

Trapa japonica Flerov Extract Attenuates Lipid Accumulation through Downregulation of Adipogenic Transcription Factors in 3T3-L1 Cells

Mi Jin Kim, Kyung Ran Im, Kyung-Sup Yoon*

R&D Center, Saimdang Cosmetics Co., Ltd., Cheongju, Republic of Korea
Email: kmj75@saimdang.co.kr, imkr@saimdang.co.kr, ksyoonjh@hanmail.net

Received 5 March 2015; accepted 21 March 2015; published 25 March 2015

Copyright © 2015 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).
<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

Obesity is a major human health problem associated with various diseases, including cardiac injury and type 2 diabetes. *Trapa japonica* Flerov (TJF) has been used in traditional oriental medicine to treat diabetes. In this study, we evaluated the inhibitory effect of and the mechanism underlying the effect of TJF extract on adipogenesis in 3T3-L1 cells. The effects of TJF extract on cell viability were analyzed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, and the anti-adipogenic effect was measured by oil red O staining. The expression of peroxisomal proliferator activated receptor (PPAR) γ , CCAAT/enhancer-binding protein- α (C/EBP) α , adenosine monophosphate-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), adiponectin, and fatty acid binding protein (FABP)4 involved in adipogenesis was determined by western blot analysis. TJF extract effectively inhibited lipid accumulation and the expression of PPAR γ and C/EBP α in 3T3-L1 cells. TJF also increased the phosphorylation of AMPK and ACC, and decreased the expression of adiponectin and FABP4. These results indicate that TJF extract exerts its anti-obesity effect through the downregulation of adipogenic transcription factors and adipogenic marker genes.

Keywords

3T3-L1 Cells, Adipogenic Transcription Factors, Lipid Accumulation, *Trapa japonica* Flerov Extract

*Corresponding author.

1. Introduction

Obesity is the result of imbalance between energy intake and energy expenditure, and greatly increases the risk of numerous associated diseases, including heart disease, hypertension, stroke, cancer, diabetes, and osteoarthritis [1] [2]. The World Health Organization (WHO) has declared obesity to be one of the most serious social and health problems today [3]. According to the Korea Health Statistics 2012, 17.3% of Koreans over the age of nineteen are obese [4]. Thus, determining the molecular mechanisms regulating adipocytes has crucial implications for the development of therapeutics targeting obesity and obesity-associated diseases.

Reducing lipid accumulation through the inhibition of adipocyte differentiation may play a crucial role in preventing obesity. Many researchers have investigated novel anti-adipogenic agents as potential therapeutics to decrease or prevent obesity. Obesity is known to be associated with excessive growth of adipose tissue mass, through increases in both the number and the size of fat cells [5]. Adipose tissue mass can be regulated by the suppression of adipogenesis, which is the development of mature fat cells from fibroblastic preadipocytes [6]. Adipokines, such as peroxisome proliferator activated receptor (PPAR) γ and CCAAT/enhancer-binding (C/EBP) α , directly regulate the development of fat cells [7]. In particular, PPAR γ is highly expressed in adipose tissue, where it plays essential roles in adipogenesis [8].

The cellular and molecular mechanisms of adipocyte differentiation have been extensively investigated using preadipocyte culture systems. The 3T3-L1 cell line is one of the best characterized and reliable models for studying the conversion of preadipocytes to adipocytes. The formation and appearance of fat droplets in these cells also mimic live adipose tissue [9]. Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail. Maximal differentiation is achieved upon treatment with the combination of insulin, a glucocorticoid, an agent that elevates intracellular cAMP levels, and fetal bovine serum [10]. Approximately 24 h after induction by adipogenic cocktail, differentiating preadipocytes undergo a postconfluent mitosis and subsequent growth arrest [11]. After the growth arrest, cells are committed to becoming adipocytes. The growth arrest is required for subsequent differentiation. Growth-arrested cells begin to express late markers of differentiation at d 3. These late markers consist of lipogenic and lipolytic enzymes, as well as other proteins responsible for modulating the mature adipocyte phenotype. The cells then round up, accumulate fat droplets and become terminally differentiated adipocytes by d 5 - 7 [12].

In the preliminary stages of differentiation, transcription factors, such as C/EBP β , are activated, followed by the activation of PPAR γ and C/EBP α in the intermediate stage. In the later stages, fatty acid binding protein (FABP)4 and adiponectin induce differentiation into mature adipocytes [12]-[14]. Adenosine monophosphate-activated protein kinase (AMPK), a sensor of cellular energy status, is also a potential molecular candidate regulating adipocyte differentiation [15]. AMPK activation requires phosphorylation at Thr172 of the AMPK α subunit, thus allowing AMPK to regulate metabolic enzymes and transcription factors [16]. Acetyl-CoA carboxylase (ACC), which is a critical enzyme for lipid biosynthesis, can also be phosphorylated and inactivated by AMPK [17].

