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Reduction of Adipogenesis by Chokeberry (*Aronia melanocarpa*) Extract in 3T3-L1 Preadipocytes

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Abstract The aim of this study was investigate the anti-obesity effect and mechanism of action of chokeberry (*Aronia melanocarpa*) extract (CBE) in 3T3-L1 cells. CBE was tested for its antioxidant compounds content (total phenol and flavonoid). Lipid accumulation levels were measured by determining the expression of genes associated with adipocyte differentiation and adipokine secretion in 3T3-L1 cells. In CBE, the total phenolic content was 212.0 ± 3.11 mg/g and the flavonoids content was 113.25 ± 2.5 mg/g. Oil Red O staining showed that CBE inhibited lipid accumulation at concentration of 0, 0.5, and 1.0 mg/mL in a dose-dependent manner. CBE decreased the expression of the key adipocyte differentiation regulator peroxisome proliferator-activated receptor-γ and the fatty acid binding protein 4 gene during the differentiation of preadipocytes into adipocytes. Moreover, CBE down regulated adipokine-specific genes such as leptin and monocyte chemoattractant protein-1 relative to non-treated adipocytes. These results demonstrated that CBE inhibited adipogenesis by down regulating peroxisome proliferator-activated receptor-γ and fatty acid binding protein 4 and by reducing adipokine expression in 3T3-L1 cells.

Keywords: *chokeberry extract, adipocyte differentiation, adipokine secretion, anti-obesity*

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1. Introduction

The number of obese people is increasing in all over the world. In Korea, a body mass index (BMI) greater than 25.0 kg/m^2 is considered to indicate obesity. According to the Korea National Health & Nutrition Examination Survey 2012, approximately one in three adults (32.8%) are obese and have increased risk of hypertension, hyperlipidemia, type 2 diabetes, and heart disease [1]. In USA, the BMI indicative of obesity is 30 kg/m^2, and 34.9% of adults are obese [2,3].

Increases in fat accumulation are associated with increased adipogenesis, which is related to a combination of factors, including genetics, metabolic, hormonal, and nutritional factors as well as physical activity. The mechanisms of adipocyte differentiation have been widely studied in preadipocyte cell lines. Particularly, the 3T3-L1 preadipocyte cell line is one of the most well-characterized and reliable models for studying the conversion of preadipocytes into adipocyte. The transcription factors involved in adipocyte differentiation include peroxisome proliferator-activated receptor γ (PPAR-γ) [4], fatty acid binding protein 4 (FABP4), fatty acid synthase, and lipoprotein lipase (LPL) [5]. Additionally, adipose tissue secretes numerous bioactive substances known as adipokines or cytokines, including tumor necrosis factor-α (TNF-α) [6], adiponectin, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6, which also regulate the inflammatory response [7,8].

Recent studies have focused on the relationship between functional foods and well-being or diseases prevention. These foods contain large amounts of polyphenolic compounds, which contribute to the prevention of cancer, cardiovascular disease, and lifestyle-related diseases [9-10]. Food phenolics are mostly present in vegetables, fruits, and leaves, and the antioxidant properties of phenolics are mainly related to their redox properties. Thus, polyphenols have beneficial effects on obesity by acting as metabolic stimulants and regulators of lipid metabolism [11].

Chokeberry (*Aronia melanocarpa*, Family: *Rosaceae*) is a popular plant that has traditionally been used to treat various diseases, including cardiovascular disease, diabetes [12], and cancer [13]. Chokeberry is botanically classified as a fruit of North America origin [14]. The taste, color, and smell of chokeberry are good, giving this fruit a high utility value in products such as jam, wine, and juice [15,16]. Chokeberry contains 84.36% water, 0.7% protein, 0.14% lipid, and 0.44% minerals and has higher beta-cryptoxanthin and polyphenols levels than other fruits and berries [13]. However, the mechanism of the anti-obesity effects of chokeberry in 3T3-L1 preadipocytes remains to be elucidated. In addition there are no reports anti-adpiogenesis activity, are only few papers related with antioxidative activity of black chokeberry cultivated in Korea [1,18], although chokeberry cultivated in North America and Europe has been investigated [19,20].

