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Original Article

Effect of modified Chung-Sim-Youn-Ja-Tang on anti-inflammatory and anti-lipogenesis in RAW 264.7 and 3T3-L1 cells

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Abstract: Jea-Ma-Chung-Sim-Youn-Ja-Tang (JCST), one of herbal formulae, is modified prescription base on Chung-Sim-Youn-Ja-Tang (CST) which treats cerebrovascular disease for the Tae-Eum-In (TE). This study was designed to determine the anti-inflammatory and anti-adipogenesis effect of CST and JCST in vitro. CST and JCST of various concentrations were added in RAW 264.7 and 3T3-L1 cell. To determine the anti-inflammatory and anti-adipogenesis effects of CST and JCST, the PGE_2 production was measured by lipopolysaccharide (LPS) treated RAW 264.7 cell. The adipocytes was determined by Oil red O staining, triglyceride (TG) production, leptin level and the protein expressions of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding proteins alpha (C/EBPα) and fatty acid binding protein 4 (FABP4) in 3T3-L1 adipocytes. Our results showed that treatment with JCST significantly decreased PGE_2 production on RAW 264.7 cell and suppressed adipocyte differentiation, lipid accumulation, TG production, leptin contents and the protein expressions of PPARγ, C/EBPα and FABP4 on 3T3-L1 adipocytes compared to CST without affecting cell viability. In conclusions, our results suggest that JCST may be useful to inhibit the effect on lipid metabolism compared to CST, and regulates lipogenesis effectively. Therefore, our data provides scientific evidence to support the clinical use of JCST in the treatment of cerebrovascular diseases such as stroke in the TE.

Keywords: Jea-Ma-Chung-Sim-Youn-Ja-Tang, Chung-Sim-Youn-Ja-Tang, anti-inflammatory, anti-adipogenesis, herbal medicine

Introduction

The prevalence of adult diseases associated with metabolic syndrome is increasing mainly due to westernized menu, aging of the society and decreased physical activities. Cerebrovascular disease, which is mainly caused by atherosclerosis, the risk of which can be significantly increased by MS, is the cause of 9.6% of all deaths in Korea, with an incidence of 50.3 per 100,000 people [1-3].

There is many research of constitution related with disease in Korea. One of them, Sasang Constitution, a medical theory that explains the constitution of the body for a Korean, was established by Jae-ma Lee. In this theory, the human body can be classified into one of four categories, namely Tae-Yang-In (TY), Tae-Eum-In (TE), So-Yang-In (SY), and So-Eum-In (SE), based on an individual's body shape, facial appearance, manner of handling tasks, and personality, in order to explain a person's organ physiology, and pharmaco- and patho-physiology [4, 5]. Several studies have shown that the prevalence of cerebrovascular disease and certain chronic diseases can depend on these constitutional types. In one study, the prevalence of MS was shown to be 46.3% in the TE group; 16.8% in the SY group; and 9.1% in the SE group in Korea. In addition, another study, in which data for adults aged between 40 and 70 were analyzed in 2006, reported the highest prevalence of MS as 43.5% in the TE group, compared to 21.1% in the SY group and 12.1% in the SE group [6, 7]. Among the four constitutional types, TE is characterized by the largest body structure, weakness of energy release,

Table 1. Composition of Jae-Ma-Chung-Sim-Youn-Ja-Tang (JCST) and Chung-Sim-Youn-Ja-Tang (CST)

Crude Drug	Componented crude drugs (g)	
Latin name	JCST	CST
Chrysanthemi Flos		6
Raphani Semen		4
Liriopis Tuber	8	8
Thujae Semen	4	6
Amomi Fructus		6
Dioscoreae Rhizoma	8	8
Zizyphi Semen	4	4
Acori Graminei Rhizoma	4	
Massa Medicata Fermentata		6
Nelumbinis Semen	8	6
Schizandrae Fructus		4
Longanae Arillus	4	1
Polygalae Radix	8	1
Asparagi Tuber	8	1
Scutellariae Radix	4	3
Total (g)	60	64
Yield (%)	22.23	26.66

and a tendency toward internal energy deposition, with the highest prevalence of stroke and obesity [8].

