ORIGINAL ARTICLES

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Effects of the oligostilbenes from *Iris lactea* Pall. var. *chinensis* (Fisch.) Koidz on the adipocytes differentiation of 3T3-L1 cells

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The dried seeds of *Iris lactea* Pall.var. *chinensis* (Fisch.) Koidz, an important traditional Chinese medicine, are regarded to have effects of clearing heat, eliminating dampness and pharyngitis and so on. It has been used in the treatment of jaundice, diarrhea, leucorrhea and carbuncles. Previous phytochemical studies of *Iris* species showed the presence of flavones, isoflavones, triterpenes and stilbenes. In our previous research, we isolated five known oligostilbenes, vitisin A, vitisin B, vitisin C, vitisin D, and cis-vitisin A were successfully isolated from *Iris lactea* for the first time. The aim of this study was to assess the effects of these oligostilbenes on the differentiation and adipogenes in 3T3-L1 cells. Our results showed that vitisin A, vitisin B, cis-vitisin A significantly inhibited adipocytes differentiation and reduced lipid accumulation in 3T3-L1 cells. In addition, vitisin A, vitisin B, cis-vitisin A strongly suppressed the expression levels of adipocyte-specific genes including peroxisome proliferator activated receptor-γ (PPARγ), CCAAT/enhancer binding protein–α (C/EBPα) and adipocyte fatty acid binding protein 4 (FABP4). In contrast, vitisin C and vitisin D significantly promoted adipogenesis and increased intracellular lipid accumulation, while the two oligostilbenes markedly increased the expression of adipocyte marker genes. In the present study, we found that vitisin A, vitisin B and cis-vitisin A inhibit the adipogenesis and adipocytes differentiation by their influence on the expression of PPARγ, which leads to subsequenet downregulation of PPARγ mediated adipocyte-specific gene during adipogenesis.

1. Introduction

The prevalence of obesity has dramatically increased worldwide over the past four decades and became a serious health problem. Obesity is closely associated with the prevalence of type II diabetes, hypertension, and severe cardiovascular disease and it induces metabolic disorders such as impairment of glucose uptake and fatty liver which ultimately produces hepatic steatosis (Kopelman 2000; Bluher 2013). Obesity is characterized by the lipid accumulation of fat tissue and cells, which is the result of increased adipocytes size (hypertrophy) and huge number of adipocytes (hyperplasia) (Bluher 2013; Spalding et al. 2008). Adipocyte hyperplasia is mimicked *in vitro* by the 3T3-L1 adipocytes differentiation process. 3T3-L1cell line is one of the classic cell types to study adipogenesis as an *in vitro* model. Preadipocytes become mature adipocytes through growth arrested, mitotic cell division, and terminal differentiation by complex molecular mechanisms regulating adipocyte-specific genes expression to synthesize, accumulate and utilize fat (Rosen and Macdougald 2006; Camp et al. 2002). Thus, it can be seen that the adipocyte differentiation process has an important role in lipid accumulation and the development of obesity. So inhibiting adipocytes differentiation is an efficient way to prevent or treat obesity. During 3T3-L1 adipocyte differentiation process, many key transcriptional factors such as peroxisome proliferator activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (C/EBPα) and adipocyte fatty acid binding protein 4 (FABP4) are involved (White and Stephens 2010). PPARγ is a member of the nuclear receptor superfamily of ligand-inducible transcription factors and is a major regulator of adipocyte differentiation and metabolism, controlling the gene networks involved in lipid metabolism. C/EBPα is a transcriptional activator, PPARγ and C/EBPα together regulate downstream target genes, which are related to lipid accumulation, fatty acid oxidation (Park et al. 2012). FABP4 (ap2) is a 14-15 KDa protein that binds with high affinity to hydrophobic ligands such as saturated or unsaturated long-chain fatty acids (Furuhashi et al 2008). FABP4 of adipocytes is highly expressed and mediated by peroxisome proliferator activated receptor γ (PPARγ) agonists, fatty acids and insulin (Kopecky et al. 1995).

