

Portulaca Oleracea L. Reduces Adipogenesis by Regulating Adipogenic Transcription Factors and Enzymes in Adipocytes

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Abstract

This study investigated whether the 80% ethanol extracts of *Portulaca oleracea* L. (POE) would inhibit adipogenesis by improving the molecular activity against adipogenic transcription factors and enzymes in 3T3-L1 adipocytes. Adipocytes treated with POE showed significantly lower triglyceride levels and higher glycerol release than did the control adipocytes. The leptin level was also significantly decreased following treatment of POE in adipocytes. POE significantly inhibited the expression of adipogenic transcription factors, such as the CCAAT/enhancer-binding protein α (C/EBP α), sterol regulatory element-binding protein 1c (SREBP-1c), and peroxisome proliferator-activated receptor γ (PPAR- γ). Results showed that POE inhibited differentiation of adipocytes and significantly inhibited adipogenic enzymes, such as acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS). Furthermore, POE reduced adipogenesis and increased the phosphorylation of AMPK, which suppresses the expression of ACC and FAS by down regulating SREBP-1c. These results show that POE could potentially prevent obesity by inhibiting adipogenesis in 3T3-L1 adipocytes.

Keywords: 3T3-L1 adipocytes; Adipogenesis; *Portulaca oleracea* L.

Introduction

Obesity is becoming an increasing health problem worldwide. It is related to various chronic diseases, including type 2 diabetes, cancer, and osteoarthritis^[1]. Obesity is characterized by the formation of lipid droplets^[2]. Lipid synthesis and accumulation in adipocytes is involved in the process of adipogenesis, which includes cell proliferation and differentiation^[3]. The differentiation of preadipocytes is regulated by the CCAAT/enhancer-binding protein α (C/EBP α), which is expressed at the adipogenic initiation stage and synergistically triggers the expression of proliferator-activated receptor γ (PPAR- γ)^[4]. The sterol regulatory element-binding protein 1c (SREBP-1c) is thought to act at an early stage of differentiation before PPAR- γ activation. Activated SREBP-1c accelerates adipogenesis through the overexpression of adipogenic enzymes, such as acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS)^[5]. Adipocytes increase the synthesis of triglycerides and acquire the behavior of adipose cells. In particular, triglycerides accumulate in lipid droplets embedded in the cytoplasm^[6].

The phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) in adipocytes inhibits lipid synthesis by regulating lipogenic enzyme activities. The phosphorylation of AMPK suppresses the expression of ACC and FAS by down regulating SREBP-1c. The inhibition of adipogenesis by controlling these transcription factors and enzymes may be used to prevent and treat obesity and obesity-mediated complications^[7].

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Portulaca oleracea L. is a succulent annual herb that belongs to the family Portulacaceae, and it has been used as a type of medicinal food for thousands of years in Asia^[8,9]. *P. oleracea* exhibits several pharmacological activities, including antibacterial^[10], antiulcerogenic^[11], anti-inflammatory^[12] and antidiabetic^[13] properties. However, the efficacy of *P. oleracea* extract on inhibiting adipogenesis has not yet been clarified. In this study, the anti-adipogenesis effects of the 80% ethanol extract of *P. oleracea* (POE) in 3T3-L1 adipocytes were investigated by assessing the accumulation of oil red O stained lipids and measuring their glycerol, triglyceride, and leptin content. Furthermore, to investigate the inhibitory mechanism of *P. oleracea* extract on adipogenesis in 3T3-L1 adipocytes, we measured the expression levels of adipogenic transcription factors and their target genes.

Materials and Methods

Plant material

P. oleracea was obtained from Hongcheon Hyosung Food, Inc. (Hongcheon, Korea). The leaves and stems of *P. oleracea* were carefully washed with distilled water, freeze-dried, and ground into a powder (Samwon Freezing Engineering Co., Busan, Korea). The powder extracted with 80 % ethanol for 3 days at room temperature (25°C). This *P. oleracea* extract filtered three times through Whatman No. 1 filter papers at room temperature. Next, the extract was concentrated in a rotary vacuum evaporator and freeze-dried to powder form. The powder was stored in a deep freezer at -80°C.