Chung-Sim-Youn-Ja-Tang (CST) is one of the most representative prescriptions for treating cerebrovascular disease for the TE [4]. Studies on its effects have been performed on various illnesses such as obesity [9], atherosclerosis [10], allergy [11], stress [12] and so forth. Jae-Ma-Chung-Sim-Youn-Ja-Tang (JCST) is currently in use in clinical treatment, as a modified prescription based on CST. Although there have been diverse reports on the pharmacological efficacy of CST, there is still a limited amount of data on the efficacy of JCST. Therefore, we aimed to evaluate the pharmacological efficacy of JCST by comparing it with that of CST. Considering the common diseases associated with TE group, we investigated the anti-inflammatory and anti-adipogenesis effect of JCST in the mouse fibroblast cell line 3T3-L1 and the murine macrophage cell line Raw 264.7.

Materials and methods

Materials

RAW 264.7 and 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbeco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (P&S), new bovine calf serum (NCS), Dulbeco's phosphate-buffered saline (DPBS), trypsin-EDTA were purchased from Gibco BRL. (NY, USA). Dimethyl sulfoxide (DMSO), formaldehyde, dexamethasone, 3-isobutyl-1-methylisobutylxanthine (IBMX), Insulin, lipopolysaccharide (LPS), indomethacine, Triton X-100, Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Antibodies against fatty acid binding protein 4 (FABP4), proliferator-activated receptor gamma (PPARγ) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies against β-actin and CCAAT/enhancer binding proteins alpha (C/EBPα) were purchased from Santa Cruz Biotechnology Inc. (CA, USA). The secondary antibody anti-mouse and anti-rabbit which attached to horse radish peroxidase were purchased from Bio-Rad Laboratories Inc. (PA, USA).

Preparation of herbal formula

Herbal plants were purchased from Omniherb (Daegu, Korea). In our study, we prepared JCST and CST with combination of 10~14 different types of herbal plant (**Table 1**). The mixture of herbs was subjected to freeze dried to make powder. Further, 12.5 g from lyophilized powder of JCST and CST was placed in 125 ml of distilled water and then mixed properly. 50 ml conical tubes containing sample were centrifuged at 3,000×g for 20 min. The extraction was repeated three times. The extracts were then filtered through a filer paper. The filtrates were collected and final concentration of the extract was calculated as 100 mg/ml. Prepared JCST and CST were stored at -70°C.

Cell culture and cell cytotoxicity

RAW 264.7 cells were grown in DMEM (containing 5.5% FBS, 1% P&S); 3T3-L1 mouse fibroblast cells were grown in DMEM (containing 10% NCS, 1% P&S) at 37°C in a humidified atmosphere of 5% CO_2. The cell viability was examined by CCK-8. RAW 264.7 and 3T3-L1 cells were seeded at 3×10^3 and 8×10^2 cells in 96-well plates. After overnight, cells were treated of various concentrations (0.10, 20, 50, 100, 200, 500 and 1000 μg/mL) of JCST and CST. The absorbance was measured at 450 nm using a Benchmark Plus microplate reader

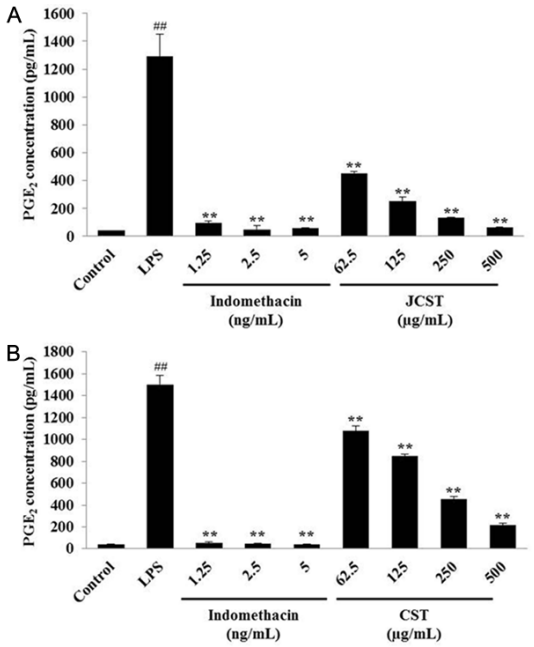

Figure 1. Inhibitory of PGE_2 for JCST and CST in LPS-stimulated RAW 264.7 cells. Cells were treated with absence or presence of various concentrations of JCST and CST with LPS (1 mg/mL) for 18 h. A: JCST and B: CST inhibited PGE_2 production in LPS-stimulated RAW 264.7 cells. The data are mean values of three experiments ± SEM; ##< 0.01 compared with control, *P < 0.05; **P < 0.01 compared with the LPS.