Iris lactea Pall.var. *chinensis* (Fisch.) Koidz, belonging to the family of Iridaceae, is an herbaceous perennid plant. The dried seeds of *Iris lactea* have, in the Chinese healing system, the effects of clearing heat, eliminating dampness and stanching bleed (State Administration of Traditional Chinese Medicine 1999). It has been used in the treatment of jaundice, vomiting blood, diarrhea, pharyngitis and carbuncles in Traditional Chinese Medicine (Shen et al. 2008). Up to now a large number of flavones and benzoquinones have been isolated from dried seeds of *Iris lactea* (Lv et al. 2016; Lin et al. 2011). Interestingly, five oligostilbenes were successfully separated and purified by our laboratory (Lv et al. 2015). Oligostibenes are a special group of polyphenolic compounds polymerized from resveratrol or other units (such as isorhapontigenin, oxyresveratrol, piceatanol, etc.) (Lin and Yao 2006). Oligostilbenes have been found to exhibit various biological activities, including antifungal, anti-inflammatory, antioxidant, anti-tumor, anti-HIV, antibacterial (Liu et al. 2013; Wang and Yao 2015). These oligostilbenes are including vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A (Fig. 1). In the present study, we investigated the effects of the oligostilbenes on the adipocytes differentiation of 3T3-L1 cells by measuring lipid accumulation and the expression levels of adipocyte marker genes.