In our previous study, we published that chokeberry attenuated the gene expression related to de *novo*

lipogenesis in the hepatocytes of fatty mice [21]. Because of that study has been confirmed lipogenesis only in the hepatocytes, we tried to verify the mechanism of the chokeberry on adipocytes related with obesity. Considering the importance of adipogenesis in obesity, we examined the mechanism of chokeberry extract (CBE)-induced adipocyte differentiation and adipogenesis in 3T3-L1 cells by measuring the viability of and lipid droplet accumulation in the cells and expression of specific adipocyte genes.

2. Materials and Methods

2.1. Preparation of CBE

Frozen chokeberry was purchased from a local farm (Okcheon-gun, Chungcheongbuk-do, Republic of Korea), was dried and ground to 16 mesh using a grinder (MF 10 Basic; IKA-WERKE, Staufen, Germany). Ground chokeberry (1 g) was refluxed with 20 mL of 70% ethanol for 8 h and the mixture was centrifuged at 1,008 x g for 20 min. The supernatant was filtered and completely evaporated in a rotary evaporator (N-21NS; EYELA, Tokyo, Japan) at 40°C before the concentrated mixture was freeze-dried.

2.2. Total Phenolic Content and Total Flavonoids Content

Total phenolic content of the CBE was determined using the Folin-Ciocalteu (FC) reagent method [14]. Briefly, CBE (2,000 μL) was mixed with 200 μL of 1 N FC reagent (Sigma, St. Louis, MO, USA) and incubated for 3 min at room temperature, and then 200 μL of 10% Na_2CO_3 solution was added. After incubation at room temperature for 1 h, the absorbance was measured at 690 nm. The total phenolic content of CBE was expressed as mg gallic acid (Sigma-Aldrich Korea, Seoul, Korea) equivalents (GAE)/g.

For total flavonoids contetn analysis, CBE (200 μL) was mixed with 10 mL of 90% diethyleneglycol and 200μL of 4 N NaOH and then incubated for 5 min at 30°C. The absorbance was measured at 420 nm. The results were expressed as mg rutin (Sigma-Aldrich Korea, Seoul, Korea) equivalents (RE)/g.

2.3. Cell Culture and Differentiation

3T3-L1 preadipocytes were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (BCS) (Invitrogen) and 1% penicillin-streptomycin. Two days after 100% confluence (day 0), to initiate differentiation, the cells were stimulated with DMEM containing 10% fetal bovine serum (Invitrogen), isobutylmethylxanthine (1:100), dexamethasone (1:1000), and insulin (1:1000) for 2 days. Cells were then maintained in DMEM with 10% FBS and insulin. CBE (100, 200, 500, and 1000 μg/mL of CBE) were added to the medium containing adipocytes at day 0. After treatment with CBE for 7 days, the 3T3-L1 adipocytes were lysed for PCR or fixed for immunocytochemistry.

2.4. Cell Viability

Cell viability was measured using the WST-1 assay according to the manufacturer instructions (LPS Solution, Daejeon, Korea). 3T3-L1 cells (4 x 10^3 cells/well) were plated into 96-well plates containing 200 μL of 10% BCS-DMEM. After the cells were incubated for 24 h, the cells were transferred to 10% BCS-DMEM containing different concentrations of CBE or 10% BCS-DMEM as a control. After 48 h, cells were further incubated with WST-1 solution for 3 h. Cell viability was determined by measuring the absorbance at 450 nm using an Epoch microplate spectrophotometer, which is an ELISA reader (BioTek, Inc., Winooski, VT). The viability of control cells cultured in DMEM containing 10% BCS was set at 100% and the viability relative to the control was determined for the experimental cells.

2.5. Oil Red O Staining

To measure the cell lipid contents, 3T3-L1 cells were stained with Oil Red O. Adherent cells were rinsed with PBS and fixed in 10% formalin and stained with 0.1% filtered Oil Red O solution (Oil Red O stock: dH_2O = 6: 4). The stained cells were imaged under a microscope (Olympus, Tokyo, Japan). The Oil Red O stain was eluted with 100% isopropanol and measured at 500 nm using an Epoch microplate spectrophotometer.

2.6. RNA Isolation and mRNA Expression Analysis

Total RNA was isolated from 3T3-L1 cells using Trizol reagent (Invitrogen). cDNA synthesis was performed with 1 μg RNA using the Maxime RT PreMix kit (iNtRON, Kyeonggi-do, Korea). The target cDNA was amplified using the AccuPower HotStart PCR PreMix (Bioneer, Daejeon, Korea). The thermal cycling program comprised 25–35 cycles of 95°C for 30 s, 48–55°C for 30 s, and 72°C for 30 s. PCR products were visualized by 1.2% agarose gel electrophoresis. The mRNA level was normalized to the GAPDH mRNA level and subsequently expressed as fold-changes relative to non-differentiated 3T3-L1 cells. The details of all primers used are shown in Table 1.