Trapa japonica Flerov (water chestnut, TJF) has been used as food and herbal medicine. TJF is an annual aquatic plant found in lakes and ponds in various parts of the world, including Korea, Japan, China, India, and North America [18]. The fruit contains approximately 80% starch, 5% protein, and a significant amount of vitamins [19]. The recent investigation revealed polyphenols, eugenin, 1,2,3,6-tetra-O-galloyl- β -D-glucopyranose, and trapain from the husk of the TJF [20]. Most studies evaluating TJF have focused on its taxonomy and ecology [18] [21]. TJF has been known to have anti-oxidative, anti-cancer and anti-diabetic effects, reduction of blood glucose levels, and inhibitory activity against α -amylase and α -glucosidase [20] [22]-[24]; however, the effects of TJF on anti-adipogenesis have not been investigated yet. In the present study, we examined whether TJF extract affects adipocyte differentiation, and the expression of adipogenic transcription factors and related proteins in 3T3-L1 cells.

2. Materials and Methods

2.1. Preparation of TJF Extract

Dried ripened TJF (containing the shell and fruit) was purchased from a Korean herbal medicine dealer in Gumiherb, Korea. Dried TJF extract was triturated, and a crude extract from the triturate was prepared by 75% aqueous ethanol extraction. Briefly, powdered TJF (30 g) was extracted twice with 75% aqueous ethanol (300 g)

under reflux at 60°C - 90°C for 4 h, and was filtered and evaporated under reduced pressure. The powdered extract was solubilized in DMSO and diluted for this experiment.

2.2. Determination of Total Polyphenol Contents

Total phenolic contents of TJF extract was determined using Folin-Ciocalteu assay [25]. Briefly, 1 mg of TJF extract was dissolved in 1 mL of DW. Then, 100 µL of this solution was mixed with 100 µL of 50% Folin-Ciocalteu reagent, and 2 mL of 4% sodium carbonate (Na₂CO₃). After incubation at RT for 30 min, the absorbance of the reaction mixtures were measured at 750 nm by a spectrophotometer (SHIMADZU, Japan). The standard calibration curve was prepared by using the gallic acid.

Polyphenols were analyzed by HPLC under the following conditions. HPLC was performed on the Agilent Technologies 1200 series coupled with UV detector, and auto-sampler with a 10 µL loop. HPLC analysis was carried out using an ZORBAX Eclipse XDB-C₁₈ (5 µm, 4.6 I.D × 250 mm, YMC Inc., USA). The separation was conducted using a linear gradient 0.1% v/v, trifluoroacetic acid in H₂O to 0.1% v/v, trifluoroacetic acid in acetonitrile for 60 min at a flow rate of 0.8 mL/min with detector at UV_{280 nm}.

2.3. Measurement of Antioxidant Activity

The antioxidant properties of TJF extract were examined using DPPH and xanthine/xanthine oxidase.

The free radical scavenging activity, based on the scavenging of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma, USA) free radical, was determined using the method described by Fujita *et al.* [26]. First, 1 mL of TJF extract was added to 2 mL DPPH solution (0.2 mM) and incubated at RT for 10 min. The absorbance was then measured at 517 nm using a microplate reader (Synergy, BioTek, USA). L-ascorbic acid (Sigma, USA) was used as a positive control.

Superoxide radicals were generated by xanthine/xanthine oxidase and measured using the method described by Noro *et al.* [27]. In brief, TJF extract was mixed with 0.05 M Na₂CO₃, 3 mM xanthine, 3 mM EDTA, BSA and 0.75 mM NBT. After 10 min at 25°C, xanthine oxidase was added. The absorbance of reaction mixture was read at 560 nm after 10 min incubation at 25°C. Tocopherol (Sigma, USA) was used as a positive control.

2.4. Cell Culture and Adipocyte Differentiation

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal calf serum (FCS, Gibco). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

3T3-L1 cells were grown to confluence in DMEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO₂. One day after confluence (designated "day 0"), cell differentiation was induced with a hormonal mixture containing 0.5 mM isobutylmethylxanthine (IBMX, Sigma, USA), 0.25 µM dexamethasone (DEX, Sigma), 10 µg/mL insulin (Sigma), and 10% fetal bovine serum (FBS, Gibco, Gaithersburg). On day 2 and day 5, the medium was replaced with DMEM containing 10% FBS and 10 µg/mL insulin only. 3T3-L1 cells were treated with DMSO vehicle and varying concentrations of TJF extract during the differentiation process.

2.5. Cytotoxicity (MTT) Assay

Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The 3T3-L1 cells were seeded at a density of 5 × 10³ cells/well in a 96-well plate, and were cultured at 37°C with 5% CO₂. The cells were then treated with TJF extract at various concentrations for 24 h. Following incubation, the cells were treated with the MTT solution (Sigma) for 4 h at 37°C. The supernatants were aspirated, and DMSO (Sigma) was added to each well. After incubation for 20 min, the absorbance was measured at 540 nm using a microplate reader (Synergy, BioTek, USA). Cytotoxicity is presented as a percentage of the optical density of the control group.

2.6. Determination of Lipid Accumulation by Oil Red O Staining

Oil red O staining was performed on day 8. Cells were washed twice with phosphate-buffered saline (PBS,

Gibco) and fixed with 10% formalin for 1 h. Oil red O was then added to stain the cells. Thereafter, the cells were washed three times with water. After oil red O staining, the lipid droplets were dissolved with isopropanol, and the optical density was measured at 540 nm with a microplate reader. Fat content was presented as a percentage of the optical density of the control group.

