Repressive effects of red bean, *Phaseolus angularis*, extracts on obesity of mouse induced with high-fat diet via downregulation of adipocyte differentiation and modulating lipid metabolism

Young Mi Park¹ · Jee In Kim^{2,3} · Dong Hyun Seo⁴ · Joo Hee Seo⁵ ·
Jae-Hwan Lim¹ · Jong Eun Lee¹ · Je-Yong Choi³ · Eul-Won Seo^{1,2}

Received: 5 March 2018 / Revised: 7 June 2018 / Accepted: 21 June 2018 / Published online: 7 July 2018
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Abstract Obesity is generally caused by quantitative changes in adipocyte differentiation and fat metabolism. Only a few studies have been determined the effect of red beans extract on obesity and plasma cholesterol concentration. We have been studied the functional activities of red-bean extracts including anti-oxidative effect against DNA and cell damages. Histological study including micro CT analysis showed that the accumulation of fat in hepatocytes and intestines was significantly decreased in red bean extract treated group. In addition, plasma cholesterol and triglyceride levels were decreased in blood samples. In addition, it was confirmed that the red bean extract inhibited the expression of PPAR γ , Fabp4 and RETN genes, which regulate total adipocyte differentiation and lipid metabolism. Red bean extract inhibits the expressions of transcription factors associated with adipocyte differentiation in a dose-dependent manner, thereby inhibiting fat

accumulation and decreasing blood lipid levels in obese mice induced by high fat diet.

Keywords *Phaseolus angularis* · Lipid metabolism · Obesity · Micro-CT · Adipocyte differentiation

Abbreviations

FAA	Formaldehyde–acetic acid–ethanol
TMB	Tetramethylbenzidine
APOE	Apolipoprotein E
Fabp4	Fatty acid binding protein 4
Scd1	Adipsin and stearyl-coenzymeA desaturase 1
PPAR γ	Peroxisome proliferator-activated receptor gamma
RETN	Resistin
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
IBMX	3-Isobutyl-1-methylxanthine
DEPC	Diethyl pyrocarbonate

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10068-018-0421-2>) contains supplementary material, which is available to authorized users.

✉ Eul-Won Seo
ewseo@andong.ac.kr

¹ Department of Biological Science, Andong National University, 1375 Gyungdong-ro, Andong 36728, South Korea

² Institute of Marine Biotechnology, Andong National University, Andong 36728, South Korea

³ Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 41566, South Korea

⁴ Biotechnology R&D Center, Cell Biotech, Gimpo 10003, South Korea

⁵ Department of Korean Neuropsychiatry, National Medical Center, Seoul 04564, South Korea

Introduction

Obesity is a complex disorder involving various causes including genetic, environmental, changed dietary patterns and other factors (Grundy, 1998). Obesity is generally induced by quantitative changes in adipocyte differentiation and an imbalance between energy intake and energy expenditure. Obesity influences normal physical and biochemical functions in the body and is associated with several diseases (The GBD 2015 Obesity Collaborators, 2017). For this reason, many studies have investigated the differentiation of pre-adipocyte and lipid metabolism to understand and cure obesity-related diseases (Rosen and Spiegelman, 2014). Many studies have explored functional

substances from diverse floras involved in biological activities to treat such chronic diseases (Cefalu et al., 2008). For example, soy protein and soy protein hydrolysates is known to be effective in improving obesity-related diseases by prohibiting weight gain and high lipid accumulation and reducing blood lipid and body fat contents (Aoyama et al., 2000; Velasquez and Bhatena, 2007). Moreover, several substances from soybean, such as isoflavones and beta-conglycinin, have a function in enhancing lipid metabolism by reducing the blood-cholesterol levels of obesity and improving metabolic syndrome by decreasing blood triacylglycerol levels and inhibiting the accumulation of visceral fat (Ali et al., 2004; Kohno et al., 2006).

Also, red bean (*Phaseolus angularis*), used as this study material, is the second most demanded pulse crop in Asia next to soybeans and it is commonly used as first choice beans for cooking with rice in various dishes, and bean pastes. Red bean contains cyanidin, a derivative type of anthocyanins, which has shown a significantly high antioxidant capability and anti-tumor activity (Knaup et al., 2009; Koide et al., 1997; Yoshida et al., 1996). The methanol and water extracts of most red bean species are known to contain large amounts of anthocyanins (Akond et al., 2011). However, only few studies are being conducted to identify physiologically active substances in red beans. Although anthocyanin-type pigments are abundant in red bean hot water extracts, produced during the red bean paste manufacturing process, all the extracts are currently discarded without supplementary studies taking place. Therefore, this study aims to investigate on the anti-obesity function of red beans by identifying the effects of red bean hot water extracts on lipid metabolism and obesity, which are usually disposed-off during food processing.

