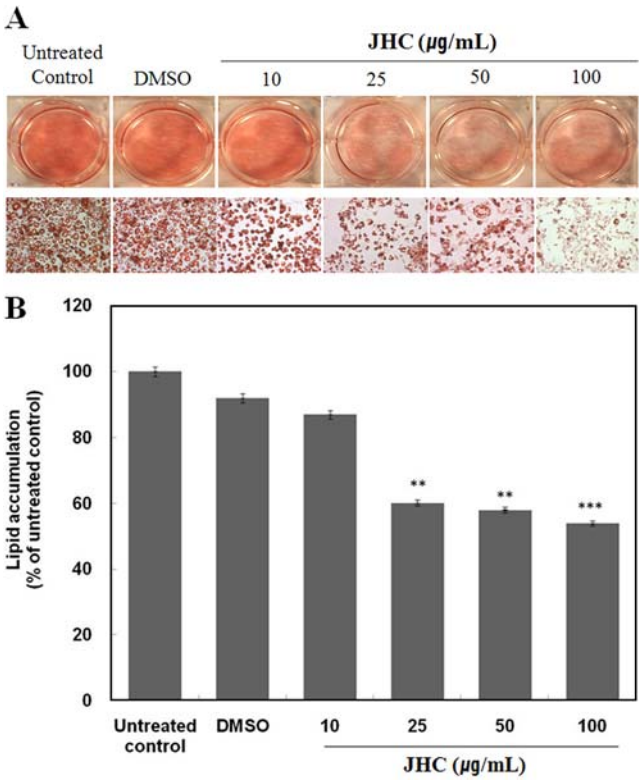

Fig. 1. The effect of JHC extract on lipid accumulation in adipocyte differentiation. 3T3-L1 preadipocytes were treated with samples for the entire differentiation process (day 0-8). Representative microscopic morphological images of the adipocytes were stained with Oil Red O after 8 days of treatment with 0, 10, 25, 50, and 100 μg/mL JHC extract and microscopic pictures were taken at 200× magnification (A). The OD values were measured from the isopropanol elution of the Oil Red O stained cells (B). Values are expressed as means±SD of 3 separate experiments. **$p<0.01$, ***$p<0.005$ compared with an untreated control.

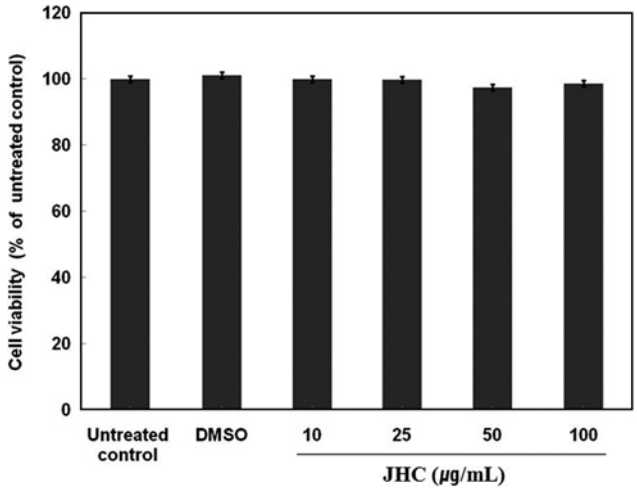

Fig. 2. The effect of JHC extract on the viability of differentiating adipocytes. Two days post confluent 3T3-L1 cells were incubated for 72 h with an adipogenic cocktail and various concentrations of JHC extract (0, 10, 25, 50, and 100 μg/mL). The CCK-8 assay for testing cell viability was performed after 72 h of treatment. Values are expressed as means±SD of three separate experiments.