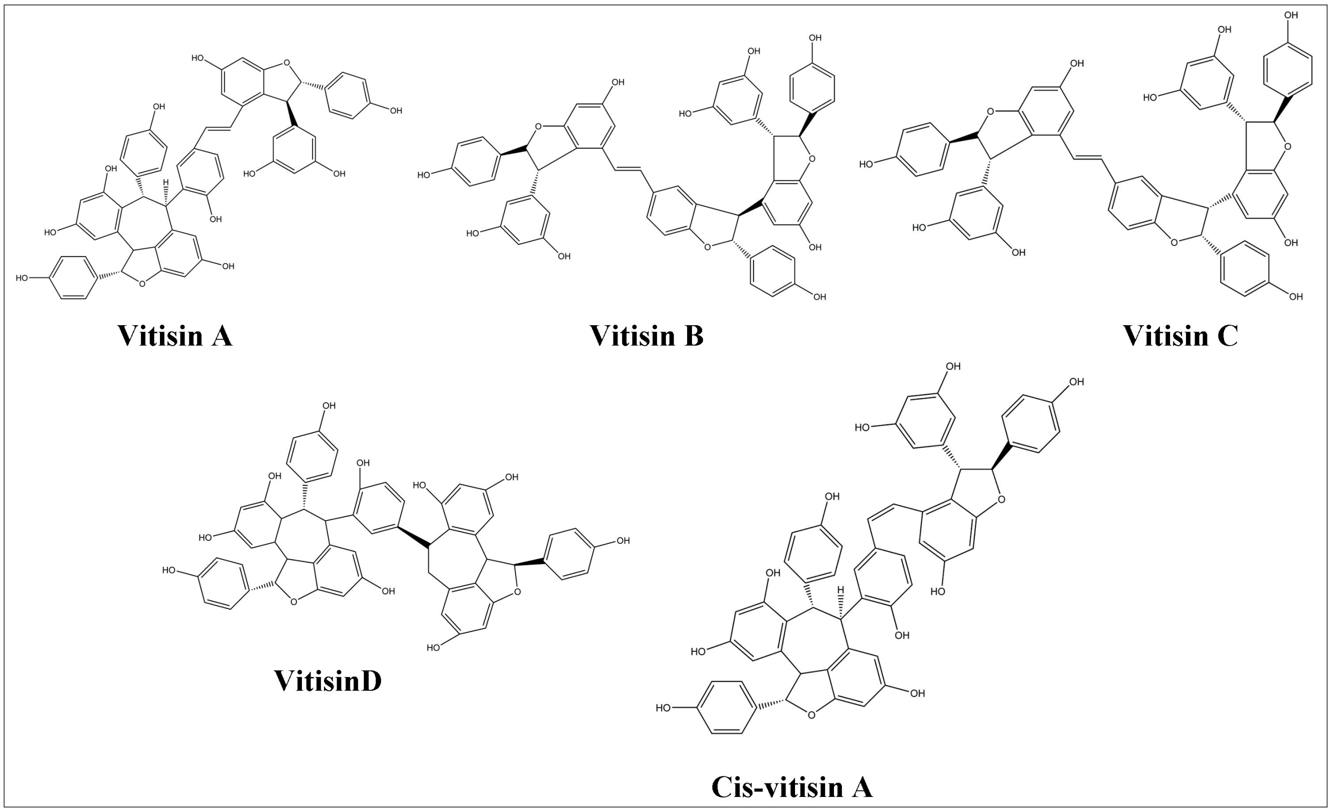

Fig. 1: Chemical structures of oligostilbenes

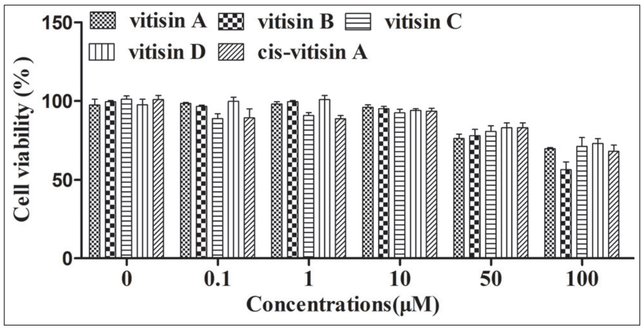

Fig. 2: Effects of oliogostilbenes on adipocytes cell viability. Pre-adipocytes were incubated with oligostilbenes for 48 h, and viability was determined by MTT assay.

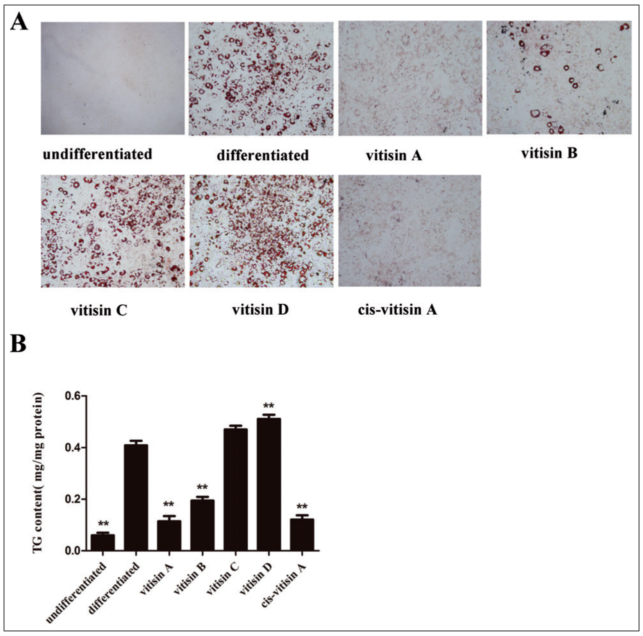

Fig. 3: Effects of oliogostilbenes on adipocytes differentiation. (A) Post-confluence 3T3-L1 cells were differentiated either in the absence or presence of oligostilbenes for ten days. Triglyceride contents were quantified by measuring absorbance. (B) Cells were stained with Oil-red O (ORO) with various compounds treatment. Adipocytes stained with ORO (magnification, 20×) were visualized by light microscopy. Date are presented as means±SD of three experiments performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. only differentiated cells.

2. Investigations and results

2.1. MTT viability

The toxic concentration of five oligostilbenes was assessed via MTT viability assay. As shown in Fig. 2, vitisin A, vitisin B, vitisin D did not affect the viability of 3T3-L1 cells at concentrations of 0.1~10 μM, and when the concentration was up to 50 μM, the five compounds began to show cytotoxicity, while vitisin B was the most sensitive, vitisin B displayed a 53% viability compared to the control at concentration of 100 μM. However, vitisin C and cis-vitisin A showed little cytotoxicity in concentrations of 0.1 μM and 10 μM, respectively. Therefore, at the concentration of 0.1-10 μM, these five compounds had little effect on the 3T3-L1 cell viability. Thus, 10 μM was selected as dose for further studies.