Cell culture and adipocyte differentiation

The 3T3-L1 preadipocyte cells (Korean Cell Line Bank, Seoul, Korea) were grown to confluence in DMEM with 10% bovine calf serum at 37°C in a humidified atmosphere of 5% CO₂. One-day post-confluence, cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 µM), and insulin (5 µg/mL) in DMEM containing 10% fetal bovine serum (FBS). After 48 h, the DMEM containing 10% FBS and insulin (5 µg/mL) were replaced every two days. The sample was added to the culture medium from days 3 to 8 to investigate its effect on lipid accumulation and triglyceride hydrolysis.

Assay of cell viability

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA). The 3T3-L1 cells seeded in 96-well plates at a density of 1×10^4 cells/well and administrated with various concentrations of the sample for 72 h. After administration, the adipocytes were incubated with MTT solution for 4 h at 7°C. The supernatants were aspirated and DMSO added to each well. After 15 min of incubation, the absorbance measured at 540 nm by using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Oil red O staining

On day 8, the 3T3-L1 cells washed twice with PBS and fixed with 10% (v/v) fresh formalin for 2 h. Cells stained with Oil red O working solution for 1 h. After the staining solution removed, stained lipid droplets in 3T3-L1 cells washed four times with PBS. The cells stained with Oil red O were visualized using mi-

croscopy with an image analysis system (Leica Microsystems, Bensheim, Germany).

Measurement of triglyceride content

The triglyceride content of the adipocyte lysates was determined by using the triglyceride quantification colorimetric/fluorometric kit (Biovision Inc., Mountain View, CA, USA) according to the manufacturer's instructions.

Measurement of glycerol content Glycerol levels in the medium measured using a free glycerol determination kit (Sigma, St. Louis, MO, USA) with glycerol standards for calibration. Briefly, 200 µL of free glycerol reagent reconstituted in distilled water was mixed with 50 µL of distilled water (blank), glycerol standard, or test samples in the presence of adipocytes. Thereafter, the mixtures incubated at 37°C for 15 min, and the absorbance of the solution was measured at 540 nm using a microplate reader.

Measurement of leptin release

The leptin release of the adipocyte lysates was determined by using the Mouse/Rat Leptin Quantikine ELISA Kit (R&D Systems Inc., MN, USA) according to the manufacturer's instructions.

Western Blot

For total protein extraction from differentiated 3T3-L1 adipocytes, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a lysis buffer (RIPA, 50 mM-Tris-HCl, 150 mMNaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mMphenylmethylsulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.1 mM sodium orthovanadate at pH 7.4) on ice with gentle shaking. After centrifugation at 13,000 ×g for 10 min at 4°C, the protein content of the resulting supernatant was determined using a BCA protein assay kit. The lysate containing 20 µg of protein subjected to electrophoresis with SDS-PAGE. Separated proteins transferred electrophoretically to a pure nitrocellulose membrane, blocked with 5% skimmed milk solution for 1 h, and then incubated with the appropriate primary antibody (Abcam, Cambridge, UK) overnight at 4°C. After washing, the blots incubated with goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody for 1 h at 25°C. Each antigen-antibody complex was visualized using ECL western blotting detection reagents, and the chemiluminescence was detected using a LAS-1000 plus instrument (FUJIFILM, Tokyo, Japan). Band densities determined by an image analyzer (Multi Gauge V3.1, FUJIFILM Corporation, Valhalla, NY, USA) and normalized to β-actin for total protein and nuclear protein analysis.

Statistical analysis

All values expressed as the mean ± standard deviation (SD). Statistical analyses performed using the SAS software 9.1 (SAS Institute, Cary, NC, USA). The values were evaluated by a one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range tests.

Results and Discussion

Effect of POE on cell viability

Obesity is a serious health problem related to various chronic diseases, including type 2 diabetes, cancer, and osteoarthritis. Obesity is a condition where there is excess body fat accumulation because of the imbalance between energy intake and energy expenditure^[1]. This imbalance promotes the differentiation of preadipocytes and adipogenesis. Currently available drugs to treat obesity cause undesirable adverse reactions^[14]. Therefore, studies using natural products to combat obesity are rapidly increasing. *P. oleracea* is reported to reduce body weight and improve lipid^[15]. However, its inhibitory effect on adipogenesis is still insufficient. In this study, we investigated the adipogenesis inhibiting effects of POE via regulating adipogenic transcription factors in 3T3-L1 adipocytes.