Table 1. Primers for Reverse Transcriptase Polymerase Chain Reaction (PCR)

Gene	Primers (5'-3')	Product size (bp)
LPL[1)]	F: TCTCAGATGCCCTACAAAGT R: GCTGAAGTAGGAGTCGCTTA	249
PPARγ	F: TGTCAGTACTGTCGGTTTCA R: TCTTTCCTGTCAAGATCGCC	238
FABP4	F: CATGATCATCAGCGTAAATG R: ACGCCTTTCATAACACATTC	249
Adiponectin	F: AGCCTCTTCAAGAAGGACAA R: TCATGGTAGAGAAGAAAGCC	206
Leptin	F: GAGCTAGAGAAGCTCACCAA R: TGTCAACAGTGTGCTACCAT	253
MCP1	F: AAGAGAGAGGTCTGTGCTGA R: TCACAGTCCGAGTCACACTA	254
GAPDH	F: AACGGATTTGGCCGTATTGG R: CGCTCCTGGAAGATGGTGAT	215

[1)]LPL; Lipoprotein lipase, PPARγ; Peroxisome proliferator activated receptor gamma, FABP4; Fatty acid binding protein, TNFα; Tumor necrosis factor alpha, MCP1; Monocyte chemoattractant protein 1, GAPDH; Glyceraldehyde-3-phosphate dehydrogenase

2.7. Statistical Analysis

Statistical analysis was performed using SAS Systems ver. 9.3 and analysis of variance (ANOVA) was used for comparisons among all groups. All data were expressed as the mean ± SD unless otherwise noted. A probability level of 5% was considered significant. Differences between two groups were analyzed using the t-test. ANOVA was conducted after Duncan's multiple comparison test.

3. Results

3.1. Total Phenolic Content and Total Flavonoids Content of CBE

The total phenolic content and total flavonoids content levels in CBE were found to be 212.00 ± 3.11 mg gallic acid equivalent (GAE)/g and 113.25 ± 2.50 mg rutin equivalent (RE)/g, respectively.

3.2. Effect of CBE on 3T3-L1 Preadipocyte Viability

3T3-L1 adipocyte viability was assessed using the WST-1 assay. CBE at the concentrations used did not affect the viability of 3T3-L1 preadipocytes (Figure 1).

3.3. Inhibitory Effect of CBE on Adipogenesis

To examine the anti-adipogenic effects of CBE, 3T3-L1 preadipocytes were treated with CBE for 7 days. The anti-adipogenic effect of CBE on the induction of differentiation markers in 3T3-L1 cells was measured at the end (day 7) of the differentiation experiment. The differentiation of preadipocytes into adipocytes is associated with an increased number of Oil Red O-stained cells due to lipid accumulation. Microscopic observations of the Oil Red O staining results revealed a gradual reduction in the number of lipid droplets at increasing CBE concentration (Figure 2A). The amount of accumulated triglycerides was analyzed on day 7, and the cells treated with 0.5 and 1.0 mg/mL CBE had significantly low lipid contents on day 7 (Figure 2B). These results indicate that CBE effectively blocked adipocyte differentiation in 3T3-L1 preadipocytes.