(Bio-Rad Laboratories Inc., PA, USA), and the percentages of viable cells were calculated.

PGE_2 inhibition

RAW 264.7 cells were seeded at 5 × 10^4 cells in 48-well plates. After confluence, the cells were stimulated with 1 μg/mL of LPS and treated with various concentrations of JCST and CST (62.5, 125, 250 and 500 μg/mL) for 18 h. Indomethacin was used as the positive control. After treatment, the cell culture media were collected and used to measure the PGE_2 production according to the manufacturer's protocols (Cayman Chemical Co., MI, USA).

Differentiation and Oil Red O staining

The mouse fibroblast cell line, 3T3-L1 cells were seeded at 3 × 10^5 cells in 6-well plates. After confluence, cells were induced by standard differentiation medium MDI (DMEM with 0.5 mM IBMX, 1 μM dexamethasone, 1 μg/ml insulin, 10% FBS and 1% P&S) and treated with JCST and CST (62.5, 125, 250 and 500 μg/mL) for 48 h (from day 0 to day 2). At this time, the medium was changed with 1 μg/ml insulin in DMEM (containing 10% FBS, 1% P&S) for the following 72 h (from day 2 to day 5). After then, the medium was replaced with DMEM medium (containing 1 μg/ml insulin) for the following 48 h (from day 5 to day 7). Cells were stained with Oil-Red O to detect fat droplets in adipocytes on day 7 after differentiation induction. Cells were washed twice with DPBS and fixed with 10% formalin for 30 min at room temperature. After washing with DPBS and distilled water three times, cells were stained with Oil Red O solution (0.3% Oil Red O in 60% isopropanol) for 30 min at room temperature. Then, cells were washed three times with distilled water and imaged with a microscope (Olympus, Tokyo, Japan). Stained fat droplets in 3T3-L1 cells were eluted with 100% DMSO and quantified by measuring the optical absorbance at 530 nm.

Triglyceride (TG) and leptin production

TG and leptin production were performed on day 7 after 3T3-L1 cell differentiation. The lysates were used to measure the TG (Bioassay Systems, CA, USA) production. The supernatant was used to determine the leptin level according to the manufacturer's protocols (R&D System Inc., MI, USA).

Western blot analysis

Cells were treated with various JCST and CST concentrations (62.5, 125, 250 and 500 μg/mL), washed twice and harvested with ice-cold DPBS. Cell lysates were prepared using RIPA cell lysis buffer. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C. Protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). 30 μg of each proteins present in cell lysates were separated on 4-20% CriterionTM TGXTM precast Gel (Bio-Rad Laboratories Inc., PA, USA) electrophoresis and transferred onto the polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane were blocked with 5% skim milk, and incubated with different primary antibodies (1:1000 dilutions) for overnight. After incubation with fluorescent-conjugated secondary antibodies (1:3000 dilutions) at room temperature, immunoreactive proteins were detected with a ECL assay kit (Thermo scientific, Rockford, UK). Bands were

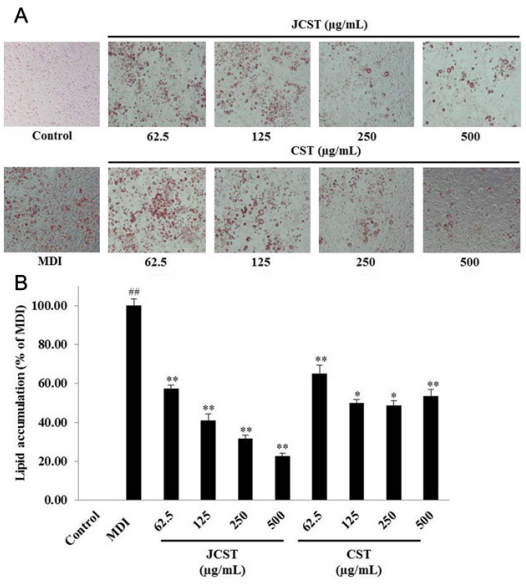

Figure 2. Inhibitory Effect of the lipid accumulation for JCST and CST on MDI-induced 3T3-L1 adipocytes. A: Fat droplets were measured by Oil red O staining and observed using microscope (at X100). B: The absorbance of lipid accumulation which was Oil red O dye was dissolved in DMSO (530 nm). The data are mean values of three experiments ± SEM; ##< 0.01 compared with control, *P < 0.05; **P < 0.01 compared with the MDI.

visualized using CemiDoc™ XRS + image analyzer (Bio-Rad Laboratories Inc., PA, USA).