2.7. Western Blot Analysis

Cells were rinsed twice with PBS and scraped into lysis buffer. Lysates were prepared with lysis buffer according to the manufacturer's instructions (Thermo, USA; iNtRON, Korea). The protein concentration was determined using a BCA Assay Reagent Kit (Thermo Fisher Scientific Inc., USA). Proteins present in the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel, transferred onto polyvinylidene fluoride membranes (GE Healthcare, UK), blocked with 5% skim milk, and treated with primary antibodies for 2 h at room temperature (1:1000 dilution, Cell Signaling, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) at room temperature, immunoreactive proteins were detected with a chemiluminescent ECL assay kit (GE Healthcare, UK) according to the manufacturer's instructions. Bands were visualized using a ChemiDoc image analyzer (BIO-RAD, USA).

2.8. Statistical Analysis

Data were analyzed using Sigma Plot. Results were expressed as the mean \pm S.D. of three independent experiments. Comparisons were performed using a one-way ANOVA followed by Duncan's multiple range test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Extraction Yield, Total Polyphenols Contents of TJF Extract

The yield obtained was 6.77%. The contents of total polyphenols in extract, were determined from regression equations of calibration curves and was expressed in gallic acid equivalents for TJF extract. TJF extract contains polyphenols of $46.58\% \pm 0.58\%$ (including eugenin and gallic acid as constituents accounting for $0.85\% \pm 0.01\%$ and $0.21\% \pm 0.02\%$, respectively).

3.2. Antioxidant Activity of TJF Extract

The antioxidant activity of *T. japonica* husk extract was studied [22] [23]. We examined the antioxidant activities of TJF extract and compared with that of L-ascorbic acid and tocopherol. The EC₅₀ values ($\mu\text{g/mL}$) of TJF extract (EC₅₀ = 107.55 ± 2.41 $\mu\text{g/mL}$) for superoxide radical scavenging activity was higher than that of tocopherol (EC₅₀ = 225.61 ± 4.56 $\mu\text{g/mL}$). TJF extract (EC₅₀ = 7.17 ± 0.86 $\mu\text{g/mL}$) showed lower DPPH radical scavenging activity compared with that of L-ascorbic acid (EC₅₀ = 4.14 ± 0.11 $\mu\text{g/mL}$) (Table 1). However, TJF extract had strong antioxidant activity.

3.3. Effect of TJF Extract on the Viability of 3T3-L1 Cells

3T3-L1 cells were exposed to various concentrations of TJF extract, and cell viability was measured by MTT assay. TJF extract did not affect cell viability at concentrations up to 50 $\mu\text{g/mL}$ (Figure 1), and there is no

Table 1. Antioxidative activities of TJF extract.

Sample	DPPH Scavenging ^a (EC ₅₀ $\mu\text{g/mL}$)	Superoxide Scavenging ^a (EC ₅₀ $\mu\text{g/mL}$)
TJF extract	7.17 ± 0.86	107.55 ± 2.41
L-ascorbic acid	4.14 ± 0.11	-
Tocopherol	-	225.61 ± 4.56

The data are presented as mean \pm standard deviation (n = 3). ^aThe antioxidative activities of the samples are expressed by the concentration that showed inhibition of 50% radical.

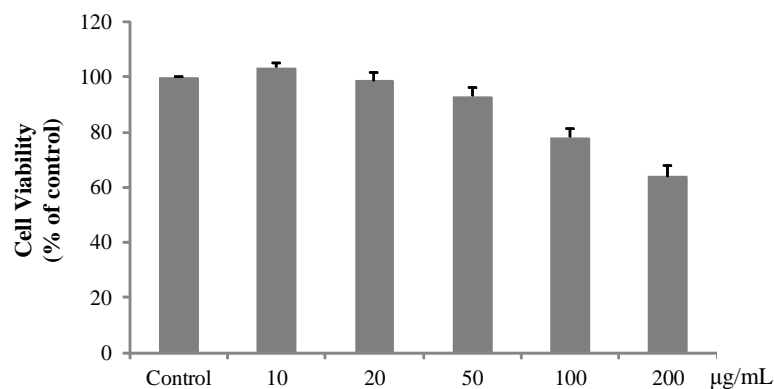


Figure 1. Effects of TJF extract on the viability of 3T3-L1 adipocytes as measured by MTT assay. 3T3-L1 cells were treated with various concentrations of TJF extract. After incubation for 24 h, the MTT assay was performed. The results represent the mean \pm S.D. of three independent experiments.

cytotoxicity during the differentiation process (data not shown). Thus, TJF extract was used in a range of non-cytotoxic concentrations (10 - 50 $\mu\text{g/mL}$) in subsequent experiments.

