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Trapa Japonica Inhibits Adipocyte Differentiation and Adipogenesis through Ampk Signaling Pathway in 3t3-L1 Pre-Adipocytes

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Abstract

Trapa japonica (water caltrop) is one of the main aquatic plant with various pharmacological functions; however, the effects associated with anti-obesity have not been fully identified. In the present study, we investigated the inhibition of adipocyte differentiation and adipogenesis by Hot-Water extracts of *T. japonica* (WETJ) in 3T3-L1 pre-adipocytes. Treatment with WETJ suppressed the terminal differentiation of 3T3-L1 pre-adipocytes in a dose-dependent manner, as confirmed by a decrease in the lipid droplet number and lipid content through Oil Red O staining. WETJ significantly reduced the accumulation of cellular triglyceride, which is associated with a significant inhibition of pro-adipogenic transcription factors, including PPARγ, C/EBPα and C/EBPβ. In addition, WETJ potentially downregulated the expression of adipocyte-specific genes, including aP2 and leptin. Furthermore, WETJ treatment effectively increased the phosphorylation of the AMPK and ACC; however, treatment with a potent inhibitor of AMPK, compound C, significantly restored the WETJ-induced inhibition of pro-adipogenic transcription factors. These results together indicate that WETJ has preeminent effects on the inhibition of adipogenesis through the AMPK signaling pathway, and further studies will be needed to identify the active compounds in *T. japonica*.

Keywords: *Trapa japonica*; Adipogenesis; AMPK; 3T3-L1 pre-adipocytes

Introduction

Obesity is a metabolic disorder caused by abnormal regulation of energy metabolism in the body when energy intake exceeds consumption for a prolonged period of time to convert the excess energy into fat that accumulates in the subcutaneous or abdominal regions. Of particular note is adipokine produced and secreted by the adipocytes, which is a known risk factor of neurodegenerative diseases such as diabetes, cardiovascular diseases, Parkinson's and Alzheimer's as well as of various cancer types including breast, endometrial, gastric, colorectal, and esophageal cancer [1-3]. According to a survey conducted by the World Health Organization (WHO), global obesity rate in 2014 was 39%, a two-fold increase since 1980. In line with this, obesity rate in Korea is reported to have steadily increased from average 29.2% in 2001 to 32.9% in 2014 due to westernization of dietary habits and changes in living conditions. Obesity is thus receiving growing attention as a grave health problem [4, 5]. Treatment of obesity generally involves drug administration and surgery in addition to the improvement of lifestyle such as dietary control and exercise. However, the two most widely used drugs for obesity treatment; Sibutramine and Osristat cause not only the side effects of cardiovascular and gastrointestinal disorders but also the symptoms of headache, severe thirst, constipation, insomnia, and palpitation, so that the development of a therapeutic agent with proven safety and effectiveness is urgently needed [6, 7]. Thus, rigorous efforts have been made in discovering and researching a material exhibiting anti-obesity efficacy among the natural substances with proven safety.

In general, obesity is known to be caused by two critical factors including adipocyte hypertrophy due to triglyceride accumulation induced by adipogenesis and adipocyte hyperplasia upon the proliferation and differentiation of adipocytes [8]. Adipogenesis is a process of differentiation that creates adipocytes, which accompanies changes in cell morphology, gene expression and hormone sensitivity. Such process of adipogenesis is known to be sequentially influenced by the adipogenic transcription factors such as sterol response element binding protein 1c (SREBP1c), peroxisome proliferator-activated receptor γ (PPARγ) and cytidine-cytidine-adenosine-adenosine-thymidine (CCAAT) / enhancer binding proteins (C/EBPs) [9, 10]. In addition, once adipocytes have been formed through adipogenesis, not only the morphological characteristics such as triglyceride accumulation seen in white adipocytes but also the expression of adipocyte-specific genes including adipocyte-specific lipid binding protein (aP2) and leptin is known to occur [11]. AMP-activated protein kinase (AMPK), in particular, is a key regulator of energy homeostasis known to control adipogenesis, which elicited numerous studies on obesity prevention and treatment with AMPK as a drug target [12]. This implicates that the regulation of adipogenesis could be applied as an important target of obesity inhibition as it is an essential method to control the mechanism leading to adipocyte production.

