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Original Article

Suppression of Lipid Accumulation in Differentiating 3T3-L1 Preadipocytes by a Standardized Commercial Juknyeok

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죽력의 3T3-L1 지방전구세포에서 지방축적 억제 효과

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Objectives: Juknyeok (JN) is natural liquor extracted from bamboo stems (*Phyllostachys bambusoides*) and has been used as a traditional Korean medicine for improving vascular function, blood glucose, and treating stroke. Until now, the JN's lipid-lowering effect and underlying mechanism in adipocytes are poorly understood. The aim of this study was to scrutinize the effect of a standardized commercial JN on lipid accumulation during the differentiation of 3T3-L1 preadipocytes.
Methods: Lipid and triglyceride (TG) accumulation in differentiating 3T3-L1 preadipocytes were measured by Oil Red O staining and AdipoRed assay, respectively. Cell count analysis was used to ascertain 3T3-L1 cytotoxicity. Immunoblotting and Reverse transcription polymerase chain reaction analysis were used to assess protein and messenger RNA (mRNA) expression levels in 3T3-L1 cells, respectively.
Results: Treatment with JN at 25 μl/ml after pH calibration with 6.35 significantly reduced lipid and TG accumulation in differentiating 3T3-L1 preadipocytes without significant cytotoxicity. On mechanistic levels, JN markedly suppressed protein expression levels of CCAAT/enhancer-binding protein (C/EBP)-β and fatty acid synthase (FAS) during the differentiation of 3T3-L1 preadipocytes. However, JN did not affect the protein expression levels of C/EBP-α, peroxisome proliferator-activated receptor-β/γ, and phosphorylation levels of signal transducer and activator of transcription-3/5 in differentiating 3T3-L1 preadipocytes. JN also reduced leptin mRNA expression levels in differentiating 3T3-L1 preadipocytes.
Conclusions: JN at 25 μl/ml lowers lipid accumulation and TG content in differentiating 3T3-L1 cells, mediated through the reduced expression levels of C/EBP-β and FAS.

Key Words: Juknyeok, CCAAT/enhancer-binding protein-beta, Fatty acid synthases, Leptin, 3T3-L1 cells

Introduction

Obesity is a major risk factor for developing a variety of chronic diseases including cardiovascular disease, gastrointestinal disorders, type 2 diabetes, joint and muscular disorders, respiratory problems, and psychological issues[1]. Obesity is featured by an increase in the number and size of (pre)adipocytes in adipose tissue[2]. Adipogenesis is a complex process that elaborates preadipocyte proliferation and adipocyte maturation in response to different stimuli and conditions[2]. Adipose tissue responds to the stimulation of extra nutrients via the hyperplasia and hypertrophy of (pre)adipocytes[3]. Given

that the adipocyte hypertrophy is achieved through the differentiation of preadipocytes into adipocytes that are rounded and filled with many lipid droplets (LDs) and excessive preadipocyte differentiation leads to the development of obesity, any substance that inhibits lipid accumulation during preadipocyte differentiation may have the potential to be an effective anti-obesity material.

The diversity of information indicates that several adipogenic transcriptional factors, such as CCAAT/enhancer-binding proteins (C/EBPs) family, peroxisome proliferator- activated receptors (PPARs), and signal transducer and activator of transcription (STAT) proteins, have the key roles in adipogenesis[4]. It is further illustrated that fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and perilipin A are also essential in preadipocyte differentiation by regulating lipogenesis and LDs maturation/stabilization[5-7].

Currently, research to discover new anti-obesity material(s) from natural products that are safe to the human body has been increasing. We have recently used several natural products including Juknyeok (JN) from different sources to screen new anti-obesity substance(s) using 3T3-L1 murine white preadipocytes, and found that JN products differentially regulates lipid accumulation in 3T3-L1 cells. JN is a natural product derived from the stems of bamboo (*Phyllostachys bambusoides*), a traditional Korean medicine, and has been widely used for improving vascular function, speech impairment, blood glucose, and also treating stroke[8]. In the last few years, several in vitro and in vivo studies have further demonstrated that JN has anti-oxidant, anti-melanogenic, and anti-obesity effects[9-11]. However, up to date, the JN's lipid-lowering effect and mode of action in adipocytes are not fully understood.