2.2. Effects of oligostilbenes on intracellular lipid accumulation during differentiation in 3T3-L1 adipocytes

To examine the effects of oligostilbenes on adipocytes differentiation and lipid accumulation, 3T3-L1 pre-adipocytes were treated with 10 μM of vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A. Cells accumulated intracellular lipids gradually from day 0 to day 8 after addition of induction medium. 3T3-L1 cells were differentiated to mature adipocytes, which were accumulated with a large number of lipid droplets, cells were stained with Oil red O and visualized (Fig. 3A).

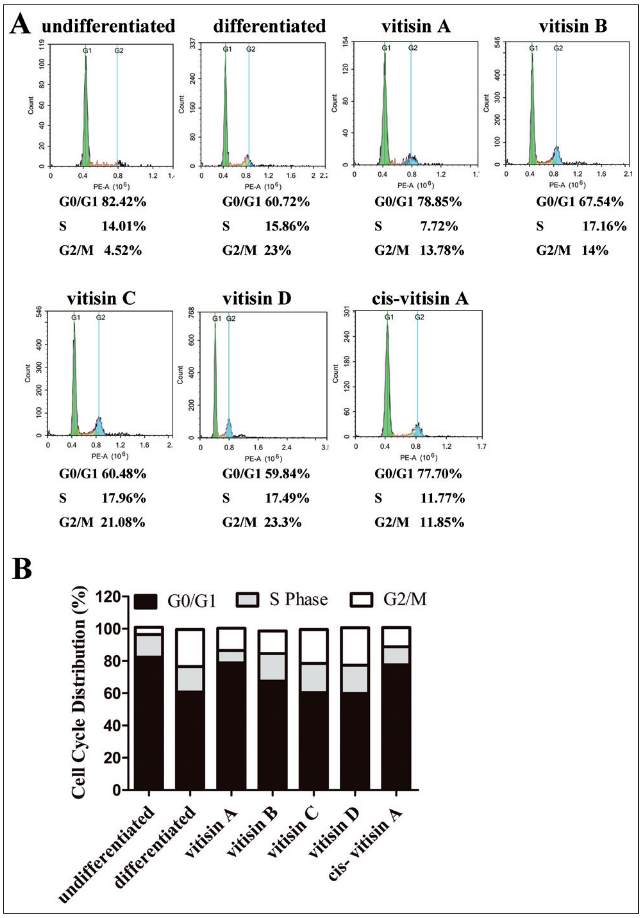

Fig. 4: Regulatory effects of oligostilbenes on cell cycle progression in 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes were cultured in differentiation medium or containing oligostilbenes for 24 h. (A) Cells were stained with PI solution and analyzed by flow cytometer. (B) The percentage of the cell distribution at each stage of the cell cycle was determined using the FCS Express 4 Flow cytometer software.

Lipid accumulation in cells was quantified by directly measuring triglyceride levels. The quantity of intracellular triglyceride contents is shown in Fig. 3B. Vitisin A and cis-vitisin A significantly reduced lipid accumulation and triglyceride contents in 3T3-L1 adipocytes by 71.07% and 69.54% (both $P < 0.01$) respectively compared to the differentiated. Vitisin B treatment reduced lipid formation by 50.96% (Fig. 3A-B, $P < 0.01$). Interestingly, vitiain C and vitisin D treatment increased lipid formation (Fig. 3A-B), concordant with the increased lipid staining, intracellular triglyceride contents were increased by 18.78% and 29.05% (Fig. 3A-B, $P < 0.01$) respectively, in vitisin C and vitisin D-treated cells.