At first, to examine whether POE had a toxic effect on 3T3-L1 adipocytes, the adipocytes were exposed to various concentrations (25, 50, 100, 250, and 500 µg/mL) of POE. Intracellular toxicity was measured using the MTT assay. The result showed that POE did not decrease cell viability, as it had no significant cytotoxic effect observed up to a maximum extract concentration of 500 µg/mL (Figure 1).

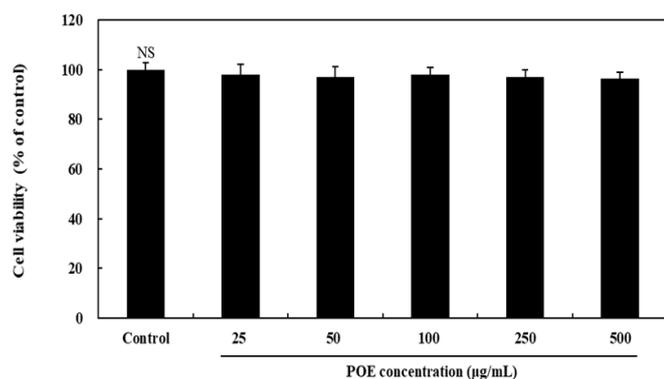


Figure 1: The effects of POE on cell viability. Differentiated 3T3-L1 adipocytes were treated with various concentrations (0, 25, 50, 100, 250, and 500 µg/mL) of POE and incubated for 72 h in incubator. Each value is expressed as mean ± SD (n = 3). NS, not significant; POE, *Portulaca oleracea* L. extract.

Effect of POE on lipid accumulation

The triglyceride accumulation in 3T3-L1 adipocytes measured as an index of adipocyte differentiation and adipogenesis. To investigate the inhibitory effects of POE on 3T3-L1 adipocytes treated with various concentrations (25, 50, 100, and 200 µg/mL) of POE. The images of Oil red O staining showed that POE suppressed triglyceride accumulation (Figure 2). MDI-treated control 3T3-L1 adipocytes observed with plenty of lipid droplets. In contrast, the adipocytes treated with POE observed with significantly less lipid droplets compared with those of the control cells. The images of Oil red O staining suggested that POE suppressed lipid accumulation in adipocytes. The adipocytes treated with POE observed with fewer lipid droplets when compared with the control adipocytes. Lipid accumulation in the adipose tissue occurs at the late stage of adipogenesis and is associated with increased triglyceride content^[16].

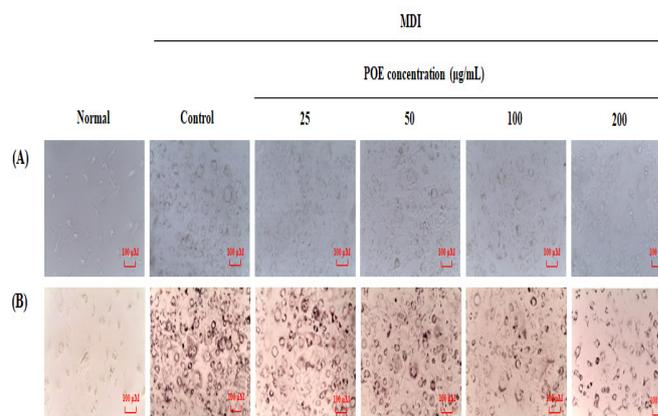


Figure 2: The effects of POE supplementation of triglyceride accumulation in differentiated 3T3-L1 adipocytes. (A) The 3T3-L1 cells were examined by light microscopy (magnification, x 100). (B) Cells were fixed and stained with Oil Red O staining to visualize the lipid droplets by light microscopy (magnification, x 100). Scale bar, 100µM.