Materials and methods

Materials and reagents

Red bean hot water extracts (RBE) used in this study has been made by boiling 1 kg of dried red bean (*Phaseolus angularis*) bought from Mungyeong Nonghyup (Mungyeong, Korea) with ten liter of water for 4 h. Subsequently, filtered extract was centrifuged at 15,000 rpm for 30 s and supernatant was freeze-dried and stored in the freezer. Antibodies used for western blotting were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Laboratory animals and diets

All 3-week-old male mice (C57BL/6) were raised in a breeding room under constant conditions (a temperature of 21.4 ± 0.05 °C, a humidity of $61 \pm 1\%$, a light-dark cycle of 12 h). Mice were domesticated for a week by feeding them with sufficient water and food (AIN-76A Purified Rodent Diet). Subsequently, twenty-one mice were classified into three groups of control group (AIN-76A diet), high-fat diet group (40% beef tallow + AIN-76A diet, HFD group) and RBE diet group (40% beef tallow + AIN-76A diet supplemented with 30% *P. angularis* extracts, H-PA30 group). Each group was fed with designed food compositions for 8 weeks (Table 1). Weight changes of mice and the amount of food and water intake were checked at indicated intervals during the experiment period. All animal testing procedures were performed in accordance with principle of laboratory animal care of Andong National University under the regulations of laboratory animal management and guidance (2014-3-1111-09-01).

Amount of adipose tissue and histological observation

After measuring the weights, adipose tissues in liver and epididymis were treated and fixated in FAA solution at 4 °C for 24 h to identify the size and the amount of fat cells in adipose tissues. After fixation, the tissues were washed and dehydrated and then, made into a paraffin block by treating with paraffin solution. Tissue blocks were cut into 4–6 µm-thick sections. Sliced tissue sections were double-stained with hematoxylin and eosin and observed under optical microscope (Olympus BX50, Japan). Photographs

Table 1 Composition of experimental diets (g/kg)

Diet composition	Control	HFD	H-PA30
Casein	200	200	200
DL-Methionine	3	3	3
Corn starch	150	150	150
Sucrose	500	345	45
Cellulose	50	50	50
Corn oil	50	–	–
Salt mix	35	35	35
Vitamin mix	10	10	10
Choline bitartrate	2	2	2
Beef tallow	–	205	205
<i>P. angularis</i> extract	–	–	300

Control normal diet, HFD high fat diet, H-PA30 high fat diet + 30% *P. angularis* extract powder

were taken using Olympus DP-71 and fat tissues in each group were observed.

Analysis of blood components

Collected blood samples were centrifuged for 10 min at 3500 rpm to separate plasma. To assess the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), plasma was added to each TMB substrate solution according to the method as described previously (Reitman and Frankel, 1957). The total cholesterol (TC) and HDL-cholesterol contents were measured at 500 nm using a detection kit (Asan, Korea) based on fermentation methods of Richmond (1976) and Noma et al. (1978) respectively. Blood cholesterol and HDL-cholesterol contents were presented as mg/dl according to standard calibration curve. LDL-cholesterol content was calculated based on an equation described method by Friedeman et al. (1991). The total lipid content was assessed with manufacturer's instruction using a kit (Asan, Korea) by methods of Frings and Dunn (1970).

Measuring abdominal fat volumes using in-vivo micro-CT

Abdominal fat volumes, colored gray, in each intra-abdominal tissue of male C57BL/6 mice were calculated using an in vivo micro-CT scanner (In-vivo Micro-CT, SkyScan 1076, SKYSCAN N.V., Belgium) and Mimics software version 13.0 (Materialise N.V., Belgium). Based on the gray values assessed using threshold methods, volumes in each classified section of lumbar vertebral, lean tissue, adipose tissue, and skin were assessed by recomposing extracted abdominal adipose tissues into 3D structures. Measured values are presented, including weight, with the volume of total abdominal fat, the volume of abdominal subcutaneous fat, and the volume of abdominal visceral fat.

Induction of preadipocyte differentiation

Purchased 3T3-L1 preadipocytes from American Type Culture Collection (ATCC) were cultured in a cell incubator by adding 10% bovine serum (Gibco Inc., USA) and 1% penicillin–streptomycin (Gibco Inc., USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco Inc., USA) under the conditions of 37 °C and 5% CO₂.

To induce the differentiation of preadipocytes, the cells were cultured for 48 h in a differentiation-inducing medium containing 1 µM dexamethasone (Sigma, USA) and 0.5 mM IBMX (Sigma, USA) and then transferred to another differentiation-inducing medium containing 1 µg/ml insulin. Subsequently, the medium had been changed

twice every 48 h whilst checking the degree of differentiation. To verify the ability to suppress preadipocyte differentiation indicated concentrations of RBE were treated during the differentiation induction process. In contrast, the control group was induced for differentiation without adding RBE. Cell survival rate was determined with MTT assay using micro plate reader (infinite 200, Tecan Trading AG, Switzerland). The measured absorbance was presented as the percentage of the average absorbance values of the control group using the following equation. (Cell viability (%) = (absorbance of extract treatment group/absorbance of control group) × 100). And adipocyte differentiation ratio was measured by Oil Red O staining method.

Total RNA isolation and cDNA synthesis

The culture media was removed from 6 well plate treated with RBE. The cultivated cells were washed with 2 ml of DPBS (WelGENE Inc., Korea) and collected to a polypropylene tube using TRIzol reagent (Gibco Inc., USA). The collected cells were kept at room temperature for 5 min and then shaken for 15 s by adding chloroform (Sigma, USA). The cells were left at room temperature for another two minutes and centrifuged at 11,000 rpm for 10 min at 4 °C to collect the supernatant. The collected supernatant was mixed with 100% isopropanol (Sigma, USA) and kept at room temperature for 10 min. Afterwards, the solution was centrifuged at 11,000 rpm for 10 min at 4 °C. After the removal of supernatant, RNA pellet was washed with 75% ethanol at 4 °C and centrifuged at 8000 rpm for 5 min. The dried RNA pellet was dissolved at 60 °C by pipetting the solution with 50 µl of RNase-free water (DEPC-treated water). Partially mixed genomic DNA was separated and eliminated using total RNA clean-up kit (Ambion, USA). The extracted RNA was quantified using micro plate reader and isolated RNA was used for the synthesis of single stranded cDNA using SuperScriptTM III RT kit (Invitrogen, USA) with template.