Statistical analysis

Data results are reported as means ± SEM and compared by ANOVA and Bonferroni multiple comparison method (SYSTAT 13.0 SPSS Inc. U.S.A). A P-values <0.05 was defined as statistically significant.

Results

Herbal formula and toxicity

The yields of JCST and CST were 22.23%, 26.66% respectively. The JCST and CST did not significantly affect cell viability up to 500 μg/mL (data not shown). Therefore, our experiment used in a range of non-cytotoxic concentrations (62.5, 125, 250 and 500 μg/mL) in experiments.

Effect of JCST and CST on PGE_2 production in RAW 264.7 cells

To confirm of PGE_2 inhibition effect for JCST and CST, we detected the supernatant on LPS induced RAW 264.7 cell. The LPS induced group increased about 34 times than control group. The positive control, indomethacin, inhibited the PGE_2 about 100%. We confirmed that the JCST and CST groups inhibited the PGE_2 production in a dose-dependent manner. The IC_{50} of PGE_2 production of JCST and CST groups were 133.55 μg/mL and 210.69 μg/mL respectively (**Figure 1**). As a result, we confirm that JCST suppressed PGE_2 than CST.

Effect of JCST and CST on lipid accumulation in 3T3-L1 cells

In this study, adipocytes undergoing induced differentiation were treated with various concentrations of JCST and CST. Staining of fat droplets with Oil Red O showed that their accumulation in cells exposed to JCST and CST were lower (**Figure 2A**). Limited metabolic activity of adipocytes following the differentiation process was accompanied by reduced fat contents in the cytoplasm. Fat contents in adipocytes were dependent on the JCST and CST. In these results, JCST group suppressed the fat droplet than CST group (**Figure 2B**).

Effect of JCST and CST on intracellular lipid accumulation in adipocytes

To confirm the effect of JCST and CST on adipogenesis, intracellular lipid accumulation was determined in mature adipocytes. Intracellular TG and leptin contents were further quantified at 7 days post differentiation of preadipocytes. As expected, TG content was significantly increased in the cells cultured with MDI alone. While the TG level of cells which were treated by JCST and CST decreased significantly. The JCST group reduced 17.68 ± 1.41% (62.5 μg/mL), 28.40 ± 0.43% (125 μg/mL), 29.33 ± 3.42% (250 μg/mL) and 53.54 ± 2.19% (500 μg/mL). CST group reduced 7.15 ± 2.28% (62.5 μg/mL), 22.86 ± 4.13% (125 μg/mL), 38.57 ± 2.71% (250 μg/mL) and 49.48 ± 4.71% (500 μg/mL) (**Figure 3A**). The leptin content was significantly increased in the cells cultured with MDI alone. While cells which were treated by JCST and CST decreased in dose dependent significantly. The JCST group suppressed 26.29 ± 1.36% (62.5 μg/mL), 44.23 ± 1.79% (125 μg/mL), 60.04 ± 3.87% (250 μg/mL) and 91.22 ± 0.74% (500 μg/mL). The CST group suppressed the leptin in 19.60 ± 5.97% (62.5 μg/mL), 37.40 ± 1.91% (125 μg/mL), 19.74 ± 5.80% (250 μg/mL) and 45.37 ± 6.22% (500 μg/mL) (**Figure 3B**). From these findings, it is suggested that JCST may