The triglyceride content was measured using a triglyceride quantification colorimetric/fluorometric kit. Triglyceride content in adipocytes treated with the POE were significantly lower than those in untreated adipocytes. Triglyceride content decreased to 93.1%, 88.8%, 78.1%, and 59.9% upon treatment with 25, 50, 100, and 200 µg/mL POE, respectively (Figure 3A). The triglyceride content in 3T3-L1 adipocytes measured as an index of adipogenesis, and the triglyceride content in adipocytes treated with POE were significantly lower than that in untreated adipocytes. This suggested that treatment with POE led to reduced adipocyte formation.

The Adipocytes treated with POE significantly increased glycerol release. The amount of released glycerol increased to 108.3%, 124.5%, 150.4%, and 175.8% after treatment with 25, 50, 100, and 200µg/mL POE, respectively (Figure 3B). Triglyceride hydrolysis in adipocytes releases glycerol and free fatty acids, which are released into the culture medium^[17]. To determine whether the reduction in triglyceride content was associated with lipolysis, the amount of glycerol was measured. Treatment with POE significantly increased glycerol amount.

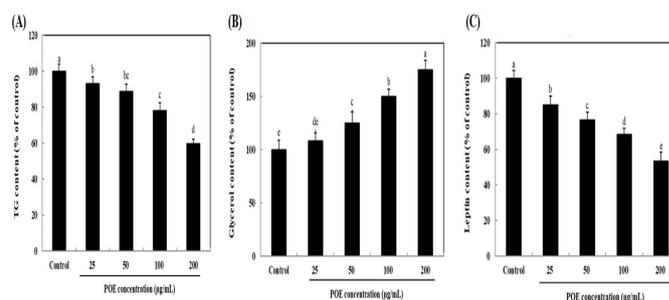


Figure 3: The effects of POE on TG, glycerol and leptin contents. (A) The effects of POE on TG content. (B) The effects of POE on glycerol content. (C) The effects of POE on leptin content. Differentiated 3T3-L1 adipocytes were treated with different concentrations (0, 25, 50, 100 and 200µg/mL) of POE and incubated for 8 days in incubator. Each value is expressed as mean ± SD (n = 3). a–e Values with different letters are significantly different (P<0.05) as analyzed using Duncan's multiple range test. POE, *Portulaca oleracea* L. extract.

As shown in Figure 3C, leptin content in the adipocytes treated with POE were significantly lower than those in untreated adipocytes. Leptin content decreased to 84.1%, 76.5%, 68.3%, and 53.3% upon treatment with 25, 50, 100, and 200 µg/mL POE, respectively. Leptin is an adipokine secreted from adipocytes, and its levels are closely associated with adipocyte size and percentage body fat^[18]. To investigate the reduction in leptin production, the amount of leptin was measured. Leptin level was significantly decreased following treatment of POE in adipocytes. These findings indicate that POE may reduce lipid accumulation during adipocyte differentiation and adipogenesis in a dose-dependent manner. It is reported that phenolic acids such as chlorogenic, caffeic, p-coumaric, ferulic, and rosmarinic acids, and flavonoids such as quercetin and kaempferol were contained in the *P. oleracea* extract^[19]. These phenolic acids and flavonoids been reported to inhibit triglyceride accumulation and increase glycerol release in 3T3-L1 adipocytes. A recent study showed that homoisoflavonoids from *P. oleracea* decreased lipid accumulation, increased the release of glycerol into the culture medium, and had inhibitory effects on adipogenic differentiation^[20]. Our previous study isolated and identified homoisoflavonoids from *P. oleracea* extract (Figure 4.)^[21]. Thus, POE might be effective in reducing lipid accumulation owing to the abundance of these compounds.

untreated adipocytes. The expression levels of SREBP-1c in adipocytes treated with 50, 100, and 200 µg/mL POE significantly decreased to 85.1%, 75.0%, and 63.9%, respectively, compared with those in untreated adipocytes.