Quantitative RT-PCR

The Quantitative RT-PCR results were analyzed using PTC-200 rev (Bio-Rad, USA). The sets of specific primers were designed using Primer Express Software version 3.0 (Applied Biosystem, USA) and the synthesis was requested to Invitrogen (USA). One of the most commonly used housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control.

PCR mixture was added with 1 µl of forward and reverse primers diluted with TaKaRa Ex TaqTM (TaKaRa, Japan) 0.2 µl, 10 × Ex Taq buffer 2 µl, dNTP mixture (2.5 mM each) 2 µl, 25 pmol, and 12.8 µl of Cell/Tissue Culture Grade Water (WelGENE Inc., Korea). The

synthesized cDNA was quantified and diluted with 50 ng/ μ l and only 1 μ l was used for the experiment. Corresponding T_m value of each gene was used for each PCR phase. Using 1.8% agarose gel containing EtBr (Fluka, USA), 5 μ l of each PCR product and 1 μ l of loading dye were mixed together in Mupid gel electrophoresis system (TaKaRa Inc., Japan). The mixture was electrophoresed at 100 V for 15 min. Upon the completion of electrophoresis, the results were verified using a UV illuminator in gel image analysis system (Core Bio iMaxTM, Korea). The degree of expression was analyzed using the Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.). Selected genes related adipocyte differentiation and primer sequences used in RT-PCR were as below, PPAR γ -F; 5'-ATGGAAGACCACTC FCATT-3', PPAR γ -R; 5'-TGGCCATGAGGGAGTTCTG-3', Fabp4-F; 5'-AGTGGGCTTTGAGGACAAA-3', Fabp4-R; 5'-GGTGATTTCATCGAATTCCA-3', Scd1-F; 5'-CGC CCCTACGACAAGAAC-3', Scd1-R; 5'-ACACCCCGAT AGCAATATCC-3', RETN-F; 5'-CGTACCCACGGGATG AA-3', RETN-R; 5'-GAAGCGACCTGCAGCTTAC-3', mGAPDH-F; 5'-ATGCCCCCATGTTTGTG-3', mGAP DH-R; 5'-GATGCAGGGATGATGTTCTF-3'.

Statistical analysis

All results acquired in each experiment were presented as average $\pm$ standard deviation. Statistical analysis was performed using the software SPSS version 12.0 (Chicago, IL, USA) and then, individual identity of variance and mean were examined using t-tests. All analyzed results were verified by performing one way analysis of variance (ANOVA) with Duncan's multiple range test. p values of < 0.05 were considered statistically significant.

Results and discussion

Changes in body-weight and food intake

Body weight changes were measured individually and food intake amounts of mice were determined for assessing the effect of RBE. After 4 weeks of feeding the test diet, the body weights of mice in high fat diet group (HFD group) gradually increased compared to control group. After 8-week dietary exposure to test diets, the body weight of control groups was 25.1 ± 2.5 g. In contrast, the body weight of HFD group was 30.3 ± 2.2 g which is 20.7% higher than control group. However, the body weight of HFD group fed with the diet containing RBE (HFD with *P. angularis* extracts group, H-PA30 group) was 28.1 ± 1.7 g on the 8th week, only 11.9% higher than control group (Fig. 1A). In contrast, the amounts of food intake were 3.1 ± 0.2 g in control group, 2.4 ± 0.1 g in HFD group,

and 2.5 ± 0.2 g in H-PA30 group, showing no significant differences among experimental groups (Fig. 1B). Experimental group fed on a high fat diet with the water extracts of corn silk (*Zea mays*), red bean sprouts (*Phaseolus angularis*), shiitake mushroom (*Lentinus edodes*), and green pepper (*Capsicum annuum*) showed a lower increase in body weight comparing to the group fed with a high fat diet only (Seo et al., 2009). Villanueva et al. (2011) suggested that mice fed on high fat diet together with okara, soybean by-product, showed a lower increase in body weight in obesity-induced animal model, indicating the diet with soybean curd by-products was effective in weight reduction. Although the amount of food intake in HFD group was relatively lower than other groups in this study, the HFD group showed a distinctive weight increase. In contrast, H-PA30 group exhibited a significant decrease in body weight compare to HFD group even though a slight increase in food intake.