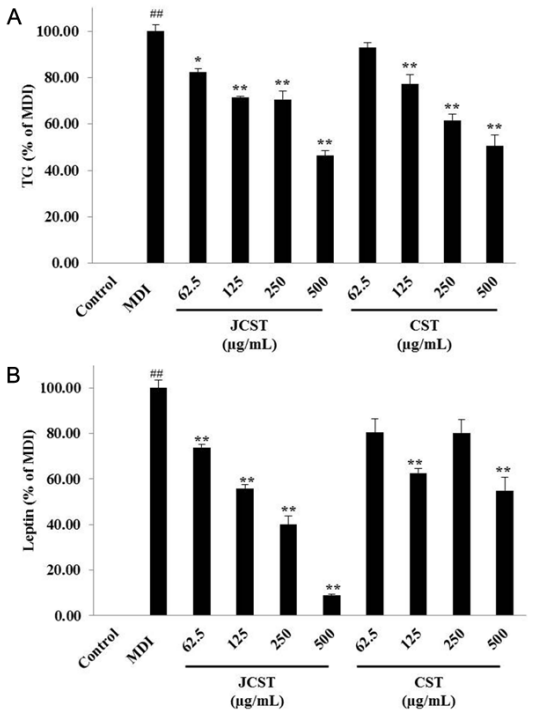

Figure 3. Inhibitory Effect of lipogenesis for JCST and CST in MDI- induced 3T3-L1 adipocytes. A: TG production of JCST and CST. B: Leptin production of JCST and CST. The data are mean values of three experiments ± SEM; ##< 0.01 compared with control, *P < 0.05; **P < 0.01 compared with the MDI.

strongly suppress differentiation of preadipocytes by decreasing of the expression of genes involved in adipocyte phenotypes.

Effect of JCST and CST on protein expressions in adipogenesis

Differentiated cells were treated with various concentrations of JCST and CST for 5 days, and the protein levels of C/EBPα, PPARγ and FABP4 were determined by western blotting. As shown in **Figure 4**, the groups which were treated by JCST and CST suppressed protein expressions of C/EBPα, PPARγ and FABP4 compared to differentiated cells. Especially, we found the inhibitory effect of protein expressions at 62.5 μg/mL in JCST group. Otherwise, CST group inhibited protein expressions at 125 μg/mL. So, we suggest that JCST have the inhibitory effect at the lower concentration than CST.

Discussion

According to the statistical record for Korean Deaths Reported in 2013, cerebrovascular dis-

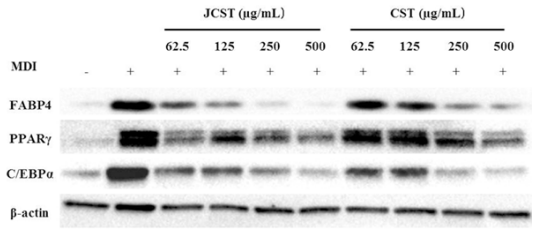

Figure 4. Protein expressions of JCST and CST in 3T3-L1 adipocytes. At day 5, adipocytes protein was isolated and protein expressions of C/EBPα, PPARγ and FABP4 were determined by Western blot analysis.

ease and heart disease were ranked the 2nd and 3rd most common causes of death in Korea, following malignant neoplasm, the number one cause of death [3]. The aging of society caused by a marked increase in the elderly population is emerging as a major problem in health care. MS; including hypertension, diabetes, obesity, hyperlipidemia, and atherosclerosis - is recognized as a high risk factor of cardiovascular and cerebrovascular diseases. Since the symptoms of MS are likely to occur concurrently rather than individually, and be accompanied by serious complications, extra attention should be focused on its prevention and treatment. One of the most outstanding organ features in the TE group which is most relevant with MS is a large liver and small lung. A study found that this group shows a higher prevalence of MS such as stroke, the most representative cerebrovascular disease, obesity, and diabetes, compared with persons with other constitutional types [13, 14].

CST, a prescription for treating cerebrovascular disease in TE patients, consists of fourteen different kinds of herbal ingredients. It is known to help improve the functioning of the lungs (Sung-Pye-Ahn-Sin), and used to treat various ailments such as stomach pain, diarrhea, apoplectic glossoplegia, upset stomach, heartburn, stress response, and neurosis diagnosed by abdominal examination by promoting gastric motility [15]. JCST, which consists of ten kinds of herbal ingredients, is a modified prescription based on CST. Although JCST has been used more often than CST at the clinical treatment in Korea, it has been the subject of only a small amount of research.

Most Korean traditional prescriptions consist of more than eight kinds of herbal ingredients.

However, due to the difficulty of quality control, they are not easy to develop into new drugs. A number of attempts have been made to reduce the ingredients and to improve the efficacy of Korean traditional prescriptions. As an extension of these efforts, JCST is a novel prescription modified from CST by reducing the number of ingredients. In the current study, based on the clinical application of JCST and CST in the treatment of cerebrovascular diseases for TE patients, we aim to justify JCST's clinical use, and compare its therapeutic efficacy with CST by investigating its effects on inflammation and lipid metabolism.