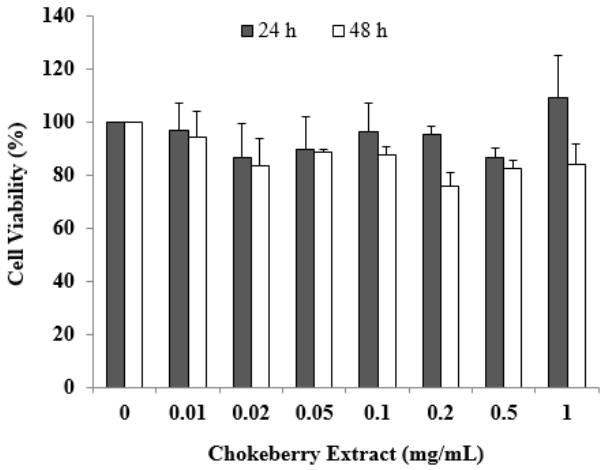

Figure 1. Cell Viability of 3T3-L1 treated with CBE

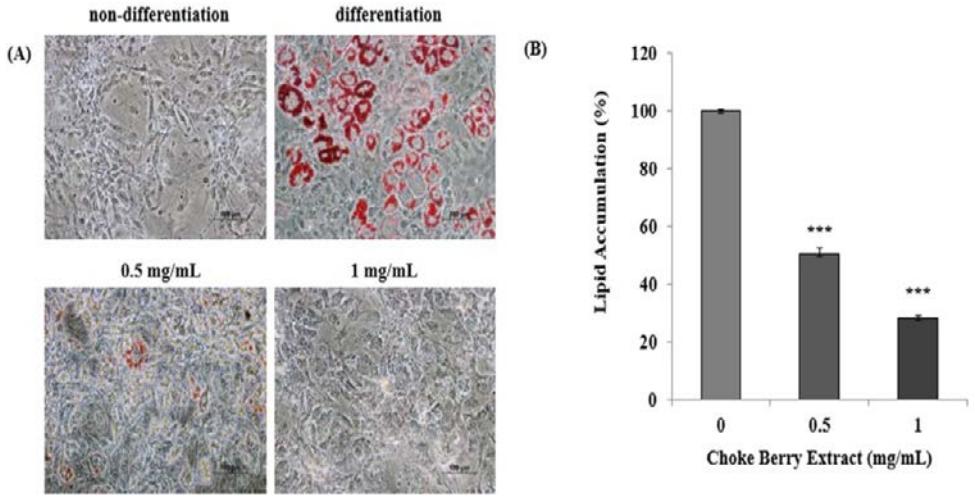

Figure 2. CBE inhibits adipocyte differentiation in a dose-dependent manner. (A) After 8 days of differentiation, lipid accumulation was detected by oil-red-O staining. (B) Quantification of oil-red-O staining. ***P < 0.001 vs. 0 mg/mL CBE group

3.4. Inhibitory Effect of CBE on the Expression of Adipogenesis-specific Genes

To investigate the effects of CBE on the differentiation of 3T3-L1 preadipocytes, 3T3-L1 cells were differentiated in FBS-DMEM medium containing CBE at 0.1, 0.2, 0.5, or 1.0 mg/mL for 7 days. The effect of CBE on the expression of LPL, PPARγ, and FABP4 was examined using PCR. We observed that the mRNA levels of LPL, PPARγ, and FABP4 decreased following treatment of differentiated 3T3-L1 preadipocytes with CBE. However, the inhibitory effects of LPL and PPARγ were not dose-dependent (Figure 3). These results suggest that CBE significantly induced the downregulation of adipogenic transcription factors, which play a critical role in adipocyte differentiation.

3.5. Effect of CBE on Adipokine Regulation during Preadipocyte Differentiation

To investigate the effects of CBE on the regulation of adipokines during 3T3-L1 preadipocyte differentiation, 3T3-L1 cells were differentiated in FBS-DMEM medium containing CBE at 0.1, 0.2, 0.5, or 1.0 mg/mL for 7 days.

The effect of CBE on the expression of adiponectin, leptin, and MCP1 was examined using PCR. We observed that the mRNA levels of adiponectin, leptin, and MCP1 decreased following treatment of differentiated 3T3-L1 preadipocytes with CBE (Figure 4).