Histological changes of fats in liver and epididymis

The weights of liver and epididymal adipose tissue in HFD group were 1.0 ± 0.1 and 1.9 ± 0.1 g, respectively, in this study. The weights were higher by 25.0 and 26.7% than those of control group. The weights of liver and epididymal adipose tissue in H-PA30 group were 0.9 ± 0.1 and 1.5 ± 0.1 g, respectively, identified to be lower than those of HFD group (Fig. 1D). Moreover, the study examined the effects of RBE on the histological changes of adipose tissues in liver and epididymis of mouse on a high fat diet. The cell nuclei of liver tissues in control group were mostly round-shaped and Kupffer cells were observed to be evenly distributed in between liver cells (Fig. 1C-①). The shapes and sizes of fat globule in liver cells of HFD group were seriously irregular and severely deformed Kupffer cells were detected due to fat infiltration (Fig. 1C-②). Severe fat infiltration and greatly enlarged fat globules were observed in epididymal adipose tissues of HFD group (Fig. 1C-⑤). Although liver cells in H-PA30 group were slightly enlarged, ballooning degeneration of liver cells were much less developed than HFD group. Most of liver and Kupffer cells in H-PA30 group were observed to be similar to those of control group (Fig. 1C-③). Although the number of fat globule was relatively greater in epididymal adipose tissue compare to HFD group, their sizes were not greatly enlarged compare to HFD group (Fig. 1C-⑥).

An increase in body fat rather than an increase in body weight is generally known to be a negative influence on health and, in particular, an increase in intra-abdominal adipose tissue masses considered as a risk factor rather than subcutaneous fat (Björntorp, 1987). Imbalance in fatty acid synthesis and oxidative balance generates fat accumulation and insulin resistance, eventually leading to

(D)

	Weight (g)	
	Liver	Epididymal fat
Control group	0.8±0.1 ^A	1.2±0.1 ^A
HFD group	1.0±0.1 ^B	1.9±0.1 ^C
H-PA30 group	0.9±0.1 ^{A, B}	1.5±0.1 ^B

Fig. 1 Changes of (A) body weight and (B) food intake in the experimental groups. All 3-week-old male mice (C57BL/6) were raised in a breeding room under constant conditions as indicated materials and methods. Mice were domesticated for a week by feeding them with sufficient water and food (AIN-76A Purified Rodent Diet) and also each group fed and measured for 8 weeks. Control group: AIN-76A purified rodent diet, HFD group: 40% beef tallow + AIN-76A diet, H-PA30 group: HFD supplemented with RBE. All points in both (A) and (B) were represented by mean $\pm$ SEM ($n = 7$). Differences of body weight among control, HFD and H-PA30 mice were analyzed using ANOVA with $p = 0.015 < 0.05$ as the criterion of significance. C Effects of *P. angularis* extracts on liver tissue and epididymal fat morphology (X20). Scale bar = 100 μ m. Adipose tissues (①, ②, ③) in liver and epididymis (④, ⑤, ⑥) were treated and fixated in FAA solution at 4 $^{\circ}$ C. In order to identify the size and the amount of fat cells, sliced tissue sections were double-stained with hematoxylin and eosin and observed under optical microscope (Olympus BX50, Japan). ①, ④: control group, ②, ⑤: HFD group, ③, ⑥: H-PA30 group. D Weights of liver and epididymal fat from each groups were measured. The results were expressed as mean $\pm$ SEM ($n = 7$). Differences of liver weight and epididymal fat weight among control, HFD and H-PA30 mice were analyzed using ANOVA with Duncan's multiple range test with $p = 0.015 < 0.05$ and $p = 0.00 < 0.05$ as the criterion of significance, respectively. Sharing same alphabet indicates no significant difference between two groups ($p < 0.05$)

chronic liver diseases and metabolic syndrome. Solloff et al. (1973) reported that high fat diet induces fat accumulation and weight gain in both the liver and epididymal adipose tissue. Moreover, the incidence of metabolic complications increases as abdominal fat and visceral fat contents increase despite the same body fat content (Despres, 1993). Recently, the hot-water extract of hog millet (*Panicum miliaceum* L.) has positive effects in curing and preventing non-alcoholic hepatic steatosis by significantly reducing fat and cholesterol accumulation in liver tissue (Despres, 1993). Furthermore, it was reported that soybean curd residue is effective in preventing obesity by reducing adiposity and cell damage generated by a high fat diet around the central veins of liver cells (Matsumoto et al., 2007). This study also verified that fat degeneration and ballooning degeneration were alleviated in the experimental group fed on a high fat diet combined with RBE. In addition, the size of fat globules was identified to be not greatly enlarged.

Changes in plasma lipid, cholesterol levels and other components

This study investigated on the effects of RBE intake on the changes of AST, ALT, TC, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and total lipid (TL) levels induced by a high fat diet. Among blood components, the levels of AST and ALT activity were 122.0 ± 5.2 and 38.3 ± 2.3 IU/l,

respectively, in HFD group and 99.8 ± 3.2 and 27.1 ± 2.1 IU/l, respectively, in H-PA 30 group. These activities of two enzymes, used as an indicator of liver abnormality, restored closely to those in control mice as showing 115.6 and 126.0% in HFD-30A group mice compared to 141.4 and 178.1% in HFD mice. The TC levels were 219.6 ± 8.7 mg/dl in HFD group and 186.0 ± 7.5 mg/dl in H-PA 30 group, exhibiting considerably high levels compared to 146.4 ± 6.0 mg/dl in control group. The HDL-C and LDL-C levels were shown to 138.2 and 122.4% higher, respectively, in HFD group and 116.3 and 110.7% higher, respectively, in H-PA 30 group than those levels in control. The TL levels were 138.8% in HFD group and 115.8% in H-PA 30 group compared to the level in control group (Fig. 2B).