Water caltrop (*Trapa japonica*) is an aquatic annual plant belonging to the Lythraceae family and a dicotyledon. It is known as a free-floating plant that inhabits the ponds, shallow marshes and swamps in tropical and subtropical regions in Asian countries. A whole variety of water

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caltrop plants exist including *Trapa japonica*, *Trapa bicornis*, *Trapa natans*, *Trapa incisa*, and naturally crossbred cultivars [13]. The fruit of water caltrop is composed of approximately 80% starch, 5% protein, and a trace amount of vitamins [14]. Its taste is similar to chestnuts and it is known to have been used as a plant resource in regions throughout Asia including Korea. The bark of water caltrop contains an abundance of dietary fibers and polyphenol [15] and it has long been used in Chinese medicine. Although functional substances with various pharmacological activities have so far been reported, studies on their inhibitory activity against obesity and the direct molecular biological mechanism behind are relatively very rare. The present study thus investigated the anti-obesity effects of Hot-Water Extracts of *Trapa japonica* (WETJ) and how WETJ influences adipogenesis, and reports significant findings.

Materials and Methods

Materials

In this study, for the differentiation of 3T3-L1 pre-adipocytes, insulin, dexamethasone and IBMX were used, and Oil Red O was used to detect the triglyceride formation in adipocytes while compound C was used to inhibit the activity of AMPK; all these materials were purchased from Sigma-Aldrich (St. Louis (MO) USA). The different types of primary antibodies used in the protein analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz (CA) USA) and Cell Signaling Technology (Beverly (MA) USA) (Table 1), and the secondary antibodies used in the immunoblotting: peroxidase-labeled donkey anti-rabbit and peroxidase-labeled sheep anti-mouse immunoglobulin, were purchased from Amersham Life Science Corp. (Arlington Heights (IL) USA).

Preparation of Hot-Water Extracts of *Trapa japonica* (WETJ)

To prepare Hot-Water Extracts of *Trapa japonica* (WETJ), *T. japonica* fruit was washed thoroughly in running water, dried, and finely ground. After adding 2,000 mL of distilled water per 100 g of *T. japonica* fruit, the mixture was boiled at 110°C for 2 h in a heater equipped with a reflux cooling device. Next, by centrifuging at 3,000 rpm for 20 min, then removing the residues and filtering the supernatant using the Whatman filter (No. 2), a solid component was produced through a decompression evaporation process. The solid component was pulverized using a mortar and pestle, then in a sealed container, stored in the -70 °C deep freezer. The so-produced WETJ, in subsequent experiments, was made to 100 mg/mL using dimethyl sulfoxide (DMSO) then diluted to a suitable concentration for the treatment.

Cell culture

The mouse fibroblast cell line 3T3-L1 pre-adipocytes used in this study was obtained from American Type Culture Collection (ATCC, USA). 3T3-L1 pre-adipocytes were cultured using the growth medium containing 90% Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island (NY) USA), 10% bovine calf serum (BCS, Gibco BRL, Grand Island (NY) USA) and 1% penicillin and streptomycin (Gibco BRL, Grand Island (NY) USA) in 37°C, 5% CO_2 condition. To prevent overcrowding due to increasing cell number, the growth medium was replaced every 48 h and an optimum number of cells was maintained.

3T3-L1 pre-adipocytes differentiation induction and morphological observation

For the differentiation of 3T3-L1 pre-adipocytes into adipocytes, the growth medium containing 10% BCS and 1% penicillin and streptomycin was used while the cells were cultured until confluence. The confluent cells were cultured using the differentiation medium containing 10% fetal bovine serum (FBS, Gibco BRL, Grand Island (NY) USA) and 1% penicillin and streptomycin for further two days, after which the medium was replaced with the one containing 10 μg/ml insulin, 1 μM dexamethasone and 0.5 μM IBMX (MDI) for another two-day culture. Afterwards, the medium was replaced with the one containing 10 μg/ml insulin every two days. In addition, to determine the level of inhibition of 3T3-L1 pre-adipocyte differentiation, the cells were treated with an adequate concentration of WETJ when the medium was replaced with the one containing insulin and MDI. The 3T3-L1 pre-adipocytes whose differentiation had been induced as illustrated, were subsequently used in various experimental analyses.