2.3. Oligostilbenes block cell cycle progression in 3T3-L1 cells

To investigate the effects of oligostilbenes on the cell cycle of adipocytes induced to differentiate to mature adipocytes. Vitisin A and cis-vitisin A blocked this cell cycle, especially at the G1/S transition, and the cell distribution was nearly the same as with undifferentiated pre-adipocytes (Fig. 4A-B). However, vitinsin C and vitisin D promoted this cell cycle at the G1/S phase (Fig. 4A-B). But, there were no significantly promoted effects compared to the differentiated cells.

2.4. Effect of oligostilbenes on the expression of adipogenic-specific genes during adipocyte differentiation

During pre-adipocytes differentiation into mature adipocytes process, active PPARγ and C/EBPα induce adipocyte-specific gene expression. To elucidate the mechanism of differention, differentiated adipocytes were treated with vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A, and the protein levels of PPARγ, C/EBPα and FABP4 were determined by Western blotting. As shown in Fig. 5, vitinsin A, vitisin B, cis-vitisin A all suppressed the expression of PPARγ, C/EBPα and FABP4 when compared with fully differentiated adipocytes and the cells treated with vitisin A significantly decreased the expression of PPARγ, C/EBPα and FABP4 by 93.95% ($P < 0.01$), 84.12% ($P < 0.01$) and 94.50% ($P < 0.01$), respectively. These results suggest that vitinsin A, vitisin B, cis-vitisin A regulate differentiated process and adipogenesis via modulation of PPARγ, C/EBPα expression. Interestingly, vitisin C and vitisin D increased the expression of adipogenic-specific genes, when vitisin D was applied, the expression of PPARγ , C/EBPα and FABP4 were enhanced by 90.35% ($P < 0.01$), 33.96% ($P < 0.01$) and 65.47% ($P < 0.01$), respectively, compared to the differentiated cells (Fig. 5).

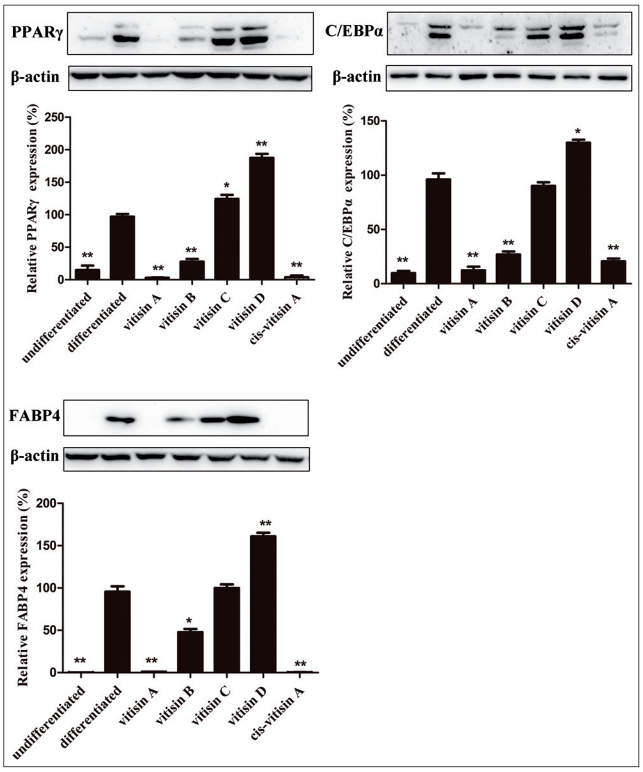

Fig. 5: Effects of vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisinA on expression of PPARγ, C/EBPα and FABP4 in 3T3-L1 adipocytes. Protein expression of PPARγ, C/EBPα and FABP4 was detected using western-blotting. Upper panel of each figure: representative blots; lower panel of each figure: results of quantitative analysis. Date were presented as means±SD of three experiments performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. only differentiated cells.