The aim of this study was to investigate the effect of a standardized commercial JN on lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes. The present study demonstrates, for the first time, that JN at 25μl/ml vastly reduces lipid accumulation and triglyceride (TG) content in differentiating 3T3-L1 preadipocytes, which is mediated through control of the expression of C/EBP-β and FAS.

Materials and Methods

1. Materials

The pH of JN used in this study was 6.35 after calibration (the original pH of JN before calibration was 3.3). Primary antibodies for anti-C/EBP-α, anti-C/EBP-β, anti-phospho (p)-STAT-3, anti-STAT-3, anti-p-STAT-5, anti-STAT-5, PPAR-γ and PPAR-β were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The primary FAS antibody was bought from BD Bioscience (San Jose, CA, USA). Primary antibodies for perilipin A and β-actin were obtained from BioVision (Milpitas, CA, USA) and Sigma Aldrich Co., Ltd. (St. Louis, MO, USA), respectively.

2. Differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were cultured in growth media containing Dulbecco's Modified Eagles' Medium (DMEM) (Welgene, Daegu, Korea) containing 10% heat-inactivated fetal calf serum (Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (Welgene) at 37°C in a humidified atmosphere of 5% CO_2. 3T3-L1 preadipocytes differentiation was induced by changing the medium to DMEM containing 10% fetal bovine serum (Welgene), 0.5 mM IBMX (M) (Sigma Aldrich Co.), 0.5 μM dexamethasone (D) (Sigma Aldrich Co.), and 5 μg/ml insulin (I) (Sigma Aldrich Co.) either with or without JN. On day 2, the first differentiation medium was replaced with DMEM supplemented with 10% fetal bovine serum (FBS) and 5 μg/ml insulin either with or without JN at the indicated doses for additional 3 days. The cells were further fed with DMEM containing 10% FBS in the presence or absence of JN for additional 3 days.

3. Oil Red O staining

On day 8, post-differentiation induction, control or JN-treated 3T3-L1 cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde for 2 h. Eventually, cells were washed with 60% isopropanol and dried. Oil Red O working solution was added to the fixed cells for 1 h and then washed with distilled water. Afterward, LDs were viewed under light microscopy (TS100; Nikon, Tokyo, Japan).

4. Cell count analysis

On day 8, post-differentiation induction, control or JN-treated 3T3-L1 cells were stained with trypan blue dye. Only cells with intact membranes can constructively exclude the dye, then dead cells with damage membranes become stained and counted using a light microscope. The cell count assay was done in triplicates. Data are mean±standard error (SE) of three independent experiments.

5. AdipoRed assay

AdipoRed Assay Reagent kit was used for assessing intracellular TG content and it was done according to the company's instructions (Lonza, Basel, Switzerland). After 10-min incubation, the plates were placed in a Victor[3] (Perkin Elmer, Waltham, MA, USA), and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm.

6. Preparation of whole-cell lysates

At the designated time point, 3T3-L1 cells were washed with PBS and lysed in a modified radioimmunoprecipitation assay buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, proteinase inhibitor cocktail [1x]). The whole-cell lysates were collected and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was saved, and protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

7. Western blot analysis

50 mg total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and then transferred to nitrocellulose membranes (Millipore, Burlington, MA, USA). The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween 20 (TBST), and blocked with blocking buffer (TBST containing 5% [w/v] non-fat dried milk). The membranes were incubated overnight with corresponding primary antibodies for C/EBP-α (1:1,000), C/EBP-β (1:1,000), PPAR-γ (1:1,000), PPAR-β (1:1,000), p-STAT-3 (1:1,000), STAT-3 (1:1,000), p-STAT-5 (1:1,000), STAT-5 (1:1,000), FAS (1:1,000) or β-actin (1:10,000) at 4°C. The membranes were washed with TBST and then incubated with secondary antibodies coupled to horseradish peroxidase for 2 h. The membranes were then washed with TBST. Enhanced chemiluminescence reagents was used to develop the image (Advansta, San Jose, CA, USA). Equal loading of proteins was verified by β-actin antibody.