3.4. Effect of TJF Extract on 3T3-L1 Differentiation

We evaluated the effects of TJF extract on adipocyte differentiation. Cultured 3T3-L1 cells were exposed to TJF extract at varying doses on day 0, and cell differentiation was induced with a hormonal mixture-containing medium. On day 8, differentiation was terminated, and lipid droplets were detected by oil red O staining. TJF extract significantly reduced lipid accumulation, as indicated by decreased oil red O staining (**Figure 2(a)**). The triglyceride content in 3T3-L1 adipocytes was decreased in a dose-dependent manner, corresponding to 94%, 83%, and 57% following treatment with 10, 20, and 50 $\mu\text{g/mL}$ TJF extract, respectively, compared to vehicle treated control cells (100%, $p < 0.05$, **Figure 2(b)**). These results indicated that TJF extract may have been efficiently blocking adipocyte differentiation and have potential of anti-obesity effects in 3T3-L1 cells.

3.5. Effects of TJF Extract on the Expression Levels of Adipogenic Transcription Factors and Markers in 3T3-L1 Cells

3T3-L1 adipocyte differentiation requires the synergistic action of multiple transcription factors and adipogenic markers, including PPAR γ , C/EBP α , and adiponectin. PPAR γ and C/EBP α alone or in cooperation with each other induce the transcription of many adipocyte genes encoding proteins and enzymes involved in creating and maintaining the adipocyte phenotype [12]. After induction of adipocyte differentiation, these transcription factors were transcriptionally induced and reached to maximum level by 3 - 4 d [12] [28]. We performed western blot analysis to determine whether TJF extract inhibited adipocyte differentiation by negatively regulating the expression of adipogenic related factors. As shown in **Figure 3**, TJF extract markedly inhibited the protein expression of PPAR γ , C/EBP α , and adiponectin.

3.6. Effect of TJF Extract on the Expression Level of Terminal Markers of Adipogenesis in 3T3-L1 Cells

Since the adipogenic transcription factors were down-regulated by TJF extract, we further determined the expression of their downstream protein targets such as FABP4. Fatty acid binding protein (FABP)4, which binds fatty acids with high affinity and transports them to various compartments within the cell, is a key mediator of intracellular transport and metabolism of fatty acids in adipose tissues. FABP4 is massively expressed during adipogenesis and comprises up to 6% of total cytosolic proteins in a mature fat cell [29]. Our western blot analysis revealed that FABP4, an important biomarker in adipogenesis, was down-regulated (**Figure 4**).

3.7. Effect of TJF Extract on the Activation of AMPK in 3T3-L1 Cells

Several reports suggest that naturally occurring compounds exert their anti-obesity effects via AMPK activation

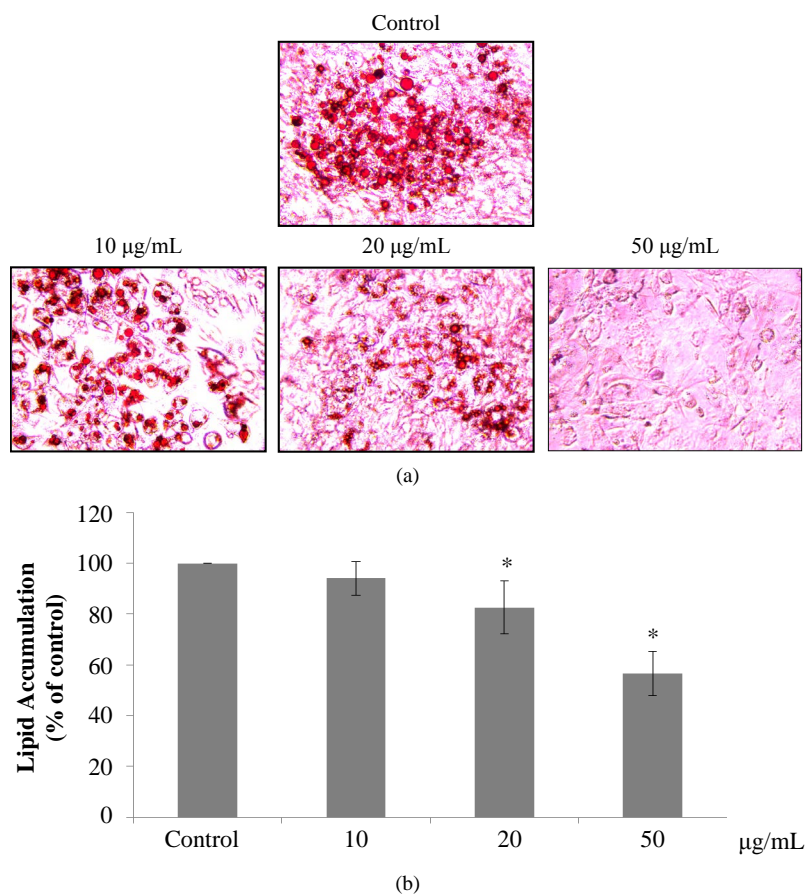


Figure 2. Effect of TJF extract on adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were exposed to a hormonal mixture for 8 d in the presence or absence of TJF extract. (a) Fat drops were stained with oil red O; (b) Stained triglyceride content was quantified by measuring absorbance. The results represent the mean \pm SD of three independent experiments. * $p < 0.05$ compared with control (DMSO vehicle) group.