2.5. Effects of oligostilbenes on the expression of PPARγ, C/EBPα and FABP4 during adipocyte differentiation

Adipocytes transcription factors such as PPARγ, C/EBPα, and FABP4 play important roles in adipocytes differentiation, adipogenesis and lipid accumulation. To prove the molecular mechanisms underlying the effects of oligostilbenes on the adipocyte differentiation of 3T3-L1 cells, the expression of PPARγ, C/EBPα, and FABP4 were detected by RT-PCR. Treatment with vitisin A, vitisin B and cis-vitisin A all reduced expression of PPARγ, C/EBPα, and FABP4, vitisin A significantly inhibited the expression of PPARγ , C/EBPα, and FABP4 by 84.45% ($P < 0.01$), 95.28% ($P < 0.01$) and 99.46% ($P < 0.01$), respectively, compared to the differentiated cells (Fig. 6). Similarly, a significant reduction in PPARγ, C/EBPα, and FABP4 mRNA was observed in vitisin B and cis-vitisin A-treated cells (Fig. 6). Amusingly, vitisin C and vitisin D exhibited opposite effects compared to the other three compounds, they increased expression of PPARγ, C/EBPα, and FABP4, when the cells treated with

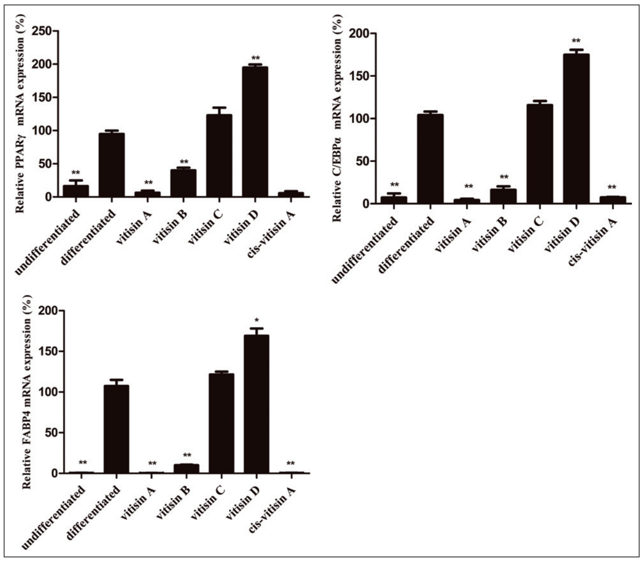

Fig. 6: Effects of vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A on expression of *PPARγ* , *C/EBPα* and *FABP4* in 3T3-L1 adipocytes. Expression mRNA of PPARγ, C/EBPα and FABP4 was detected by RT-PCR. Relative mRNA expression was normalized to β-actin. Date were presented as means±SD of three experiments performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. only differentiated cells.

vitisin D, which strongly increased expression of PPARγ, C/EBPα and FABP4 by 99.47% ($P < 0.01$), 73% ($P < 0.01$) and 69.08% ($P < 0.01$), respectively, compared to the differentiated cells (Fig. 6).

3. Discussion

In the past four decades, obesity has become the leading metabolic disease and is a significant health problem owing to the increased risk of the occurrence of other diseases. Adipocytes differentiation and intracellular lipid accumulation are very closely associated with the development of obesity (Daquinag et al. 2015). Lipid accumulation reflects the process of adipogenesis and the programmed differentiation of pre-adipocytes into mature adipocytes is related to obesity (Wu et al. 1999). Commonly, anti-obesity strategies are classified into four categories: reducing nutrition and food intake, suppressing nutrient absorption, increasing energy metabolism and fat or protein metabolism or storage (Bray and Tartaglia 2000). Moreover, many researchers advise that inhibiting of adipocytes differentiation is one of the anti-obesity manners, and many studies have strived to reduce obesity by focusing on blocking pre-adipocyte differentiation and inhibiting lipid accumulation. Currently, more and more studies have explored novel therapeutic agents derived from natural plants against adipocytes differentiation, intracellular lipid accumulation and gene expression closely related to obesity (Jung et al. 2014; Cassidy et al. 2000). Plants are a precious source of curing various diseases, and there is an emerging trend to use potential plants constituents, especially stilbenes (Chong et al. 2009). Hence, resveratrol, as the most well-known stilbene, has attracted much attention because of its multiple pharmacological activities, particularly concerning obesity (Chen et al.2011). It is reported that resveratrol inhibits lipogenesis and adipocyte differentiation. It also reduces accumulated lipid or triglyceride contents in adipocytes (Zhang et al. 2012). It was demonstrated that vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A, a resveratrol tetramer, have bioactivities similar to resveratrol (Huang et al. 2001; Ito et al. 1998). Vitisin A and vitisin B were isolated from *Vitis thunbergii*, and displayed significant PGE2 inhibition activities (Wang et al. 2011). In addition, the mixture of vitisin A and cis-vitisin A showed powerful hepatoprotective activity in vitro in primary cultured rat hepatocytes (Oshima et al. 1995). Concerning a protection against brain cell dysfunctio vitisin C, isolated from *V. vinifera* stems, demonstrated strong inhibitory effects on Aβ aggregation (Waffo-Teguo et al. 2013).