8. Reverse transcription polymerase chain reaction (RT-PCR) analysis

At the designated time point, the total RNA from control or JN-treated 3T3-L1 cells was extracted using RNAiso Plus (TaKaRa, Kusatsu, Japan). Random hexadeoxynucleotide primer and reverse transcriptase were used for reverse transcribed the total RNA (3 mg). The single-strand cDNA was amplified by PCR with primers of leptin sense 5'-CCAAAACCCTCATCAAGACC-3'; antisense 5'-CTCAAAGCCACCACCTCTGT-3'; actin sense 5'-GGTGAAGGTCGGTGTGAACG-3'; antisense 5'-GGTAGGAACACGGAAGGCCA-3'. Expression levels of actin messenger RNA (mRNA) were used to evaluate the relative mRNA expression of leptin.

9. Statistical analysis

Cell count analysis was measured in triplicate and repeated three times. The results were expressed as mean±SE. One-way analysis of variance was used to compare the difference significance. All significance testing was established on a p value of <0.05. The statistical software used in this study was the IBM SPSS statistics 25 software (IBM Co., Armonk, NY, USA).

Results

1. JN reduces intracellular lipid accumulation during 3T3-L1 preadipocyte differentiation in a concentration-dependent manner

The experimental scheme for 3T3-L1 preadipocyte differ-

entiation is depicted in Fig. 1A. In this study, the pH of original and adjusted JN were 3.3 and 6.35, respectively. Due to limited studies regarding the use of JN for its anti-obesity effect, the effects of two different concentrations (50 and 100 μl/ml) of the original and pH adjusted JN on lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes were initially investigated by a phase-contrast microscopic observation. Of note, the JN with adjusted pH at 50 μl/ml vastly suppressed lipid accumulation during 3T3-L1 preadipocyte differentiation with low cytotoxicity (data not shown). On the other hand, although the original JN at 50 and 100 μl/ml also greatly inhibited lipid accumulation during 3T3-L1 preadipocyte differentiation, they were highly cytotoxic to the cells. Because of this, the JN with adjusted pH (called JN thereafter) at 50 μl/ml was chosen for further works. To next see the lipid-lowering effect of JN, 3T3-L1 preadipocytes were grown in the differentiation induction media in the absence or presence of JN at different concentrations (0, 10, 25, and 50 μl/ml) for 8 days, followed by measurement of intracellular lipid deposition in the control or JN-treated 3T3-L1 cells by using Oil Red O staining. Of note, as shown in Fig. 1B (upper panels), without JN treatment, there was a high deposition (above >50%) of LDs on day (D)8 of differentiation in 3T3-L1 cells compared with undifferentiated cells at D0. However, treatment with JN resulted in a concentration-dependent reduction of intracellular LDs on D8 of differentiation in 3T3-L1 cells. It was shown that JN treatment at 10 μl/ml caused about 25-50% reduction of intracellular LDs, whereas JN at 25 and 50 μl/ml led to below 25% reduction of those in 3T3-L1 cells on D8 of differentiation compared with undifferentiated cells at D0. The JN's suppressive effects on intracellular LDs in 3T3-L1 cells on D8 of differentiation were also confirmed by phase-contrast images (Fig. 1B, lower panels).