The differentiation of preadipocyte to adipocyte includes the activation of adipogenic gene expression. It is regulated by a cascade of transcription factors, including the PPAR-γ, C/EBPα, and SREBP family of proteins, which together contribute to adipocyte differentiation. The expression of PPARγ is elevated relatively early during differentiation. Ectopic expression and ligand activation of PPARγ in non-adipogenic fibroblasts promotes their conversion into excess accumulation of lipids in the adipocytes and induces a series of adipogenic gene expression. Thus, regulation of PPAR-γ activity may be effective in treating obesity^[22]. PPARγ cooperates with C/EBPα to activate gene expression, and the full program of adipogenesis and C/EBPα is induced relatively late during adipogenesis in culture, after the induction of PPARγ^[7]. SREBP-1c, another important transcription factor in adipogenesis, cross-activates a ligand binding domain of PPAR-γ and regulates the expression of enzymes involved in adipogenesis^[23]. In this study, control adipocytes showed an increase of PPAR-γ, C/EBPα, and SREBP-1c. However, the adipocytes treated with POE considerably reduced the expression levels of PPAR-γ, C/EBPα, and SREBP-1c. These results suggest that POE inhibited differentiation and adipogenesis of adipocytes regulating adipogenic transcription factors.

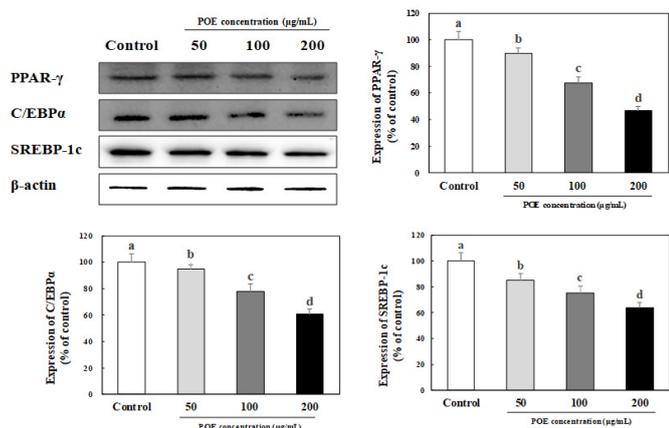


Figure 4: The effects of POE supplementation on PPAR-γ, C/EBPα, SREBP-1c, β-actin protein expression in 3T3-L1 adipocytes. Western blotting and signal intensities were determined by densitometric analysis using Multi Gauge ver. 3.1 software. Each value is expressed as mean ± SD in triplicate experiments. ^{a-d}The mean values that are not indicated by a common letter are significantly different among the groups (P<.05). PPAR-γ; peroxisome proliferator-activated receptor γ, C/EBPα; CCAAT/enhancer binding protein α, SREBP-1c; sterol regulatory element binding protein 1c

Effect of POE on the protein expression of adipogenic transcription factors

In this study, 50, 100, and 200 µg/mL POE treatment significantly reduced the expression levels of PPAR-γ in 3T3-L1 adipocytes to 89.5%, 67.6%, and 46.9%, respectively, compared with those in untreated adipocytes (Figure 4). POE treatment also significantly decreased the expression of C/EBPα in adipocytes. The C/EBPα expression levels in adipocytes treated with 50, 100, and 200 µg/mL POE significantly decreased to 95.1%, 77.9%, and 60.5% respectively, compared with those in

Effect of POE on the protein expression of adipogenic enzymes

The inhibitory effects of POE on adipogenesis were investigated. The FAS expression levels in adipocytes treated with 50, 100, and 200 µg/mL POE significantly decreased to 94.0%, 84.1%, and 40.2%, respectively, compared with those in untreated adipocytes. ACC phosphorylation levels in adipocytes treated with 50, 100, and 200 µg/mL POE significantly increased to 150.3%, 184.8%, and 215.1%, respectively, compared with those in untreated adipocytes (Figure 5).