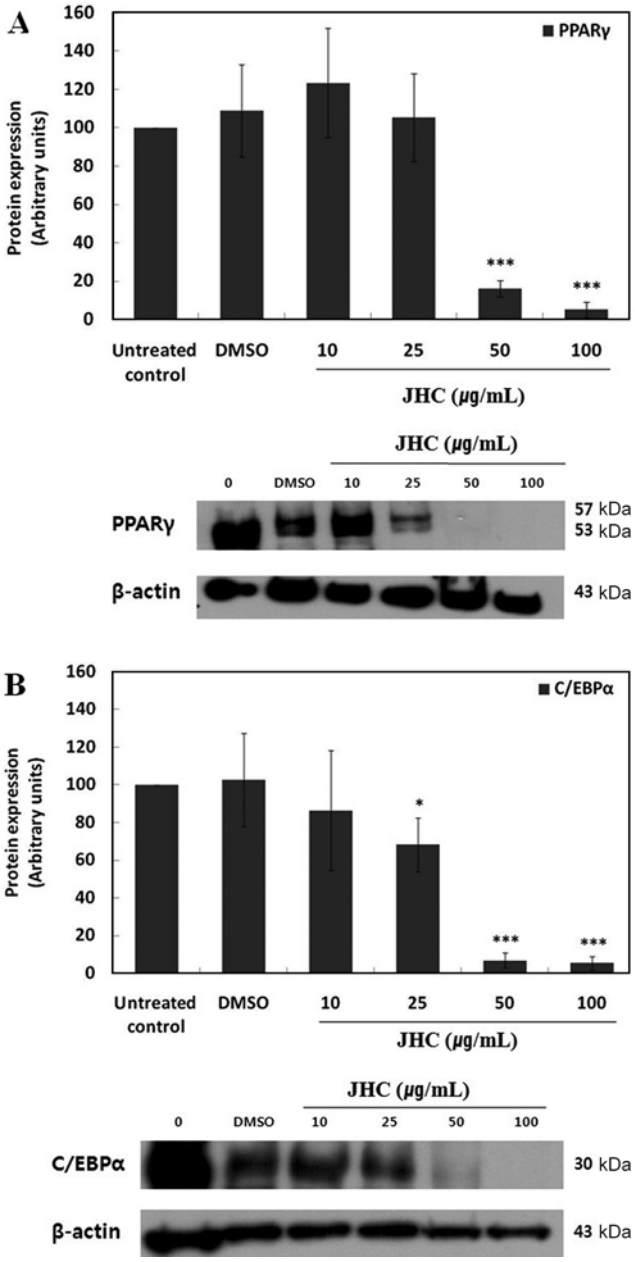

Fig. 3. Effects of JHC extract on the expression of adipogenic-related proteins by Western blot analysis. The results showed that the expression levels of PPARγ (A) and C/EBPα (B) were reduced in 3T3-L1 cells treated with JHC extract on day 8 of differentiation. These signals were normalized to β-actin. Values are expressed as means±SD of 3 separate experiments. *$p<0.05$, ***$p<0.005$ compared with an untreated control.

3T3-L1 preadipocytes as an *in vitro* assay system, the 70% ethanol crude extract of JHC showed significant inhibitory activity (Fig. 1). The inhibitory effect of JHC extract on adipogenesis was observed after Oil Red O staining. As shown in morphological observations, lipid accumulation in JHC extract treated cells was lower than in untreated control cells (Fig. 1A). In the OD values of JHC extract treated cells, a significant decrease of lipid accumulation

was detected as compared to the untreated control cells (Fig. 1B). The results showed that 25, 50, and 100 μg/mL concentrations of JHC extract were effective in inhibiting the lipid content up to 40, 42, and 46% of the untreated control cells, respectively. However, this JHC extract barely showed inhibitory activity when used on differentiated adipocytes (data not shown). These results suggest that JHC extract might have an anti-adipogenic effect.

Effect of JHC extract on cell viability To investigate the effect of JHC extract on the viability of 3T3-L1 cells in the early stage of adipocyte differentiation, the differentiating 3T3-L1 cells were treated with various concentrations (0, 10, 25, 50, and 100 μg/mL) of JHC extract for 72 h. As shown in Fig. 2, treating cells with JHC did not have any impact on cell viability when compared with control cells.