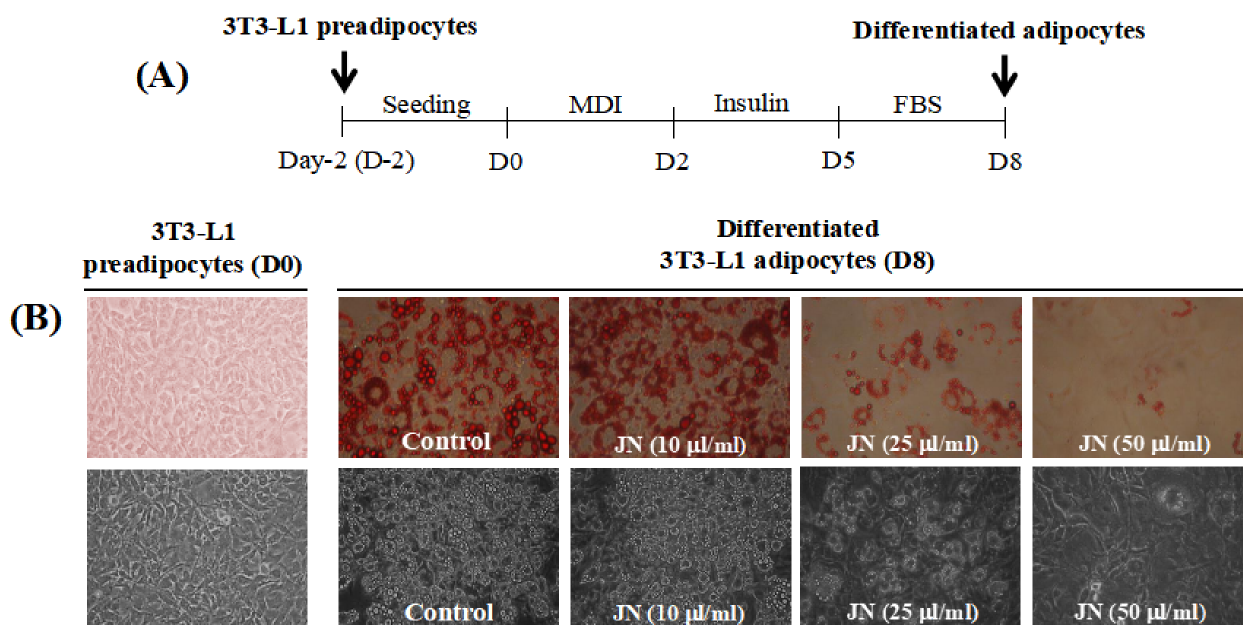

Fig. 1. Effects of JN at different concentrations on intracellular lipid accumulation in differentiating 3T3-L1 cells. (A) The experimental scheme of 3T3-L1 preadipocytes differentiation. (B) 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS in the presence or absence of JN at the indicated concentrations for 8 days. On D8, the intracellular LDs were assessed by Oil Red O staining (upper panels in [B]). Phase-contrast images of the control or JN-treated cells on D8 were also taken (lower panels in [B]). JN: Juknyeok, MDI: 0.5 mM IBMX, 0.5 μM dexamethasone, and 5 μg/mL insulin, FBS: fetal bovine serum, D: day, LD: lipid droplets.

2. JN at 25 μl/ml significantly reduces intracellular TG content in differentiating 3T3-L1 preadipocytes without significant cytotoxicity

AdipoRed assay was next carried out to investigate whether the JN also reduces intracellular lipid (TG) content during 3T3-L1 preadipocyte differentiation. To this end, 3T3-L1 preadipocytes were grown in the differentiation induction media with or without of JN at different concentrations for 8 days. As shown in Fig. 2A, JN treatment also led to a dose-dependent decrease in the intracellular TG content in 3T3-L1 cells on D8 of differentiation. Next, a cell count assay was performed to study whether JN at the doses tested has cytotoxicity in 3T3-L1 cells. As shown in Fig. 2B, treatment with the JN at 10 and 25 μl/ml had no cytotoxicity in 3T3-L1 cells on D8 of differentiation, but the JN at 50 μl/ml was significantly cytotoxic to these cells. These results indicate that the strong lipid-lowering effect of the JN at 50 μl/ml might be due to its cytotoxicity. Thus, in consequence of strong inhibitory effects on lipid accumulation and TG content with no significant cytotoxicity, the concentration of 25 μl/ml of JN was selected for further studies.

3. JN at 25 μl/ml significantly reduces protein expression levels of C/EBP-β in differentiating 3T3-L1 cells

To next determine molecular mechanisms underlying the JN's lipid-lowering effects, 3T3-L1 preadipocytes were grown in the differentiation induction media in the absence or presence JN at 25 μl/ml for 8 days, followed by measurement of protein expression and phosphorylation levels of known adipogenesis-related transcription factors in control or JN-treated 3T3-L1 cells by using Western blot analysis. Distinctly, as shown in Fig. 3, while treatment with JN had no effect