Hyperlipidemia is the condition characterized by abnormally increased level of lipid contents such as cholesterol, triglyceride, free fatty acid, and others in blood. And it is known widely as a risk factor for coronary artery disease by affecting the expression of the metabolic sensor proteins including acetyl-CoA carboxylase and, stearoyl-CoA desaturase-1 (Wat et al., 2009). According to a recent study, the levels of cholesterol and triglyceride were reduced in mice after treatment with soybean oligosaccharides, indicating the health improvement by down regulation in plasma lipid concentration (Chen et al., 2003). Furthermore, the administering 10% soybean solvent fractions to a Wistar mouse in the growth period was effective in preventing obesity by declining cholesterol and triglyceride levels. In this study, we also verified that RBE was also effective in decreasing plasma lipid and cholesterol levels induced by a high fat diet.

Measurement of abdominal fat volumes by micro-CT scanning

Distribution of the abdominal fat of a mouse was determined using an in vivo micro-CT scanner (Skyscan 1076, SKYSCAN N.V., Belgium). Extensive amounts of subcutaneous fat and visceral fat were found to be accumulated in the abdomen of HFD group. In contrast, relatively less volume of the abdominal fat was identified in H-PA 30 group compare to HFD group (Fig. 2A). Comparing the abdominal fat volumes of each group, the highest volumes of visceral fat and subcutaneous fat were exhibited by HFD group with 2002.9 ± 33.5 and 524.7 ± 17.5 mm³, respectively. On the other hand, the volumes of visceral fat and subcutaneous fat were significantly lower in H-PA 30 group with 1962.2 ± 29.9 and 453.1 ± 11.8 mm³, respectively. Considering that the abdominal fat volume of HFD group was 2527.6 ± 51.0 mm³, the total abdominal fat volumes of H-PA 30 group was reduced to 2415.3 ± 41.7 mm³, exhibiting similar total abdominal fat

Fig. 2 Representative 3D (①, ②, ③) and 2D (①', ②', ③') images of total volume and partial area of abdominal adipose tissue (A). (Colored grey; visceral adipose tissue, colored yellow; subcutaneous adipose tissue). Abdominal fat volumes in each intra-abdominal tissue of male C57BL/6 mice were calculated using an in vivo micro-CT scanner (in-vivo Micro-CT, Skyscan 1076, Belgium) and Mimics software version 13.0 (materialise N.V., Belgium). ①, ①': control group, ②, ②': HFD group, ③, ③': H-PA30 group. Serum components (B) and volume of abdominal and subcutaneous adipose tissues (C) of the experimental groups were determined by the methods as described in materials and method. The results were expressed as mean $\pm$ SEM ($n = 7$). Differences between control, HFD and H-PA30 mice were analyzed using ANOVA with Duncan's test with $p < 0.05$ as the criterion of significance. Sharing same alphabet indicates no significant difference between two groups ($p < 0.05$). Sharing same alphabet indicates no significant difference between two groups ($p < 0.05$). (Color figure online)

(A)

(B) Serum components of the experimental groups

	Control group	HFD group	H-PA30 group
AST ¹⁾ (IU/L)	86.3 $\pm$ 3.4 ^A	122.0 $\pm$ 5.2 ^B	99.8 $\pm$ 3.2 ^{A,B}
ALT ²⁾ (IU/L)	21.5 $\pm$ 3.2 ^A	38.3 $\pm$ 2.3 ^A	27.1 $\pm$ 2.1 ^A
TC ³⁾ (mg/dL)	146.4 $\pm$ 6.0 ^A	219.6 $\pm$ 8.7 ^B	186.0 $\pm$ 7.5 ^{A,B}
HDL-C ⁴⁾ (mg/dL)	105.1 $\pm$ 4.4 ^A	145.3 $\pm$ 6.2 ^B	122.2 $\pm$ 6.3 ^{A,B}
LDL-C ⁵⁾ (mg/dL)	29.0 $\pm$ 3.1 ^A	35.5 $\pm$ 4.1 ^A	32.1 $\pm$ 3.4 ^A
TL ⁶⁾ (mg/dL)	432.9 $\pm$ 15.1 ^A	600.9 $\pm$ 24.6 ^B	501.2 $\pm$ 19.2 ^{A,B}

¹⁾ AST : Aspartate aminotransferase.. ²⁾ ALT : Alanine aminotransferase. ³⁾ TC : Total cholesterol.

⁴⁾ HDL : High density lipoprotein cholesterol. ⁵⁾ LDL : Low density lipoprotein cholesterol. ⁶⁾ TL : Total lipid.

(C) Volume of abdominal and subcutaneous adipose tissues

	Control group	HFD group	H-PA30 group
Weight (g)	25.1 $\pm$ 2.5	30.3 $\pm$ 2.2	28.1 $\pm$ 1.7
Total (mm ³)	2290.5 $\pm$ 66.9	2527.6 $\pm$ 51.0	2415.3 $\pm$ 41.7
Visceral (mm ³)	1890.3 $\pm$ 54.4	2002.9 $\pm$ 33.5	1962.2 $\pm$ 29.9
Subcutaneous (mm ³)	400.2 $\pm$ 12.5	524.7 $\pm$ 17.5	453.1 $\pm$ 11.8

volume of 2290.5 ± 66.9 mm³ in control group (Fig. 2C). This study also analyzed the total volume of subcutaneous fat and visceral fat distribution in mice using micro-CT scan. Here we verify that the abdominal fat distribution was not increased in H-PA 30 group unlike HFD group, indicating that red bean is effective in suppressing body fat accumulation.