Furthermore, to examine the effects of WETJ on morphological changes caused by the differentiation of 3T3-L1 pre-adipocytes into adipocytes, the same methods as above were used for the differentiation and WETJ treatment, then the cells were observed in 200× magnification using an inverted microscope (Carl Zeiss, Gottingen, Germany). Images were taken using the Axio Vision program.

Cell viability measurement

To examine the effects of WETJ on the cell viability of 3T3-L1 pre-adipocytes, the cells were aliquoted to a 6-well culture plate and cultured until confluence. The confluent cells were then treated with an adequate concentration of WETJ. After 72 h, the supernatant was removed, and the cells were treated with 0.05% trypsin-EDTA for detachment from the plate. Next, phosphate-buffered saline (PBS) and 0.5% trypan blue solution (Gibco BRL, Grand Island (NY) USA)

Table 1: Antibodies used in the present study.

Antibody	Origin	Company	Catalogue no.
PPARγ	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-7196
C/EBPα	Rabbit polyclonal	Cell Signaling Technology, Inc.	2295
C/EBPβ	Rabbit polyclonal	Cell Signaling Technology, Inc.	3087
SREBP-1c	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-366
aP2	Goat polyclonal	Santa Cruz Biotechnology, Inc.	SC-18661
Leptin	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-842
AMPK	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-25792
pAMPK	Rabbit polyclonal	Cell Signaling Technology, Inc.	2535
ACC	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-30212
pACC	Rabbit polyclonal	Cell Signaling Technology, Inc.	3661
Actin	Mouse monoclonal	Santa Cruz Biotechnology, Inc.	SC-47778

were added to each well. After around two minutes, hemocytometer was used to count the live cells in the sample under an inverted microscope and the relative values of cell counts were compared. As an alternative way to measure cell viability, the cells were prepared in the same method described above, then after removing the medium, 0.5 mg/ml tetrazolium bromide salt (MTT, Amresco, Solon (OH) USA) was aliquoted and the cells were cultured in complete darkness in a CO_2 incubator. After 3 h, MTT reagent was removed and an adequate amount of DMSO was aliquoted to the wells to dissolve the produced formazin completely. The cells were then transferred to a 96-well plate by 200 µl each, to measure the OD at 540 nm using the ELISA reader (Molecular Devices, Sunnyvale (CA) USA).

Oil Red O staining and triglyceride quantification

Oil red O staining is a dye binding method used to quantify total lipids. The lipid-specific binding allows frequency analysis of the lipids produced by adipocytes. Thus, Oil red O staining was carried out to examine the effects of WETJ on the lipid droplets produced inside 3T3-L1 adipocytes following the differentiation induction. For the control and the 3T3-L1 adipocytes treated with varying concentrations of WETJ, the medium was removed and the cells were washed with PBS then fixed for 1 h using 3.7% formalin. After fixing, the cells were washed with 60% isopropanol and treated with Oil Red O solution for the staining at room temperature for 20 min. With the completion of staining, Oil Red O solution was removed and the cells were washed with distilled water three times. The stained cells were observed under an inverted microscope, and images were taken using the Axio Vision program. In addition, for quantitative analysis of the level of inhibition on WETJ-induced triglyceride production, the cells were treated with 100% isopropanol to dissolve and remove Oil Red O solution then transferred to a 96-well plate by 200 µl, after which the OD was measured at 500 nm using the ELISA reader.

Western blot analysis

To examine the effects of WETJ on the expression of adipogenic transcription factors and adipocyte-specific genes, western blot analysis was carried out. First, for the cells prepared in the same method as previously described, an adequate amount of lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM phenymethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)] was added and the reaction was left at 4°C for 1 h. After centrifugation at 14,000 rpm for 30 min, the total proteins in the supernatant were isolated. The concentration of proteins in the supernatant was quantified using the Bio-Rad protein quantification agent (Bio-Rad, Hercules (CA) USA) and following the method of use. By mixing with an equal amount of Laemmli sample buffer (Bio-Rad), the protein sample was prepared. The sample was then applied to electrophoresis using a sodium dodecyl sulphate (SDS)-polyacrylamide gel so that the proteins separated according to molecular weight, and by electro-blotting, the separated proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene (NH) USA). The protein-transferred nitrocellulose membrane was treated with 5% skim milk for the blocking of non-specific proteins, followed by the treatment with respective primary antibodies at 4 °C for 12 h, washing with PBS-T, and the treatment with secondary antibodies for each of the primary antibodies at room temperature for 1 h. When the reaction was complete, Enhanced Chemiluminescence (ECL) solution (Amersham Life Science Corp.) was added in a dark room and following the exposure to an X-ray film, the expression levels of specific proteins were analyzed.