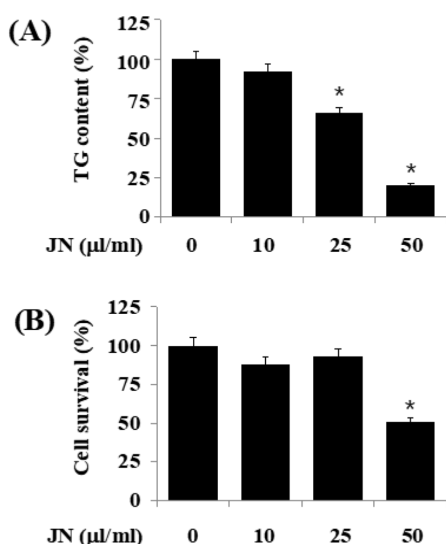

Fig. 2. Effects of JN at different concentrations on intracellular TG content and cell growth in differentiating 3T3-L1 cells. (A, B) 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS in the presence or absence of JN at the indicated concentrations for 8 days. On D8, intracellular TG content was measured by AdipoRed assay (A). Values are mean±standard error of data from three independent experiments with triplicate. *P<0.05 vs control. On D8, the control or JN-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, were counted under a microscope (B). The cell count assay was done in triplicates. Data are mean±SE of three independent experiments. *P<0.05 vs control. JN: Juknyeok, TG: triglyceride, MDI: 0.5 mM IBMX, 0.5 μM dexamethasone, and 5 μg/mL insulin, FBS: fetal bovine serum, D: day, LD: lipid droplets.

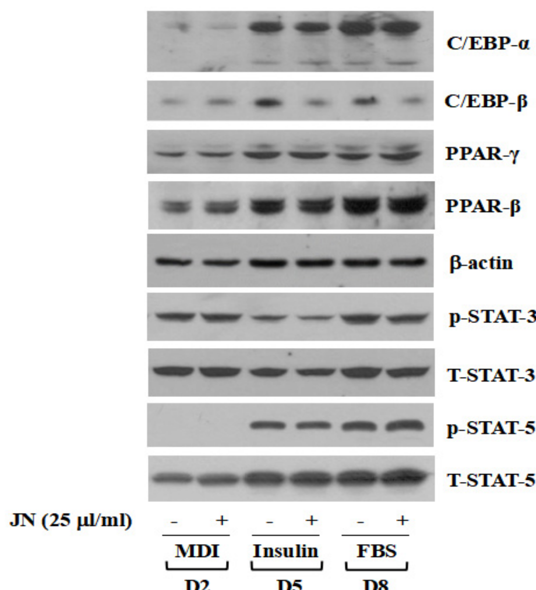

Fig. 3. Effects of JN at 25 μl/ml on expression and phosphorylation levels of C/EBP-α, C/EBP-β, PPAR-γ, PPAR-β, STAT-3, and STAT-5 in differentiating 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS in the presence or absence of JN at 25 μl/ml, and harvested at D2, D5, and D8, respectively. Whole-cell lysates at the indicated time point were extracted and analysed by Western blot analysis with respective antibodies. p-STAT-3, phosphorylated STAT-3; T-STAT-3, total STAT-3; p-STAT-5, phosphorylated STAT-5; T-STAT-5, total STAT-5. JN: Juknyeok, C/EBP: CCAAT/enhancer-binding proteins, PPAR: peroxisome proliferator-activated receptors, STAT: signal transducer and activator of transcription, MDI: 0.5 mM IBMX, 0.5 μM dexamethasone, and 5 μg/mL insulin, FBS: fetal bovine serum, D: day.

on protein expression levels of C/EBP-α in 3T3-L1 cells on D2, D5, and D8 of differentiation, it highly reduced C/EBP-β protein expression levels. Treatment with JN also did influence not only the protein expression levels of PPAR-β and PPAR-γ but also the protein phosphorylation levels of STAT-3 and STAT-5 in 3T3-L1 cells on D2, D5, and D8 of differentiation. Total expression levels of STAT-3, STAT-5, and β-actin proteins remained constant under these experimental conditions.

4. JN at 25 μl/ml down-regulates FAS protein and leptin mRNA expression levels in differentiating 3T3-L1 cells

Next, the JN's regulation of protein expression levels of FAS, a lipogenic enzyme, in 3T3-L1 cells on D2, D5, and D8 of differentiation was investigated. As shown in Fig. 4A, in the absence of JN, there was a time-dependent increase in the FAS protein expression levels during 3T3-L1 preadipocyte differentiation on D2, D5, and D8. However, JN at 25 μl/ml greatly suppressed protein expression levels of FAS in 3T3-L1 cells on D5 and D8 of differentiation. The ability of JN at 25 μl/ml to regulate mRNA expression levels of leptin, one of adipokines, during 3T3-L1 preadipocyte differentiation on D2, D5, and D8 was also evaluated by using RT-PCR. As shown in Fig. 4B, treatment with JN led to substantial reduction of leptin mRNA levels in differentiating 3T3-L1 cells on D2. However, JN treatment did not affect expression levels of leptin mRNA in differentiating 3T3-L1 cells on D5 and D8. Protein and mRNA expression levels of control b-actin remained unchanged under these experimental conditions.