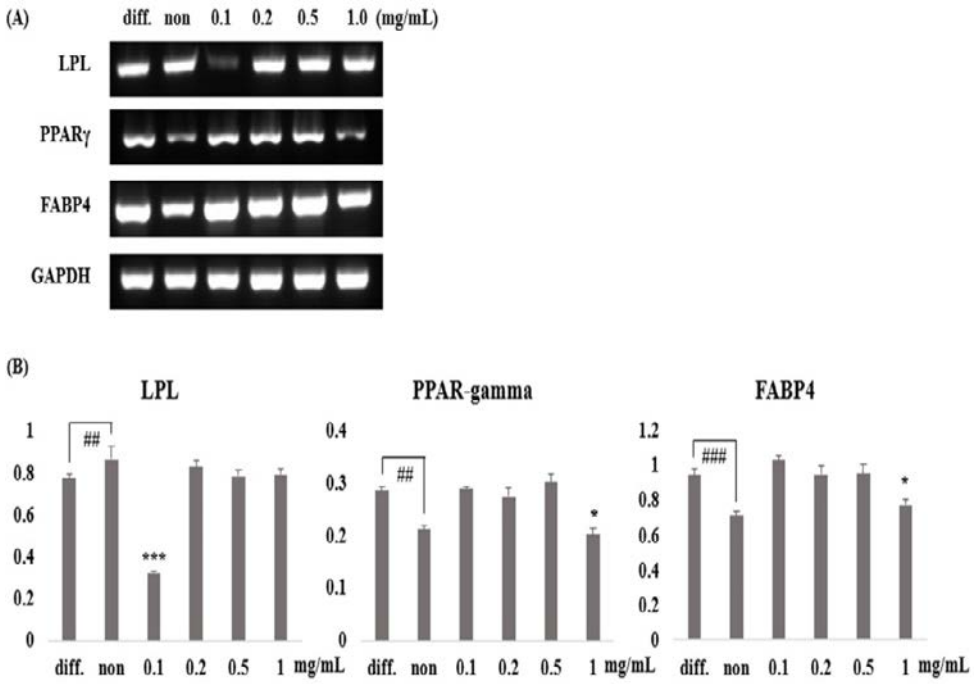

Figure 3. The effect of CBE on the expression of adipogenic marker genes. ##P<0.01 / ###P<0.001 vs. 0 mg/mL CBE group. *P <0.05 / ***P < 0.001 vs. 0 mg/mL CBE group

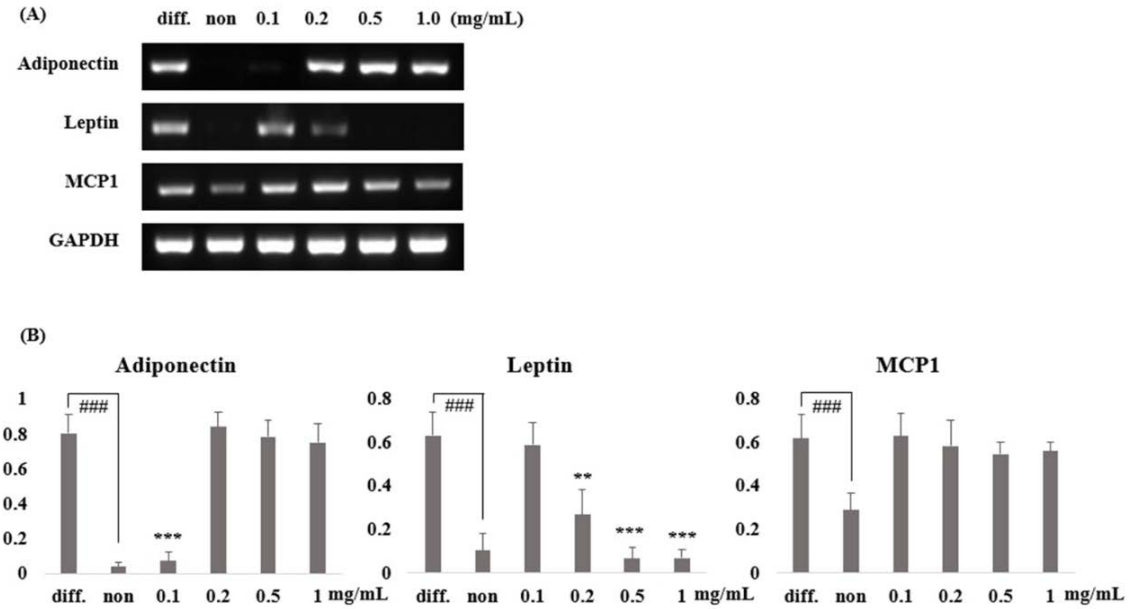

Figure 4. The effect of CBE on the expression of adipokine genes. ###P<0.001 vs. 0 mg/mL CBE group. **P <0.01 / ***P < 0.001 vs. 0 mg/mL CBE group

4. Discussion

In this study, we analyzed the effects of CBE on adipocyte differentiation and its inhibitory mechanisms on adipogenesis in 3T3-L1 cells. Before evaluating the anti-obesity effects of CBE, this research team evaluated that CBE exhibited the level of total phenolic and flavonoid contents. Next, we examined whether CBE exerted its anti-adipogenic effects by inhibiting PPARγ and FABP4 expression and adipokine secretion in 3T3-L1 cells.