Inflammation caused by accumulated lipids on the vessel wall can result in hyperlipidemia and arteriosclerosis, leading to cerebrovascular disease such as stroke. Adipocytes, accumulated in the body mainly due to an imbalance of the lipid metabolism, function as an energy reservoir by restoring excessive energy in the form of TG, and providing the restored energy when needed. Therefore, adipocytes play an important role in maintaining homeostasis in terms of the energy level of the body [16-18].

During the biosynthesis of eicosanoids, inflammation results from prostaglandin (PG), which is produced by cycooxygenase (COX). COX-2 is known to be selectively expressed in the brain and the macula densa of the kidneys, and found to be up-regulated in certain conditions such as rheumatoid arthritis and colon cancer. PGE_2 can be immediately derived by a variety of stimulators including lipopolysaccharide (LPS), phorbol esters (TPA), tumour necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), and so forth, which indicates that COX-2 gene is a member of the immediate early gene family [19, 20]. In addition, based on a growing interest in the role of leptin in inflammatory response, and a recent studies result revealing the correlation between obesity and inflammation, a study reports demonstrated that PGE_2, an inflammatory mediator produced during adipogenesis, can regulate the blood level of leptin [21-23]. Leptin, secreted by adipocytes, is known to be a signaling molecule that helps maintain the energy balance between the brain and adipose tissue. The signal emitted by leptin is recognized by leptin receptors distributed in the arcuate nucleus, and the neurons of the ventromedial, lateral, and dorsomedial hypothalamus. It also plays a role as a hormone for regulating bodyweight, lipid storage and energy homeostasis by acting on the central nervous system. The blood level of leptin is known to be closely associated with the TG level and the amount of adipose tissue in the body, and to be influenced by environmental factors, hormones such as insulin and dexamethasone, and so forth [24-26]. In our current study, we confirmed that JCST and CST inhibited the production of PGE_2, TG, and leptin. Conclusively, we suggested that leptin release was regulated by PGE_2 and JCST and CST inhibited adipogenesis via PGE_2 inhibitory efficacy.

In the early phase of differentiation of preadipocyte induced by MDI treatment, certain groups of genes including *c-fos*, *c-jun*, and *s-myc* are up-regulated, and transcription factors such as C/EBPβ and C/EBPδ are activated by IBMX and dexamethasone, respectively [27]. The activation of C/EBPβ and C/EBPδ induces the expression of PPARγ, an adipogenic inducer. At a later stage, the decreased activity of C/EBPβ and C/EBPδ results in the activation of C/EBPα which leads to the promotion of adipogenic differentiation [28]. During the mid-phase of adipogenesis, activated C/EBPα and PPARγ act separately or together to promote the differentiation of preadipocytes and their maturation into adipocytes by inducing the expression of adpogenic genes, which affect biosynthesis, trafficking, and storage of lipid, such as fatty acid synthase (FAS), acyl-CoA synthase (ACS1), fatty acid binding protein 4 (FABP4), and fatty acid binding protein 1 (FABP1) [29, 30]. In our study, we analyzed the changes in the expression profile of C/EBPα, PPARγ, and FABP4 after adipogenic stimulation for 5 days to investigate the effects of JCST and CST on the activity of major transcription factors associated with adipogenesis. We found that both JCST and CST inhibited the protein expressions of C/EBPα, PPARγ, and FABP4 that is a marker for differentiated cells. This result indicates that JCST and CST may regulate on the initial stage of adipogenesis and inhibit the differentiation of preadipocyte. Especially JCST groups significantly suppressed the protein expressions of C/EBPα, PPARγ and FABP4 as compared to CST groups from lower concentration. Although JCST consists of fewer ingredients compared with CST, inhibitory effects of PGE_2 level, lipid accumulation, TG production, leptin content and adipogenic differentiation more effective than CST.

Conclusively, our data suggest that JCST has a superior inhibitory effect on lipid metabolism compared to CST, and regulates lipogenesis effectively by decreasing the expression level of the genes and transcription factors associated with adipogenesis. Therefore, our results provide scientific evidence to support the clinical use of JCST in the treatment of cerebrovascular diseases such as stroke in the TE group.

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Disclosure of conflict of interest

None.

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