The aim of this study was to define the effects of the isolated oligostilbenes on 3T3-L1 adipocyte differentiation and underlying mechanisms. The 3T3-L1 cell line was used as an adipocytes differentiation classic model for research into the molecular mechanism of adipogenesis. To study the effects of oligostilbenes on the differentiation of pre-adipocytes into mature adipocytes, 3T3-L1 pre-adipocytes were treated with vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A, respectively. On day 8, the lipid droplets of differentiated adipocytes were stained with Oil-red O, and triglyceride contents were quantified. Vitisin A, vitisin B and cis-vitisin A were found to significantly reduce lipid accumulation and lower triglyceride contents in 3T3-L1 adipocytes compared with differentiated cells. However, vitisin C and vitisin D obviously increased intracellular lipid accumulation and triglyceride levels in 3T3-L1 adipocytes compared with differentiated cells. This phenomenon was observed for the first time.

Subsequently, we investigated the effects of oligostilbenes on the adipocyte differentiation of 3T3-L1 cells by determining the cell cycles. To the best of our knowledge, differentiated medium caused the grown-arrested pre-adipocytes to initiate cell cycle progression. FACS date on cell cycle analysis showed that vitisin A, vitisin B and cis-vitisin A inhibited the cell cycle at the G1/ S transition compared to the differentiated cells. However, vitisin C and vitisin D accelerated cell cycle progression at the G1/ S phase compared to the differentiated cells. Previous studies have also exhibited that vitisin A has anti-adipogenic activity through cell cycle arrest and reduce lipid accumulation in a dose-dependent manner in 3T3-L1 cells (Kim et al. 2008). Our dates combined with previous results demonstrated that vitisin A, vitisin B and cis-vitisin A showed potenial anti-adipogenic activity.

The study also examined the effects of oligostilbenes on the adipocyte differentiation of 3T3-L1 cells by examining the expression levels of adipogenic-specific genes PPARγ, C/EBPα, and FABP4. Adipocyte differentiation is associated with multifunctional cellular pathways and requires a series of transcription factors involved in this process (Gwo et al. 2013). It is well known that the crucial adipogenic transcription factors, PPARγ, C/EBPα, play in cooperation to accelerate adipogenesis and lipid accumulation (Madsen et al. 2014; Ramji and Foka 2002). Generally, the first stage of adipogenesis needs the transient induction of C/EBP-β and C/EBP-δ and can be activated in vitro by induction medium (Ramji and Foka 200). Next, C/EBP-β and C/EBP-δ begin to accumulate and both directly induce expression of PPARγ and C/EBPα (Tang et al. 2003). PPARγ expression is necessary for pre-adipocyte differentiation during the early phase and. C/EBPα is expressed at the later stage of differentiation and is involved in a positive feedback loop with PPARγ to induce their own expression and also activate a number of downstream target genes expressing and sustaining adipocyte maturation (Tong and Hotamisligil 2001). FABP4 is a member of the intracellular fatty acid binging with protein family, and is significantly expressed in adipocytes. Studies have shown that *FABP4-/-* mice have significantly reduced triglyceride contents and cholesterol, but increased FFA (Uysal et al. 2000).