Statistical analysis

All experimental data were expressed in mean ± standard deviation (SD) using the statistics program SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). For testing the statistical significance of each experimental group per analysis item, the Analysis of Variance (ANOVA) was performed, then the Student t-test was used for the verification at $*p<0.05$.

Results and Discussion

Effects of WETJ on cell growth in 3T3-L1 pre-adipocytes

To determine the effects of WETJ in 3T3-L1 pre-adipocytes with respect to cytotoxicity, the growth inhibition and cell viability of 3T3-L1 pre-adipocytes according to WETJ treatment were examined. First, to find out the level of growth inhibition caused by WETJ, the cultured cells were treated with varying concentrations of WETJ for 72 h, then the hemocytometer counts of live cells were compared. The result is presented in Figure 1A. As shown, no significant growth inhibition appeared up to 100 µg/ml treatment group but a dose-dependent increase in growth inhibition was observed from 120 µg/ml treatment group. To determine the effects of WETJ on the cell viability of 3T3-L1 pre-adipocytes under the same conditions described above, MTT assay was carried out and the result is presented in Figure 1B. For cell survival inhibition, likewise, WETJ did not induce a significant change up to 100 µg/ml treatment group but a strong inhibitory effect on cell survival was observed from 120 µg/ml treatment group. Based on these

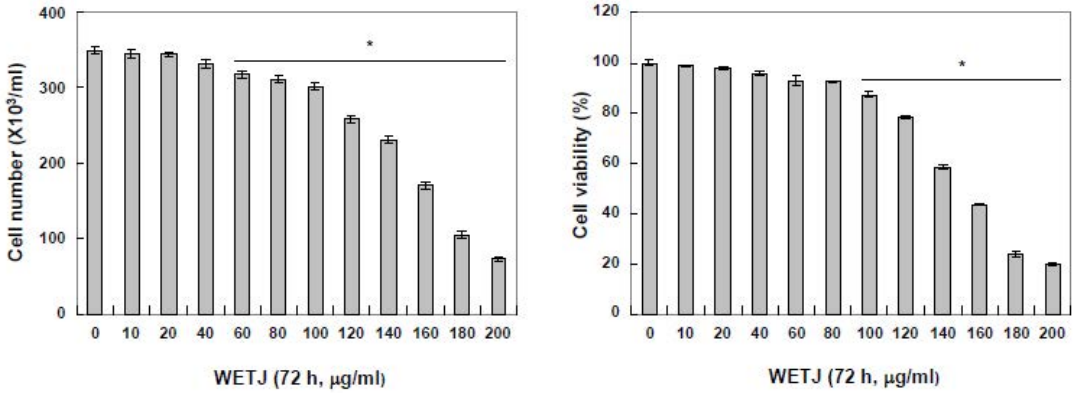

Figure 1: Effects of WETJ on the cell growth in 3T3-L1 mouse pre-adipocytes. Cells were treated with the indicated concentrations of WETJ for 72 hr. Cell number (A) and viability (B) were determined by hemocytometer counts of trypan blue excluding cells and MTT assay, respectively. The data are expressed as the mean ± SD of three independent experiments. The significance was determined by the Student's t-test (*$p<0.05$ vs. untreated control).

results, WETJ was determined to exhibit negligible cytotoxicity up to 100 μg/ml treatment group; hence, subsequent experiments were performed within a concentration range up to 100 μg/ml.

Effects of WETJ on the production of lipid droplets

The lipid droplets produced during the process of differentiation into adipocytes are in an inactive vesicle surrounded by a phospholipid monolayer. They begin to form when triglyceride and cholesterol ester accumulate in the bilayer of the endoplasmic reticulum so that the layers start to separate [16]. The lipid droplets in mature adipocytes are known to be controlled by the influx of triglycerides due to LPL and release of triglycerides mediated by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). Such lipid droplets have been reported to participate in the induction of obesity as well as metabolic diseases such as cancer, arteriosclerosis, and type II diabetes [17, 18]. Thus, to examine the effects of WETJ on the production of lipid droplets during adipogenesis in 3T3-L1 pre-adipocytes, the cells upon differentiation induction were treated with an adequate concentration of WETJ and the level of intracellular production of lipid droplets was observed under an inverted microscope before and after Oil red O staining. As the result in Figure 2 shows, lipid droplets were not produced when differentiation was not induced, but when MDI treatment induced the differentiation, intracellular production of lipid droplets was seen to have been activated. The so produced lipid droplets were substantially inhibited in a dose-dependent manner upon WETJ treatment. This indicated that WETJ inhibited the differentiation of 3T3-L1 pre-adipocytes into adipocytes.