Effect on adipocyte differentiation

The cell survival rates of 3T3-L1 adipocytes were 95.9% at 1.6 μ g/ml, 94.1% at 8 μ g/ml, 93.3% at 40 μ g/ml, and 90.6% at 200 μ g/ml of specimen concentration (Fig. S1). In order to examine the suppressive effects of RBE on 3T3-L1 adipocyte differentiation, the study treated cultured adipocytes with differentiation-inducing substance (1 μ M Dexamethasone, 0.5 mM IBMX, 1 μ g/ml Insulin) by different specimen concentrations. Upon the completion of differentiation induction, the cultured cells were dyed with

Oil Red-O solution and observed with a microscope to quantify fat contents. The cultured adipocytes treated with or without a substance inducing differentiation were established as positive and negative control groups, respectively. Compared to the adipocytes treated with differentiation-inducing substance, the fat contents of H-PA 30 group were 98.7% at 1.6 $\mu\text{g/ml}$, 95.2% at 8 $\mu\text{g/ml}$, 94.5% at 40 $\mu\text{g/ml}$, and 89.7% at 200 $\mu\text{g/ml}$, showed a decreasing tendency with dose-concentration dependent manner. Moreover, the fat contents of dyed adipocytes also showed a decreasing tendency as RBE concentration increased (Fig. 3A, B).

Measurement of transcription factor PPAR γ expression

To examine the effects of RBE on the expression of genes related to 3T3-L1 adipocyte differentiation induction, this study compared the expression of PPAR γ genes in experimental groups treated with different specimen concentrations with differentiation-induced control group. The expression rates of PPAR γ mRNA in treated cell line with RBE were 75.5% at 1.6 $\mu\text{g/ml}$, 44.5% at 8 $\mu\text{g/ml}$, 25.8% at 40 $\mu\text{g/ml}$, and 14.3% at 200 $\mu\text{g/ml}$, showing a decreasing tendency of expression with increasing concentrations of extracts (Fig. 3C). As adipocyte differentiation induction progresses, the fat metabolism-related gene expression increases with the regulation of transcription factor PPAR γ gene. An increase in PPAR γ gene expression is known to generate increased insulin absorption and adipocyte

differentiation (Spiegelman and Flier, 2001). According to recent studies, the formation of fat cells was prevented when preadipocytes differentiated to adipocytes and PPAR γ gene expression was suppressed (Ferre, 2004, Moller and Berger, 2003). They also reported that the removal PPAR γ gene resulted in insulin resistance. Moreover, Jin et al. (2012) reported that decreased PPAR γ gene expression could be effective in preventing fat formation when andrographolide (AG) was treated during early stage of adipocyte differentiation. We also examined aspects in the expression of PPAR γ mRNA in the groups treated with RBE. The extract was effective in decreasing PPAR γ gene expression that suppresses fat accumulation during adipocyte differentiation. Therefore, adipocyte differentiation suppression may be acquired through the inhibition of PPAR γ gene expression.

Analysis of lipid metabolism gene expression

The study analyzed the effects of RBE in the expression of Fab4 and Scd1 genes expressed during adipocyte differentiation induction. The Fabp4 genes expression levels in the experimental group were 99.5% at 1.6 $\mu\text{g/ml}$, 83.4% at 8 $\mu\text{g/ml}$, 29.1% at 40 $\mu\text{g/ml}$, and 28.7% at 200 $\mu\text{g/ml}$, showing a gradually decreasing tendency (Fig. 4A). However, the Scd1 mRNA expression remained almost unchanged compared to the differentiation-induced group (Fig. 4B). Adipocyte differentiation is known to be regulated by transcription factors involved in lipid metabolism, including apolipoprotein E (APOE), fatty acid binding

Fig. 3 Inhibitory activity of extracts from *P. angularis* on 3T3-L1 cell differentiation. Cultured 3T3-L1 cells were stained with oil-red O dissolved with isopropanol (A). All samples, control group (①), experimental group without extracts (②) and treated with RBE (③), were photographed under microscopy. And relative value ratio of differentiation was presented after quantification by spectrophotometrical analysis at 520 nm (B). The results were expressed as mean $\pm$ SEM of at least triplicate experimental results. Differences between control group and RBE treated groups were analyzed using Student's *t* test with $p = 0.001 < 0.05$ as the criterion of significance.

Suppressive effect of *P. angularis* extracts on adipogenic transcription factor, PPAR γ (peroxisome proliferator-activated receptor- γ), gene expression showed under extract dose dependent manner (C). Gene expression levels were normalized with GAPDH, and shown relative ratio to its expression in differentiated cells. The results were expressed as mean $\pm$ SEM of at least triplicate experimental results. Differences between control group and RBE treated group were analyzed using ANOVA with Duncan's test with $p < 0.05$ as the criterion of significance. Sharing same alphabet indicates no significant difference between two groups ($p < 0.05$). (Color figure online)

Fig. 4 Suppression of gene expression relating lipid metabolism and adipokine by extracts from *P. angularis*. Gene expression of (A) fatty acid binding protein, Fabp4, (B) stearyl- coenzyme A desaturase 1, Scd1 and (C) resistin, TETN were determined by quantitative RT-PCR with selected primers and calculated by gel image analysis using UV illuminator system (Core Bio iMaxTM, Korea). All results acquired in each experiment were presented as mean $\pm$ SEM of at least triplicate experimental results. Differences between control group (undifferentiated as a negative control and differentiated as a positive) and RBE treated group analyzed using ANOVA with Duncan's test with $p < 0.05$ as the criterion of significance. Sharing same alphabet indicates no significant difference between two groups

protein 4 (Fabp4), adipsin and stearyl-coenzymeA desaturase 1 (Scd1), PPAR γ , and others. Among these, Fabp4 gene facilitates the synthesis of long chain fatty acid to triglyceride. In addition, Scd1 gene promotes the conversion of stearic acid to oleic acid in the pathway of fat cell

formation as a reaction speed regulatory enzyme involved in the formation of monounsaturated fatty acid (Ntambi 1999; Von Eynatten et al., 2010).