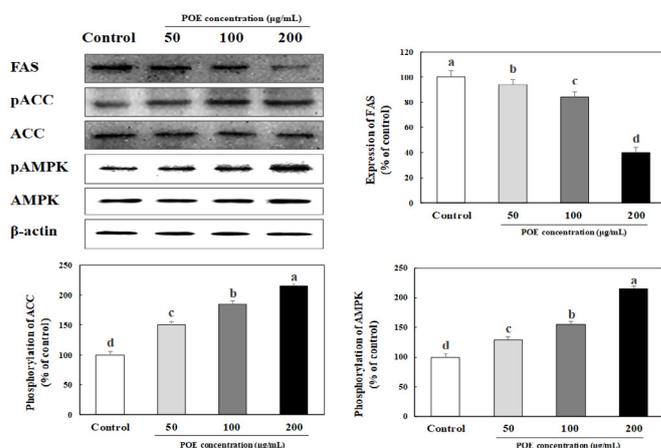


Figure 5: The effects of POE supplementation on FAS, pACC, ACC, pAMPK, AMPK, β-actin protein expression in 3T3-L1 adipocytes. Western blotting and signal intensities were determined by densitometric analysis using Multi Gauge ver. 3.1 software. Each value is expressed as mean ± SD in triplicate experiments. ^{a-d}The mean values that are not indicated by a common letter are significantly different among the groups (P<.05). FAS; fatty acid synthase, ACC; acetyl-coenzyme A

carboxylase, pAMPK; phosphorylated adenosine monophosphate-activated protein kinase, AMPK; adenosine monophosphate-activated protein kinase

Furthermore, SREBP-1c regulates the activation of the key enzymes for fatty acid synthesis, FAS and ACC. The role of FAS in lipogenesis is to regulate fatty acid synthesis. The function of ACC is to convert acetyl-CoA, an essential substrate of fatty acids, to malonyl-CoA^[24]. POE significantly inhibited the expression of these enzymes. POE treatment significantly increased phosphorylation of ACC. Since the phosphorylation of ACC inhibits the activity of the enzyme, increased levels of phosphorylated-ACC by POE treatment results in the inhibition of fatty acid synthesis. These results show the inhibition of lipogenesis in 3T3-L1 adipocytes by POE. Lipogenesis is the enzymatic process by which glycerol is esterified with free fatty acids to form triglyceride^[25,26]. Therefore, POE treatment would inhibit activation of lipogenesis enzymes and decrease triglyceride content.

Effect of POE on the expression of pAMPK

To examine whether POE had an effect on the activity of AMPK, the level of phosphorylation of AMPK was examined by western blot analyses. AMPK phosphorylation levels in adipocytes treated with 50, 100, and 200 µg/mL POE significantly increased to 128.6%, 154.5%, and 214.8%, respectively, compared with those in untreated adipocytes (Figure 5).

AMPK mediates the regulation of body weight, glucose homeostasis, lipid metabolism, mitochondrial biogenesis, and insulin signaling. Activation of AMPK by phosphorylation may potentially inhibit adipocyte differentiation and control obesity^[27]. POE significantly upregulated the phosphorylation of AMPK, which might be related to the negative regulation of proliferation key molecules in adipogenesis, such as PPAR-γ. The result indicates that POE treatment might inhibit differentiation and lipogenesis in 3T3-L1 adipocytes by activating AMPK.

These anti-adipogenic effects seem to appear because of various components of POE. It is reported that phenolic acids such as chlorogenic, caffeic, p-coumaric, ferulic, and rosmarinic acids, and flavonoids such as quercetin and kaempferol are contained in the *P. oleracea* extract (Erkan, 2012)^[19]. It is also reported that kaempferol, a flavonoid in *P. oleracea*, downregulated adipogenic transcription factors such as C/EBPα, SREBP-1c, and PPAR-γ, and enzymes such as ACC and FAS in adipocytes^[28]. In addition, p-coumaric acid belonging to the phenolic acids in *P. oleracea* inhibits expression of these adipogenic transcription factors and enzymes in 3T3-L1 cells by activating the AMPK pathway^[29]. These phenolic acids and flavonoids may have reduced adipogenesis owing to the regulation of adipogenic transcription factors and enzymes in *P. oleracea*.

In conclusion, this study has demonstrated that POE treatment inhibits adipocyte differentiation and adipogenesis by regulating adipogenic transcription factors and enzymes in 3T3-L1 adipocytes. POE downregulated adipogenic transcription factors, including C/EBPα, SREBP-1c, and PPAR-γ as well as enzymes, including FAS and ACC expression. POE also significantly increased phosphorylated-AMPK expression. As a result, cells treated with POE showed significantly lower triglyceride content as well as higher glycerol release than did control cells.

The results of the present study suggest that POE could be used as a functional food to control obesity, and further studies are necessary to identify the active components with anti-adipogenic effects in POE.

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