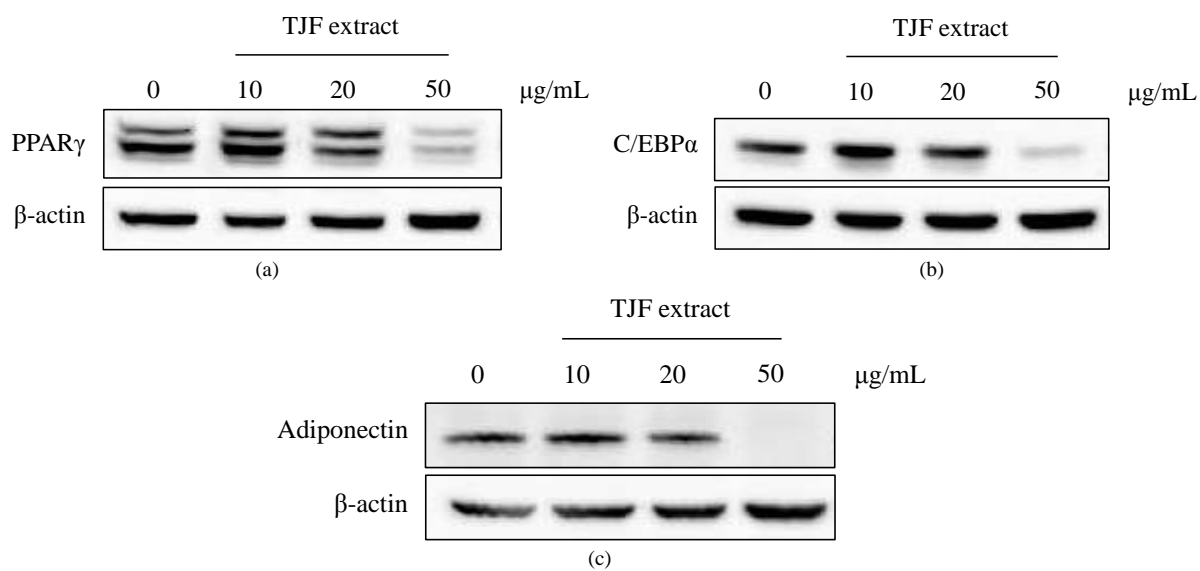


Figure 3. Effect of TJF extract on PPAR γ (a), C/EBP α (b), and adiponectin (c) expression in 3T3-L1 adipocytes. 3T3-L1 cells were exposed to a hormonal mixture for 5 d in the presence or absence of TJF extract. Cell lysates were prepared and subjected to western blot analysis to detect PPAR γ , C/EBP α , and adiponectin.

[30]. Therefore, we investigated the involvement of AMPK in the process of TJF extract induced inhibition of adipocyte differentiation. AMPK activation and its substrate ACC phosphorylation were examined. AMPK activation was directly observed by examining increases in phosphorylated AMPK, and indirectly by observing the phosphorylation level of ACC-Ser79, the best-characterized phosphorylation site on AMPK. As shown in **Figure 5**, AMPK phosphorylation increased at 20, 50 $\mu\text{g/mL}$, and phosphorylation of its substrate, ACC-Ser79 was also enhanced in a dose dependent manner. These results indicated that AMPK is involved in the inhibition process of adipocyte differentiation.

4. Discussion

Adipogenesis is the process by which preadipocytes differentiate to mature adipocytes and is accompanied by coordinated changes in cell morphology, gene expression, and hormone sensitivity. The 3T3-L1 cell line is one of the best characterized and reliable models for studying the conversion of preadipocytes into adipocytes [12], and is useful to rapidly screen and assess the adipogenic potential of various agents.

In this study, we observed that TJF extract significantly inhibited 3T3-L1 adipogenesis and fat accumulation (**Figure 2**). Several transcription factors have been identified that act in a sequential fashion to promote adipocyte differentiation. PPAR γ and the C/EBP family serve as early markers of adipocyte differentiation. The C/EBP families include C/EBP α , C/EBP β , and C/EBP δ . Transient expression of C/EBP β and C/EBP δ occurs during the early stages of differentiation, followed by induced expression of PPAR γ and C/EBP α . These proteins induce the expression of multiple adipocyte specific genes and are important for terminal adipocyte differentiation [31]. TJF extract inhibits 3T3-L1 adipogenesis at a relatively early stage by regulating the expression of PPAR γ and C/EBP α (**Figure 3(a)** & **Figure 3(b)**). Moreover, TJF extract significantly suppressed FABP4, a late adipogenesis marker downstream of PPAR γ (**Figure 4**). Our results also show that TJF extract markedly inhibits the expression level of adiponectin (**Figure 3(c)**). Thus, the current study demonstrates that overexpression of adiponectin can enhance 3T3-L1 preadipocyte proliferation, accelerate adipocyte differentiation, and, in fully differentiated adipocytes, augment both lipid accumulation and insulin-responsive glucose transport [32].