A recent study assessed fatty acid metabolism-related gene expression in the liver tissues of fatty liver-induced mice. As a result, the study verified that the expression of L-Fabp and Scd1 decreased in the group treated with hog millet hot water extract, indicating that the extract is effective in suppressing fatty acid synthesis (Park et al., 2012). Moreover, Scd1 expression in mouse pre-adipocyte cell treated with soybean curd by-products during adipocyte differentiation, were decreased to the range between 50 and 85% depending on the treatment concentration in contrast to Fabp4 gene expression was almost unchanged compared to the control (Park et al., 2012). This could be attributable to soybean curd by-products which involve in lipid metabolism and regulate adipocyte differentiation (Choi et al., 2011). In this study, although RBE did not have much impact on Scd1 expression, it was identified to decrease the expression of Fabp4 genes effectively.

Analysis of adipokine gene expression

Here we examined the effects of RBE on the expression of adipokine genes. The expression rates of resistin genes in specimen-treated group were 36.6% at 1.6 μ g/ml, 5.2% at 8 μ g/ml, 4.0% at 40 μ g/ml, and 2.5% at 200 μ g/ml, showing a gradually decreasing tendency (Fig. 4C). Adipokine is known as a regulatory substance involved in body metabolism and it is produced and secreted from fat tissues (Lyon et al., 2003). Resistin (RETN), one of the most commonly known adipokines, is uniquely present in the cytoplasm of adipocytes and expressed only in adipocytes. It is also reported to induce diabetes by hampering insulin's metabolic effects. Also, the resistin gene expression is forecasted to be effective in regulating body metabolism since the expression level was reduced to 86% at the concentration of 100 nM endothelin-1 in 3T3-L1 adipocytes (Zhong et al., 2002). In this study, we also identified that the expression level of adipokine resistin was reduced to 2.5% with the treatment of RBE.

Briefly, we examined the effects of RBE on adipocyte differentiation in obesity of a mouse on a high fat diet. The fat accumulation in adipose tissues in liver and epididymis in H-PA 30 group was remarkably less than HFD group. Moreover, the activation levels of AST and ALT and plasma lipid level were found to be maintained at the similar levels of control group. According to micro-CT analysis, abdominal and subcutaneous fat in H-PA 30 group were much less developed compared with those of HFD group. Adipocyte differentiation showed a gradually decreasing tendency as the concentration of RBE increased, indicating the suppression effects of the extracts

on adipocyte differentiation. RBE are found to inhibit the expression of Fabp4 and RETN genes which are known to regulate transcription factor PPAR γ gene expression and energy and fat metabolism. In conclusion, the extracts are verified to reduce body toxicity generated by oxidative stress, suppress fat accumulation, and improve plasma lipid level. Red bean hot water extracts also inhibit adipocyte differentiation by decreasing the expression of differentiation-inducing transcription factor and the formation of fat cells.

Acknowledgements This work supported by a Grant from 2016 Research Funds of Andong National University.

Compliance with ethical standards

Ethical approval This study protocol was reviewed and approved by the institutional review board of the Andong National University (2014-3-1111-09-01).

Conflict of interest None of the authors of this study has any financial interest or conflict with industries or parties.