Effects of WETJ on the production of triglycerides

Triglycerides are in the form of one glycerol molecule connected to three fatty acid molecules via an ester bond. They account for over 95% of fat ingested as food, and along with glucose, they are used as an important energy source by the cells [19]. However, an excess amount of triglycerides are absorbed and stored by adipocytes where they take a part in inducing obesity and thereby cause various diseases [20]. The triglyceride storage by adipocytes is known to be mediated by triglyceride-rich lipoproteins including chylomicron and very low density lipoprotein (VLDL) [21]. Thus, to examine the effects of WETJ on the production of triglycerides, the Oil Red O-stained lipid droplets were extracted using isopropanol and triglyceride content was measured. The result is presented in Figure 3. As shown, along the process of MDI-induced differentiation into adipocytes, triglyceride production markedly increased; however, WETJ treatment led to a gradual decrease in triglyceride production, until the group treated with a maximum concentration 100 μg/ml of WETJ displayed approximately 80% inhibition of triglyceride production. The result is indicative of the inhibitory effects of WETJ on the production of triglycerides, and such effects are thought to be correlated with the inhibition of lipid droplet formation.

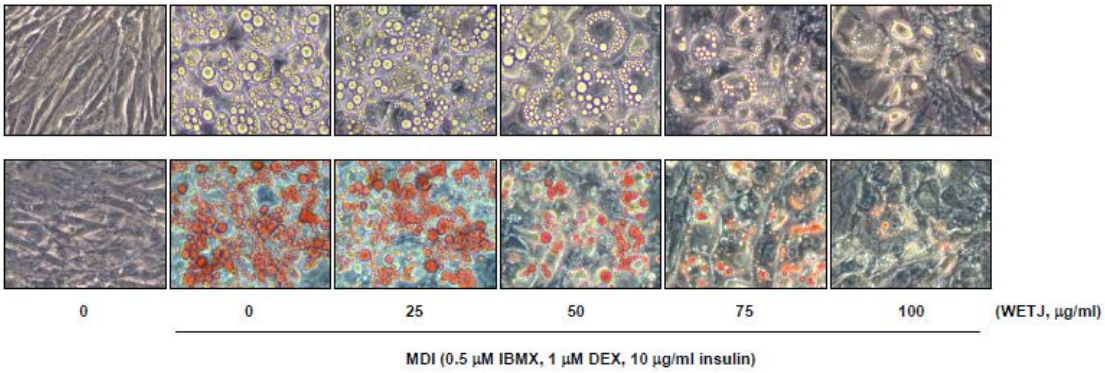

Figure 2: Effects of WETJ on the microscopic morphological changes and lipid droplet accumulation of differentiated 3T3-L1 mouse pre-adipocytes. Differentiation of confluent 3T3-L1 mouse pre-adipocytes was initiated with MDI (0.5 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin) and maintained DMEM-5% FBS medium (maintenance differentiation medium) in the absence or presence of WETJ for 8 days. (A) Differentiating 3T3-L1 cells were visualized by light microscopy. Magnification, ×200. (B) Cells were fixed and stained with Oil Red O to visualize lipid droplets by light microscopy. Magnification, ×200.