One possible mechanism of TJF extract action could involve the activation of AMPK. AMPK, an energy sensor, plays a key role in the regulation of fatty acids. Several groups have demonstrated that AMPK activation reduced body weight gain and decreased liver and plasma triglyceride levels *in vivo* [33]. Furthermore it was reported that AMPK affects adipocyte differentiation *in vitro*. Zhou *et al.* reported that A-769662, which is an

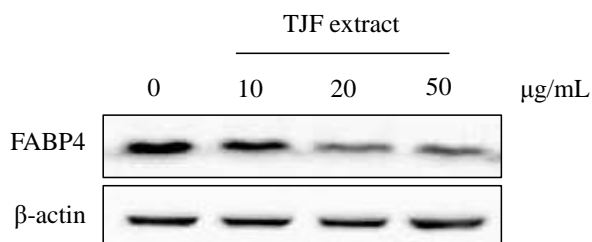


Figure 4. Effect of TJF extract on FABP4 expression in 3T3-L1 adipocytes. 3T3-L1 cells were fully exposed to a hormonal mixture for 8 d in the presence or absence of TJF extract. Cell lysates were prepared and subjected to western blot analysis to detect FABP4.

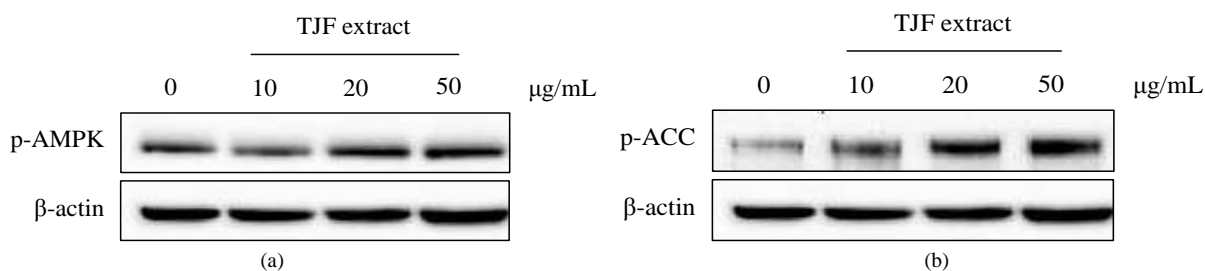


Figure 5. Effect of TJF extract on AMPK activation in 3T3-L1 adipocytes. 3T3-L1 cells were fully exposed to a hormonal mixture for 8 d in the presence or absence of TJF extract. Phosphorylation of AMPK and its substrate acetyl-CoA carboxylase (ACC) were detected by western blot analysis.

AMPK activator, inhibited 3T3-L1 adipogenic differentiation by downregulating the expression of C/EBP β and C/EBP δ , thereby inhibiting clonal expansion. Moreover, AMPK activation blocked the expression of PPAR γ and C/EBP α , and their downstream adipogenic genes. TJF extract also activated AMPK via phosphorylation and phosphorylated and inactivated ACC, which is essential for lipid biosynthesis (Figure 5) [34]. The AMPK cascade is an important therapeutic target for obesity because it promotes the expression or phosphorylation of downstream proteins involved in energy metabolism.

5. Conclusion

TJF extract inhibits adipocyte differentiation by downregulating the expression of PPAR γ and C/EBP α and their downstream adipogenic target genes that are essential for adipocyte differentiation, and by activating AMPK. Other approaches will be necessary to identify the transcription factors and target genes during adipocyte differentiation.

Acknowledgements

This work was supported by Chungcheong Institute for Regional Program Evaluation Promotion Project (R0002893) of the Ministry of Trade, Industry and Energy Republic of Korea.