References

- Akond ASMGM, Khandaker L, Berthold J, Gates L, Peters K, Delong H, Hossain K. Anthocyanin, Total polyphenols and antioxidant activity of common bean. *Am. J. Food Technol.* 6: 385–394 (2011)
- Ali AA, Velasquez MT, Hansen CT, Mohamed AI, Bhathena SJ. Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. *J. Nutr. Biochem.* 15: 583–590 (2004)
- Aoyama T, Fukui K, Takamatsu K, Hashimoto Y, Yamamoto T. Soy protein isolate and its hydrolysate reduce body fat of dietary obese rats and genetically obese mice (yellow KK). *Nutrition.* 16: 349–354 (2000)
- Björntorp P. The associations between obesity, adipose tissue distribution and disease. *J. Int. Med.* S723: 121–134 (1987)
- Cefalu WT, Ye J, Zuberi A, Ribnicki DM, Raskin I, Liu Z, Wang ZQ, Brantley PJ, Howard L, Lefevre M. Botanicals and the metabolic syndrome. *Am. J. Clin. Nutr.* 87: 481S–487S (2008)
- Chen JR, Chiou SF, Suetsuna K, Yang HY, Yang SC. Lipid metabolism in hypercholesterolemic rats affected by feeding cholesterol-free diets containing different amounts of non-dialyzed soybean protein fraction. *Nutrition.* 19: 676–680 (2003)
- Choi MS, Kim JI, Jeong J B, Lee SB, Jeong JN, Jeong HJ, Seo EW, Kim TY, Kwon OJ, Lim JH. Suppressive effects of by-product extracts from soybean on adipocyte differentiation and expression of obesity-related genes in 3T3-L1 adipocytes. *J. Life Sci.* 21: 358–367 (2011)
- Despres JP. Abdominal obesity as important component of insulin-resistance syndrome. *Nutrition.* 9: 452–459 (1993)
- Ferre P. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes.* 53: S43–S50 (2004)
- Friedman JE, Neuffer PD, Dohm GL. Regulation of glycogen resynthesis following exercise. Dietary considerations. *Sport. Med.* 11: 232–243 (1991)
- Frings CS, Dunn RT. A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am. J. Clin. Pathol.* 53(1): 89–91 (1970)
- Grundy SM. Multifactorial causation of obesity: implications for prevention. *Am. J. Clin. Nutr.* 67: 563S–572S (1998)
- Jin L, Fang W, Li B, Shi G, Li X, Yang Y, Yang J, Zhang Z, Ning G. Inhibitory effect of andrographolide in 3T3-L1 adipocytes differentiation through the PPAR γ pathway. *Mol. Cell. Endocrinol.* 358: 81–87 (2012)
- Knaup B, Oehme A, Valotis A, Schreier P. Anthocyanins as lipoxygenase inhibitors. *Mol. Nutr. Food Res.* 53: 617–624 (2009)
- Kohn M, Hirotsuka M, Kito M, Matsuzawa Y. Decreases in serum triacylglycerol and visceral fat mediated by dietary soybean beta-conglycinin. *J. Atheroscler. Thromb.* 13: 247–255 (2006)
- Koide T, Hashimoto U, Kamei H, Kojima T, Hasegawa M, Terabe K. Antitumor effect of anthocyanin fractions extracted from red soybeans and red beans in vitro and in vivo. *Cancer Biother. Radiopharm.* 12: 277–280 (1997)
- Lyon CJ, Law RE, Hsueh WA. Adiposity, inflammation, and atherogenesis. *Endocrinology.* 144: 2195–2200 (2003)
- Matsumoto K, Watanabe Y, Yokoyama S, Okara, soybean residue, prevents obesity in a diet-induced murine obesity model. *Biosci. Biotechnol. Biochem.* 71: 720–727 (2007)
- Moller DE, Berger JP. Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *Int. J. Obes. Relat. Metab. Disord.* 27: S17–S21 (2003)
- Noma A, Nezu-Nakayama K, Kita M, Okabe H. Simultaneous determination of serum cholesterol in high- and low-density lipoproteins with use of heparin, Ca²⁺, and an anion-exchange resin. *Clin. Chem.* 24: 1504–1508 (1978)
- Ntambi JM. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J. Lipid Res.* 40: 1549–1558 (1999)
- Park MY, Jang HH, Lee JY, Lee YM, Kim JH, Park JH, Park DS. Effect of hog millet supplementation on hepatic steatosis and insulin resistance in mice fed a high-fat diet. *J. Korean Soc. Food Sci. Nutr.* 41: 501–509 (2012)
- Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.* 28: 56–63 (1957)
- Richmond W. Use of cholesterol oxidase for assay of total and free cholesterol in serum by continuous flow analysis. *Clin. Chem.* 22: 1579–1588 (1976)
- Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell.* 156: 20–44 (2014)
- Seo DJ, Chung MJ, Kim DJ, Choe M. Antiobese effects of diet containing medicinal plant water extracts in high fat diet-induced obese mice. *J. Korean Soc. Food Sci. Nutr.* 38: 1522–1527 (2009)
- Soloff LA, Rutenberg HL, Lacko AG. Serum cholesterol esterification in patients with coronary heart disease: Importance of initial rate of esterification expressed as a function of free cholesterol. *Am. Heart J.* 85: 153–161 (1973)
- Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. *Cell.* 104: 531–543 (2001)
- The GBD 2015 Obesity Collaborators. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *N. Engl. J. Med.* 377: pp. 13–27 (2017)
- Velasquez MT, Bhathena SJ. Role of dietary soy protein in obesity. *Int. J. Med. Sci.* 4: 72–82 (2007)
- Villanueva MJ, Yokoyama WH, Hong YJ, Bartley GE, Ruperez P. Effect of high-fat diets supplemented with okara soybean by-product on lipid profiles of plasma, liver and faeces in Syrian hamsters. *Food Chem.* 124: 72–79 (2011)

- Von Eynatten M, Baumann M, Heemann U, Zdunek D, Hess G, Nawroth PP, Bierhaus A, Humpert PM. Urinary L-FABP and anaemia: Distinct roles of urinary markers in type 2 diabetes. *Eur. J. Clin. Invest.* 40: 95–102 (2010)
- Wat E, Tandy S, Kapera E, Kamili A, Chung RW, Brown A, Rowney M, Cohn JS. Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet. *Atherosclerosis*. 205: 144–150 (2009)
- Yoshida K, Sato Y, Okuno R, Kameda K, Isobe M, Kondo T. Structural analysis and measurement of anthocyanins from colored seed coats of *Vigna*, *Phaseolus*, and *Glycine* legumes. *Biosci. Biotechnol. Biochem.* 60: 589–593 (1996)
- Zhong Q, Lin CY, Clarke KJ, Kemppainen RJ, Schwartz DD, Judd RL. Endothelin-1 inhibits resistin secretion in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 296: 383–387 (2002)