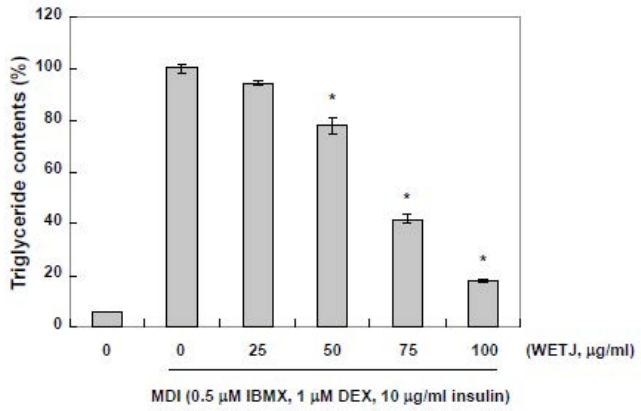

Figure 3: Inhibitory effects of WETJ on triglyceride accumulation of differentiated 3T3-L1 mouse pre-adipocytes. Triglyceride contents were determined by Oil Red O staining after treatment of the absence or presence of WETJ. The rates of triglyceride contents were measured at λ=500 nm wavelength by the ELISA reader. The data are expressed as the mean ± SD of three independent experiments. The significance was determined by the Student's t-test (*p<0.05 vs. untreated control).

Effects of WETJ on the expression of adipogenic transcription factors and adipocyte-specific genes

Myriads of adipogenic transcription factors are known to engage in each step of adipogenesis. First, the expression of C/EBPβ in early stage of differentiation promotes the expression of other adipogenic transcription factors C/EBPα and PPARγ so as to play a part in enhancing the characteristics of mature adipocytes such as insulin-sensitive glucose uptake. For SREBP1c whose expression is induced by insulin, a direct participation in PPARγ expression has been reported [22, 23]. The C/EBPα and PPARγ activated by the adipogenic transcription factors in early stage of differentiation are known as the key regulators of adipogenesis. The interaction between the two genes with a consequent synergistic effect completes the morphological characterization such as lipid droplet production and cell size increase and enhances the characteristics of adipocytes, through the expression of adipocyte-specific genes including aP2 and leptin [24, 25]. This study thus examined, on the protein level, the effects of WETJ on the expression of adipogenic transcription factors and adipocyte-specific genes in relation to adipogenesis inhibition. First, the expression level of adipogenic transcription factors according to WETJ treatment was determined, and as Figure 4A shows, substantially increased expression levels of PPARγ, C/EBPα, C/EBPβ and SREBP1c were observed when the differentiation was induced without WETJ treatment; however, upon WETJ treatment, a dose-dependent decrease in expression levels was induced. WETJ treatment was also shown to have significantly decreased the expression levels of adipocyte-specific genes aP2 and leptin as Figure 4B shows. The results collectively indicate that WETJ leads to the inhibition of adipogenesis through the inhibitory effects on the expression of adipogenic transcription factors, whereby the production of lipid droplets and triglycerides and the expression of adipocyte-specific genes were reduced.

Effects of WETJ on the inhibition of adipogenesis through the AMPK signaling pathway

AMPK, as a key regulator and energy sensor in the energy homeostasis, is known to be activated by phosphorylation caused by an increase in AMP/ATP ratio due increased level of intracellular AMP upon increased energy consumption, and by the activation of CaMKKβ caused by a change in intracellular Ca^{2+} level and LKB1, a serine/threonine protein kinase and a known tumor suppressor [26, 27]. The activated AMPK increases the level of GLUT4 in muscle cells to act on increasing the glucose transport while also increasing the lipid production in the body based on the inhibition of fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) in synthesizing fatty acids and of HMG-CoA reductase in limiting the cholesterol biosynthesis. In this way, AMPK is known to promote adipogenesis [28 – 30]. This study thus determined whether the AMPK signaling pathway was involved in WETJ-induced adipogenesis inhibition. First, the expression of ACC acting on AMPK and the step downstream was examined for changes, and as shown in Figure 5A, the phosphorylation of AMPK and ACC substantially increased. Based on the result, a role of AMPK activation in WETJ-induced adipogenesis inhibition was predicted. Next, suppressing the AMPK pathway through the pretreatment with compound C, an AMPK inhibitor, led to the inhibition of WETJ-induced phosphorylation of AMPK and ACC as shown in Figure 5B, while the expression of adipogenic transcription factors PPARγ, C/EBPα, C/EBPβ and SREBP1c were shown to have increased. What the results so far have indicated was a crucial role played by the AMPK activation and the subsequent reduced expression of adipogenic transcription factors in WETJ-induced adipogenesis inhibition. The findings of this study are anticipated to prove highly useful as valuable data for continuing research as they suggest a potential use of Hot-Water Extracts of *Trapa japonica* as an anti-obesity agent or drug. Nevertheless, an additional biochemical interpretation of the anti-obesity mechanism associated with energy metabolism and signaling system and an analysis of the active compounds contained in WETJ, seem essential in future studies.