Effect of JHC extract on protein expression of PPARγ and C/EBPα PPARγ and C/EBPα are major transcriptional factors during adipocyte differentiation (7). Thus, to investigate the molecular mechanism underlying the anti-adipogenic effect of JHC extract on 3T3-L1 cells, we analyzed the expression of the key transcription markers involved in adipogenesis. After 3T3-L1 preadipocytes were exposed to JHC extract for 8 days, we measured the protein levels of PPARγ and C/EBPα. JHC extract treated cells significantly suppressed the expression of these proteins compared with untreated control cells (Fig. 3). In conclusion, our study indicated that the 70% ethanol extract of JHC significantly inhibited adipogenesis as a result of down-regulating PPARγ and C/EBPα without altered cell viability. Although further studies are needed to explain the relative mechanisms of this anti-obesity effect, our results suggest that JHC extract might be a potential candidate for novel agents for the prevention and/or treatment of obesity.

Acknowledgments This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A6A1048184) and by Hallym University Specialization Fund (HRF-S-41).

References

1. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, Singh GM, Gutierrez HR, Lu Y, Bahalim AN, Farzadfar F, Riley LM, Ezzati M. National, regional, and global trends in body-mass index since 1980: Systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet 377: 557-567 (2011)
2. Reaven G, Abbasi F, McLaughlin T. Obesity, insulin resistance, and cardiovascular disease. Recent Prog. Horm. Res. 59: 207-223 (2004)
3. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. Am. J. Clin. Nutr. 83: 461S-465S (2006)
4. Nam Y, Jung H, Karuppasamy S, Lee JY, Kang KD, Hwang KY, Seong SI, Suh JG. Anti-hyperlipidemic effect of soybean extract fermented by Bacillus subtilis MORI in db/db mice. Lab. Anim. Res. 28: 123-129 (2012)
5. Zhang Y, Huang C. Targeting adipocyte apoptosis: a novel strategy for obesity therapy. Biochem. Bioph. Res. Co. 417: 1-4 (2012)
6. Garaulet M, Hernandez-Morante JJ, Lujan J, Tebar FJ, Zamora S. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. Int. J. Obes. 30: 899-905 (2006)
7. Park DY, Ahn YT, Huh CS, Jeon SM, Choi MS. The inhibitory effect of *Lactobacillus plantarum* KY1032 cell extract on the adipogenesis of 3T3-L1 cells. J. Med. Food. 14: 670-675 (2011)
8. Sato I, Kofujita H, Suzuki T, Kobayashi H, Tsuda S. Antiinflammatory effect of Japanese horse chestnut (*Aesculus turbinate*) seeds. J. Vet. Med. Sci. 68: 487-489 (2006)
9. Kimura H, Ogawa S, Katsube T, Jisaka M, Yokota K. Antiobese effects of novel saponins from edible seeds of Japanese horse chestnut (*Aesculus turbinata* BLUME) after treatment with wood ashes. J. Agr. Food Chem. 56: 4783-4788 (2008)
10. Sato I, Kofujita H, Suzuki T, Kobayashi H, Tsuda S. Antiinflammatory effect of Japanese horse chestnut (*Aesculus turbinata*) seeds. J. Vet. Med. Sci. 68: 487-489 (2006)
11. Sato I, Suzuki T, Kobayashi H, Tsuda S. Antioxidative and antigenotoxic effects of Japanese horse chestnut (*Aesculus turbinata*) seeds. J. Vet. Med. Sci. 67: 731-734 (2005)
12. Kim MJ, Park MH, Jeong MK, Yeo MK, Yeo JD, Cho WI, Chang PS, Chung JH, Lee JH. Radical scavenging activity and anti-obesity effects in 3T3-L1 preadipocyte differentiation of Ssuk (*Artemisia princeps* Pamp.) extract. Food Sci. Biotechnol. 19: 535-540 (2010)
13. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254 (1976)