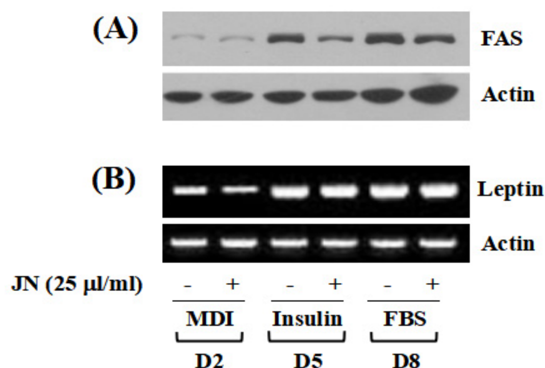

Fig. 4. Effects of JN at 25 μl/ml on protein and mRNA expression levels of FAS and leptin in differentiating 3T3-L1 cells. (A, B) 3T3-L1 preadipocytes were induced to differentiate with an induction medium containing MDI, insulin, and FBS in the presence or absence of JN at 25 μl/ml, and harvested at D2, D5, and D8, respectively. Whole-cell lysates and total cellular mRNA at the indicated time point were extracted and analysed by Western blot (A) and RT-PCR (B) analysis, respectively. JN: Juknyeok, mRNA: messenger RNA, FAS: fatty acid synthase, MDI: 0.5 mM IBMX, 0.5 μM dexamethasone, and 5 μg/mL insulin, FBS: fetal bovine serum, D: day, RT-PCR: reverse transcription polymerase chain reaction.

Discussion

Mature adipocytes in the white adipose tissue (WAT) store excess energy in the form of lipids (mainly TG) through preadipocyte differentiation process. Evidence strongly illustrates that excessive preadipocyte differentiation leads to hypertrophic adipocytes, contributing to an abnormal lipid accumulation and expansion in the WAT and further the development of obesity[12]. Besides from energy storage, adipocytes release a wide range of cytokines called adipokines that play crucial roles in (patho)physiology[13]. Therefore, any inhibitor of excessive preadipocyte differentiation and adipokine production may have the potential as an anti-obesity agent.

Aforementioned, JN is a traditional Korean medicine that has been known over generations to improve and treat numerous symptoms, including vascular function, speech impairment, blood glucose, and stroke[8]. JN comes in a form of liquid condensed from the steam resulting from heating bamboo at a very high temperature in an airtight vessel and is reported to have anti-inflammatory, anti-apoptotic, and anti-obesity properties[8]. JN used in this study is a standardized commercial bamboo stem vinegar. However, at present, the anti-obesity effect and mode of action of JN in fat cells are still poorly understood. The present study demonstrates, for the first time, that JN at 25 μl/ml has strong anti-adipogenic effect on differentiating 3T3-L1 cells, which is mediated through control of the expression levels of C/EBP-β and FAS.

It has been previously demonstrated that bamboo stem extract or JN consumption reduces lipid profiles levels, exhibiting its lipid-lowering effect[9]. In agreement with it, treatment with JN at 25 μl/ml herein vastly inhibits intracellular

lipid accumulation and TG content with no cytotoxicity in 3T3-L1 cells on D8 of differentiation, as determined by Oil Red O staining, AdipoRed assay, and cell count analysis. These results advocate that JN at 25 μl/ml has strong lipid-lowering effects during 3T3-L1 preadipocyte differentiation.