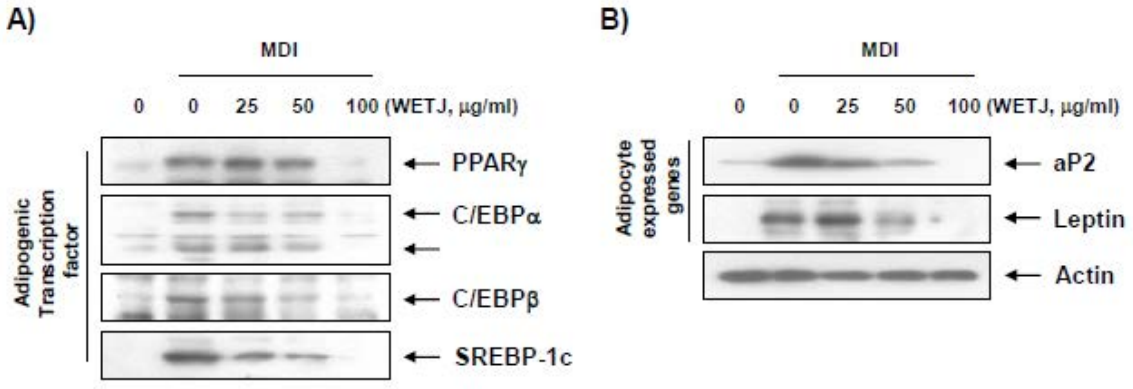

Figure 4: Effects of WETJ on the levels of adipogenic transcription factors (A) and adipocyte-specific genes (B) expression in differentiated 3T3-L1 mouse pre-adipocytes. Differentiation of confluent 3T3-L1 mouse pre-adipocytes was incubated with the absence or presence of WETJ for 8 days after initiated with MDI. Cells were lysed and cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

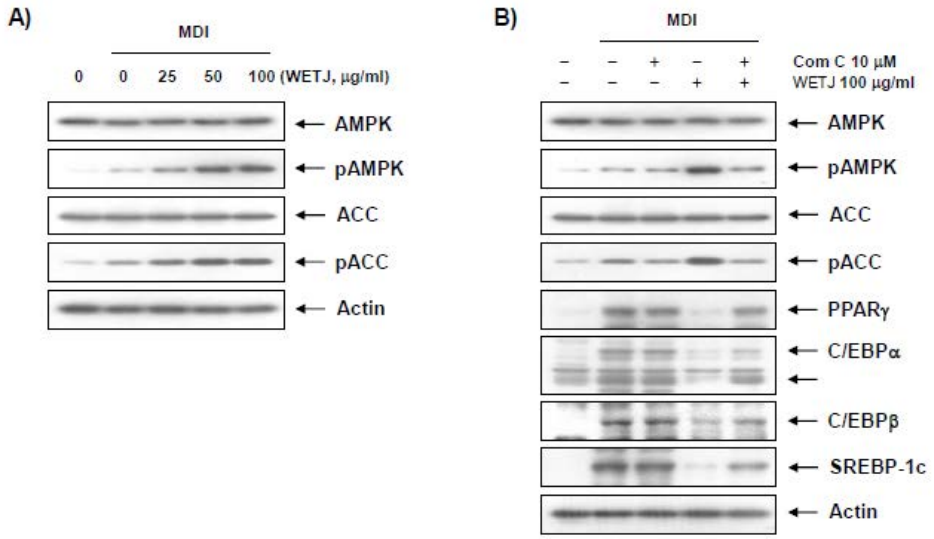

Figure 5: Roles of AMPK signaling pathway on the WETJ-induced inhibition of adipogenesis in differentiated 3T3-L1 mouse pre-adipocytes. (A) Effects of WETJ on AMPK and ACC phosphorylation in 3T3-L1 cells. Confluent cells were treated with various concentrations of WETJ. (B) Effects of AMPK inhibitor, Compound C, on the levels of adipogenic transcription factors in 3T3-L1 cells. Cells were pre-treated with compound C for 1 h, and then treated with 100 μg/ml of WETJ. On day 8, completely differentiated cells were lysed and cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

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