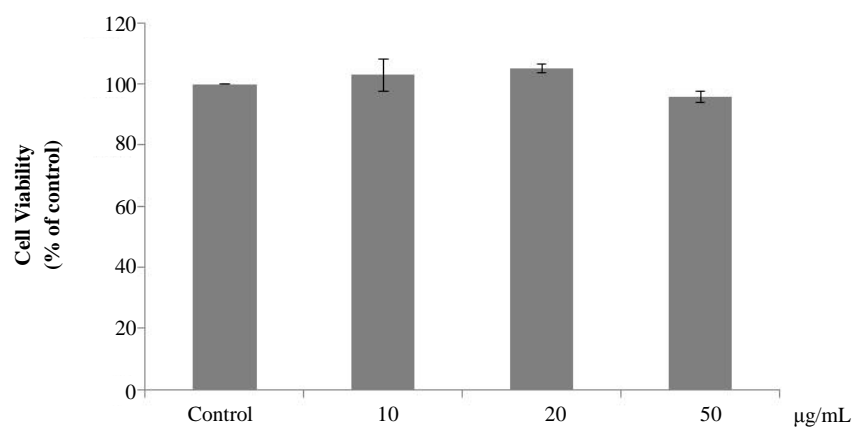
References

- [1] Grundy, S.M. (1998) Multifactorial Causation of Obesity: Implication for Prevention. *American Journal of Clinical Nutrition*, **67**, 563S-572S.
- [2] Kopelman, P.G. (2000) Obesity as Medical Problem. *Nature*, **404**, 635-643.
- [3] World Health Organization; International Association for the Study of Obesity; International Obesity Task Force (2000) The Asia-Pacific Perspective: Redefining Obesity and Its Treatment. Health Communication Australia Pty Ltd., Sydney, 15-21.
- [4] Ministry of Health and Welfare (2012) Korea Health Statistics 2012. Ministry of Health and Welfare, Seoul.
- [5] Yang, J.Y., Della-Fera, M.A., Rayalam, S., Ambati, S., Hartzell, D.L., Park, H.J. and Baile, C.A. (2008) Enhanced Inhibition of Adipogenesis and Induction of Apoptosis in 3T3-L1 Adipocytes with Combination of Resveratrol and Quercetin. *Life Sciences*, **82**, 1032-1039. <http://dx.doi.org/10.1016/j.lfs.2008.03.003>
- [6] Roncari, D.A., Lau, D.C. and Kindler, S. (1981) Exaggerated Replication in Culture of Adipocyte Precursors from Massively Obese Persons. *Metabolism*, **30**, 425-427. [http://dx.doi.org/10.1016/0026-0495\(81\)90174-8](http://dx.doi.org/10.1016/0026-0495(81)90174-8)
- [7] Rosen, E.D., Walkey, C.J. Puiqserver, P. and Spiegelman, B.M. (2000) Transcriptional Regulation of Adipogenesis. *Genes & Development*, **14**, 1293-1307.
- [8] Moller, D.E. and Greene, D.A. (2001) Peroxisome Proliferator-Activated Receptor (PPAR) Gamma Agonists for Diabetes. *Advances in Protein Chemistry*, **56**, 182-212. [http://dx.doi.org/10.1016/S0065-3233\(01\)56006-4](http://dx.doi.org/10.1016/S0065-3233(01)56006-4)
- [9] Green, H. and Kehinde, O. (1974) An Established Pre-Adipose Cell Line and Its Differentiation in Culture. *Cell*, **1**, 113-116. [http://dx.doi.org/10.1016/0092-8674\(74\)90126-3](http://dx.doi.org/10.1016/0092-8674(74)90126-3)
- [10] Student, A.K., Hsu, R.Y. and Lane, M.D. (1980) Induction of Fatty Acid Synthetase Synthesis in Differentiating 3T3-L1 Preadipocyte. *Journal of Biological Chemistry*, **255**, 4745-4750.
- [11] Bernlohr, D.A., Bolanowski, M.A., Kelly Jr., T.J. and Lane, M.D. (1985) Evidence for an Increase in Transcription of Specific mRNAs during Differentiation of 3T3-L1 Preadipocytes. *Journal of Biological Chemistry*, **260**, 5563-5567.
- [12] Ntambi, J.M. and Kim, Y.C. (2000) Adipocyte Differentiation and Gene Expression. *Journal of Nutrition*, **130**, 3122S-3126S.
- [13] Distel, R.J., Robinson, G.S. and Spiegelman, B.M. (1992) Fatty Acid Regulation of Gene Expression. Transcriptional and Post-Transcriptional Mechanisms. *Journal of Biological Chemistry*, **267**, 5937-5941.
- [14] Farmer, S.R. (2005) Regulation of PPAR γ Activity during Adipogenesis. *International Journal of Obesity*, **29**, S13-S16. <http://dx.doi.org/10.1038/sj.ijo.0802907>
- [15] Erbayraktar, Z., Yilmaz, O., Artmann, A.T., Cehreli, R. and Coker, C. (2007) Effects of Selenium Supplementation on Antioxidant Defense and Glucose Homeostasis in Experimental Diabetes Mellitus. *Biological Trace Element Research*, **118**, 217-226. <http://dx.doi.org/10.1007/s12011-007-0037-5>
- [16] Hardie, D.G. (2007) AMP-Activated/SNF1 Protein Kinase: Conserved Guardians of Cellular Energy. *Nature Reviews Molecular Cell Biology*, **8**, 774-785. <http://dx.doi.org/10.1038/nrm2249>