Obesity has been described as a state of chronic oxidative stress. Furthermore, oxidative stress has been defined as the link between obesity and its major associated disorders, such as insulin resistance and hypertension, among others [22,23]. Recent studies have suggested the potential therapeutic role of dietary antioxidant supplementation in the reduction of body weight and its beneficial effect on several obesity-related disorders [24,25]. Flavonoids are potential antioxidants that can remove oxidant material; its main sources include vegetables and fruits. The blueberry, which is commonly

recognized antioxidant fruit, contained 42.26 mg GAE/g and 26.39 mg rutin of total phenolic content and total flavonoids content, respectively [26]. These results suggest that CBE has high level of antioxidant contents and potent biological activities. Taken together, the antioxidant capability of CBE was considered excellent, and CBE may have a beneficial effect on obesity and associated disorders.

Adipocyte differentiation and fat accumulation are associated with the occurrence and development of obesity [27]. Obesity is caused by an increase in the number of fat cells relative to the increase in adipose tissue mass [28]. Reduced adiposity is related to the inhibition of angiogenesis along with a reduction in adipocyte numbers and lipid content in adipocytes [29]. During adipocyte differentiation, the accumulation of lipids is regulated by a complex network of various transcription factors and adipocyte-specific genes.

In the present study, CBE treatment (1.0 mg/mL) suppressed LPL, PPARγ, and FABP4 mRNA expression compared with expression in differentiated control cells. Furthermore, treatment with CBE reduced lipid accumulation as determined by Oil Red O staining and a triglyceride accumulation assay. PPARγ acts as master regulator that controls the growth arrest of preadipocyte proliferation and induces differentiation and adipogenesis by regulating a large number of adipocyte-specific genes [30]. Particularly, PPARγ is critical to the progression through the stages of adipocyte differentiation. Thus, these results indicate that CBE reduced lipid accumulation by downregulating the adipocyte differentiation. Moreover, PPARγ is activated by fatty acids, and fat accumulation is associated with PPARγ activation. PPARγ activates the expression of genes involved in adipogenesis, such as LPL and FABP4 [31]. During the terminal phase of differentiation, adipocytes dramatically increase lipogenesis and become sensitive to insulin. The activation of genes involved in TG metabolism increases by 10–100-fold [32]. In our study, the expression of LPL was lower in 3T3-L1 cells treated with CBE compared with in terminally differentiated 3T3-L1 adipocyte control cells. Taken together, the reductions in LPL expression resulted from the down-regulation of PPARγ, which not only slows the de novo synthesis of fatty acids and TG, but also inhibits the differentiation of early differentiating preadipocytes and lipogenesis in mature adipocytes.

Adipokine secretion is related to adipocyte triacylglycerol content. Particularly, lipid accumulation leads to decreased adiponectin secretion and to greater secretion of leptin and MCP1 [33]. Previous studies reported increased serum levels of leptin in obese people [34]. Obesity was found to be associated with reduced levels of serum adiponectin, and obesity may regulate the serum levels of leptin in favor of pro-inflammation [35,36]. In our study, the expression of leptin and MCP1 was lower in 3T3-L1 cells treated with CBE than in terminally differentiated 3T3-L1 adipocyte control cells. In particular, the expression of leptin in cells treated with CBE significantly decreased in a dose-dependent manner. Accordingly, CBE upregulated the gene expression of adiponectin and downregulated that of leptin, thereby reducing lipid accumulation.

The relationship between adipogenesis-related genes and adipokines has been examined in many previous studies [37,38]. The expression of leptin was regulated by several transcriptional factors, including CEBP, PPAR, and SREBP [39]. These results suggest that reduced expression of the transcription factor PPARγ induced by CBE contributed to the reduction of leptin expression. Thus, the changes observed in leptin expression in maturing pre-adipocytes may be related to the effect of CBE on this parameter as well as to the effects derived from changes in PPARγ expression.

In conclusion, the present study demonstrated the effect of CBE on adipogenesis-related genes and adipokine expression in 3T3-L1 cells. CBE, which contains high levels of total phenolic and flavonoids, may have beneficial effects against obesity by regulating the expression of genes involved in adipogenesis. Further studies should be performed to fine anti-obesity effect from chokeberry that is effective *in vivo*.

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