Mounting evidence indicates that the differentiation of 3T3-L1 preadipocytes into adipocytes is mostly impacted by the expression and activation of multiple adipogenesis-related transcription factors, including C/EBP-α, C/EBP-β, PPAR-α, PPAR-γ, STAT-3, and STAT-5[4,14-19]. Numerous studies have further indicated that the expression of C/EBP-β and C/EBP-δ occurs at early stage of preadipocyte differentiation and leads to the induction of the expression of C/EBP-α and PPAR-γ, which are the central positive regulators of middle and late stages of adipogenesis[4,14-19]. PPAR-γ can induce adipogenesis in C/EBP-α deficient mouse embryonic fibroblasts. Meanwhile, C/EBP-α is unable to promote adipogenesis in the absence of PPAR-γ[19]. This finding suggests that C/EBP-α and PPAR-γ contribute to a single pathway of adipogenesis, where PPAR-γ is a lead factor[19]. It also has been demonstrated that the expression and phosphorylation of STAT family members are increased during 3T3-L1 preadipocyte differentiation, and the hyperphosphorylation of STAT-3 is important for early stage of the cell differentiation[18,20-23]. In addition to these, there is a wealth of information illustrating that FAS, a lipogenic enzyme involved in fatty acid synthesis, has an essential part in lipid accumulation and TG synthesis during 3T3-L1 cell differentiation[5-7]. However, up to date, the JN regulation of C/EBP-α, C/EBP-β, PPAR-α, PPAR-γ, STAT-3, STAT-5, and FAS in adipocytes is unknown. Of importance, the present study has demonstrated the ability of JN at 25 μl/ml to selectively down-regulate the protein expression levels of C/EBP-β and FAS in differentiating 3T3-L1 cells on D5 and D8. These results thus point out that the JN's lipid-lowering effect on differentiating 3T3-L1 cells is not through regulation of the expression and phosphorylation levels of PPAR-γ, PPAR-β, STAT-3, and STAT-5 but via the reduced expression levels of C/EBP-β and FAS.

To my best knowledge, it is the first reporting the JN's lipid-lowering mechanism through down-regulation of C/EBP-β and FAS in fat cells. Of note, there is a previous study addressing that bamboo vinegar decreases inflammatory mediator expression by inhibiting reactive oxygen species (ROS) production and protein kinase C-α/δ activation[24]. It also has been shown that bamboo extract inhibits the palmitic acid-induced increase in interleukin-6 secretion by an adipose cell line, which is mediated through inactivation of nuclear factor-kappa B and activator protein 1[25]. Given that adipocyte hypertrophy and the resultant adipose tissue expansion is also related with abnormal production of inflammatory mediators and ROS generation and hyperactivation of signaling proteins and kinases[3], it will be interesting to test, in future, whether JN interferes with production and expression of these markers, which may further provide new molecular, cellular, and signaling mechanisms and factors underlying the JN's anti-obesity (lipid-lowering) effect in adipocytes.

Accordingly, differentiating and mature adipocytes in adipose tissue secrete a variety of adipokines, including leptin[26]. Of note, a number of in vitro and in vivo studies have reported the role of leptin in regulating body weight[27]. Moreover, it is further established that mice lacking the gene encoding leptin are very obese and diabetic[28,29]. As a result, decreased leptin expression is thought to be an alternative target as opposed to obesity and its associated diseases. In the present study, it is also of importance demonstrated that the JN at 25 μl/ml substantially inhibits leptin mRNA expression in differentiating 3T3-L1 cells on D2. These results may further suggest that the JN may be used to prevent or treat obesity and related diseases where leptin overexpression is problematic.

It is documented that JN is acidic with a pH of 2.5 to 2.8 by nature[30] and the JN's biological and therapeutic effects might be closely related to its acidic characteristic[31]. Supporting this, the pH of a standardized commercial JN is 3.3. However, because of strong cytotoxicity of the original JN due to its low pH (3.3), in this study, it was inevitably necessary to adjust the pH of JN with 6.35 for in vitro experiments to evaluate the adjusted JN's lipid-lowering effect with no cytotoxicity. Although the pH adjusted JN used herein has strong anti-adipogenic effect on adipocytes, it should be noted that this may be a critical change that can impair or reduce the effi-

cacy of the herb extract clinically.

It is known that JN is composed of acetic acid, butyric acid, and many other organic components, which contribute to its biological effects[8,30]. It is worthy to state previous studies that butyric acid has anti-obesity effects in vitro and in vivo[32,33], though the effects are somewhat controversial. Until now, whether the JN's lipid-lowering effects in this study is also due to butyric acid is unknown. It will be interesting to analyze, in future, the major components in JN and the role of these in the JN's lipid-lowering effects in vitro and in vivo animal models.

Conclusions

The present study demonstrates firstly that JN strongly reduces lipid accumulation and TG content on differentiating 3T3-L1 cells, which are in part mediated through the reduced expression of C/EBP-β and FAS. This study suggests JN as an alternative material to prevent or treat obesity by targeting lipid accumulation and TG synthesis in fat cells.

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