- [17] Sim, A.T. and Hardie, D.G. (1998) The Low Activity of Acetyl-CoA Carboxylase in Basal and Glucagon-Stimulated Hepatocytes Is Due to Phosphorylation by the AMP-Activated Protein Kinase and Not Cyclic AMP-Dependent Protein Kinase. *FEBS Letters*, **233**, 294-298. [http://dx.doi.org/10.1016/0014-5793\(88\)80445-9](http://dx.doi.org/10.1016/0014-5793(88)80445-9)
- [18] Suriyagoda, L.D.B., Arima, S. and Suzuki, A. (2006) Canopy and Fruit Characters with Morphological Relationships of European and Asian Water Chestnuts (*Trapa* spp.). *Bulletin of the Faculty of Agriculture, Saga University*, **92**, 45-51.
- [19] Bargale, M., Samwarkar, N.J. and Sharma, Y.K. (1987) Mineralogical Composition of Water Chestnut as Compared to Sweet Potato. *Indian Journal of Nutrition and Dietetics*, **24**, 78-82.
- [20] Yasuda, M., Yasutake, K., Hino, M., Ohwatari, H., Ohmagari, N., Takedomi, K., Tanaka, T. and Nonaka, G. (2014) Inhibitory Effects of Polyphenols from Water Chestnut (*Trapa japonica*) Husk on Glycolytic Enzymes and Postprandial Blood Glucose Elevation in Mice. *Food Chemistry*, **165**, 42-49. <http://dx.doi.org/10.1016/j.foodchem.2014.05.083>
- [21] Chung, Y.H., Choi, H.K., Suh, K.H. and Shin, H.C. (1987) Numerical Taxonomic Study of the Nuts of Genus *Trapa* in Korea. *Korean Journal of Plant Taxonomy*, **17**, 45-54.
- [22] Kang, M.J., Lee, S.K., Song, J.H., Kim, M.E., Kim, M.J., Jang, J.S., Lee, J.H. and Kim, J.I. (2009) Water Chestnut (*Trapa japonica* Flerov.) Exerts Inhibitory Effect on Postprandial Glycemic Response in Rats and Free Radical Scavenging Activity *in Vitro*. *Food Science and Biotechnology*, **18**, 808-812.
- [23] Kim, Y.S., Hwang, J.W., Han, Y.K., Kwon, H.J., Hong, H., Kim, E.H., Moon, S.H., Jeon, B.T. and Park, P.J. (2014) Antioxidant Activity and Protective Effects of *Trapa japonica* Pericarp Extracts against Tert-Butylhydroperoxide-Induced Oxidative Damage in Chang Cells. *Food and Chemical Toxicology*, **64**, 49-56. <http://dx.doi.org/10.1016/j.fct.2013.11.018>
- [24] Ministry of Agriculture, Food and Rural Affairs (2006) Anti-Diabetic and Anti-Cancer Agents from *Trapa japonica* Flerov. GOVP1200711645.
- [25] Meda, A., Lamien, C.E., Romito, M., Millogo, J. and Nacoulma, O.G. (2005) Determination of the Total Phenolic, Flavonoid and Proline Contents in Burkina Fasan Honey, as Well as Their Radical Scavenging Activity. *Food Chemistry*, **91**, 571-577. <http://dx.doi.org/10.1016/j.foodchem.2004.10.006>
- [26] Fugita, Y., Uehara, I., Morimoto, Y., Nakashima, M., Hatano, T. and Okuda, T. (1988) Studies on Inhibition Mechanism of Autoxidation by Tannins and Flavonoids. II. Inhibition Mechanism of Caffeetannins Isolated from Leaves of Artemisia Species on Lipooxygenase Dependent Lipid Peroxidation. *Yakugaku Zasshi*, **108**, 129-135.
- [27] Noro, T., Oda, Y., Miyase, T., Ueno, A. and Fukushima, S. (1983) Inhibition of Xanthine Oxidase from the Flowers and Buds of Daphne Genkwa. *Chemical and Pharmaceutical Bulletin*, **31**, 3984-3987. <http://dx.doi.org/10.1248/cpb.31.3984>
- [28] Habinowski, S.A. and Witters, L.A. (2001) The Effects of AICAR on Adipocyte Differentiation of 3T3-L1 Cells. *Biochemical and Biophysical Research Communications*, **286**, 852-856. <http://dx.doi.org/10.1006/bbrc.2001.5484>
- [29] Spiegelman, B.M. and Green, H. (1980) Control of Specific Protein Biosynthesis during the Adipose Conversion of 3T3-L1 Cells. *Journal of Biological Chemistry*, **255**, 8811-8818.
- [30] Hwang, J.T., Park, I.J., Shin, J.I., Lee, Y.K., Baik, H.W., Ha, J. and Park, O.J. (2005) Genistein EGCG, and Capsaicin Inhibit Adipocyte Differentiation Process via Activating AMP-Activated Protein Kinase. *Biochemical and Biophysical Research Communications*, **338**, 694-699. <http://dx.doi.org/10.1016/j.bbrc.2005.09.195>
- [31] Kudo, M., Sugawara, A., Uruno, A., Takeuchi, K. and Ito, S. (2004) Transcription Suppression of Peroxisome Proliferator Activated Receptor γ 2 Gene Expression by Tumor Necrosis Factor α via an Inhibition of CCAAT/Enhancer-Binding Protein δ during the Early Stage of Adipocyte Differentiation. *Endocrinology*, **145**, 4948-4956. <http://dx.doi.org/10.1210/en.2004-0180>
- [32] Fu, Y., Luo, N., Klein, R.L. and Garvey, W.T. (2005) Adiponectin Promotes Adipocyte Differentiation, Insulin Sensitivity, and Lipid Accumulation. *Journal of Lipid Research*, **46**, 1369-1379. <http://dx.doi.org/10.1194/jlr.M400373-JLR200>
- [33] Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M.W. and Guarente, L. (2004) Sirt1 Promotes Fat Mobilization in White Adipocytes by Repressing PPAR-Gamma. *Nature*, **429**, 771-776. <http://dx.doi.org/10.1038/nature02583>
- [34] Zhou, Y., Wang, D., Zhu, Q., Gao, X., Yang, S., Xu, A. and Wu, D. (2009) Inhibitory Effects of A-769662, a Novel Activator of AMP-Activated Protein Kinase, on 3T3-L1 Adipogenesis. *Biological and Pharmaceutical Bulletin*, **32**, 993-998. <http://dx.doi.org/10.1248/bpb.32.993>

Supplementary Data

1) Effect of the TJF extract on the viability of differentiated 3T3-L1 adipocytes at d 8.



2) Relative intensity of p-AMPK (**Figure 4(a)**).