Our results showed that vitisin A, vitisin B and cis-vitisin A could attenuate the expression of PPARγ, C/EBPα, and FABP4 and the gene expression of PPARγ, C/EBPα, and FABP4 compared with differentiated cells. Especially, vitisin A and cis-vitisin A significantly reduced the adipocyte-specific gene expression such as PPARγ, C/EBPα. The analysis of the adipogenic transcription factors at protein and gene levels show that vitisin A and cis-vitisin A inhibited pre-adipocytes transformation into mature adipocytes associated with decreased PPARγ and C/EBPα expression. In addition, intracellular triglyceride contents reduced by vitisin A, vitisin B, and cis-vitisin A may be through regulating FABP4 expression. The FABP4 gene is the terminal differentiation marker of adipocytes and plays important roles in fatty acid metabolism (Palmer et al. 2002). Thus, we determined that oligostilbenes, vitisin A, vitisin B and cis-vitisin A, blocked adipocytes differentiation by downregulation of adipocyte-specific genes in the following order vitisin A= cis-vitisin A > vitisin B. However, vitisin C and vitisin D significantly enhanced the expression of PPARγ, C/EBPα, and

FABP4 compared with differentiated cells. Moreover, vitisin C and vitisin D strongly accelerated intrallular lipid accumulation. Among them, vitisin D was the most active accelerator and vitisin C was the less active.

In our previous work, five known oligostilbenes, vitisin A, vitisin B, vitisin C, vitisin D, and cis-vitisin A were isolated from *Iris lactea* for the first time. Though vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A are resveratrol tetramers, their structures are different. So, vitisin A and cis-vitisin A are cis/trans isomers. Their structural difference, did not lead to different bioactivities in 3T3-L1 cells. Vitisin B and vitisin C are epimerides, and our results showed that their effects on differentiation of 3T3-L1 cells are distinctive. Vitisin B significantly reduced lipid accumulation, and downregulated adipocyte-specific genes. Compared with vitisin B, vitisin C strongly increased lipid accumulation and upregulated the expression levels of PPARγ, C/EBPα, and FABP4. Vitisin B and vitisin C share the same construction pattern, but the difference in their relative stereo configurations may explain why these compounds displayed different bioactivities in 3T3-L1 cells. Compared with the other four compounds, vitisin D possesses two seven membered rings. The subtle structural differences in oligostilbenes may explain why these compounds exhibited different activities in 3T3-L1 cells. More studies on the molecular mechanism of vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A in 3T3-L1 cells are needed to elucidate the relationship between the structure and bioactivities of these compounds.

In conclusion, this study demonstrated that five oligostilbenes isolated from seeds kernel of *Iris lactea* Pall. var. *chinensis* (Fisch.) Koidz, which exhibited different bioactivities on adipocytes differentiation. Vitisin A, vitisin B and cis-vitisin A efficiently inhibited adipogenesis and differentiation in 3T3-L1 adipocytes as indicated by a strong reduction in intracellular lipid accumulation and triglyceride contents. However, vitisin C and vitisin D could promote the differentiation of 3T3-L1 pre-adipocytes and increase fat accumulation and triglyceride synthesis in 3T3-L1 adipocytes by facilitating the expression of adipocytes differentiation genes (PPARγ, C/EBPα, and FABP4). Therefore, these finding suggest that vitisin A, vitisin B and cis-vitisin A from *Iris lactea* can be useful for treatment of obesity and obesity –related metabolic diseases.

4. Experimental

4.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin and penicillin were obtained from Gibco (Grand island, NY, USA). Isobutyl-3-methyl-xanthine (IBMX) and dexamethasone (DEX), (3,4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Oil-red O, sodium dodecyl sulphate (SDS), Cell Cycle and Apoptosis Analysis Kit were purchased from Sigma-Aldrich chemical CO, Ltd. (St. Louis, MO, USA). Biosynthetic Human Insulin Injection (INS) was purchased from Novo Nordisk A/S (Novo Alle, DK-2880 Bagsvaerd). TG assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientic Inc (Rockford, IL, USA). Antibodies directed against: PPARγ, C/EBPα, FABP4 were purchased from Cell Signaling Technology (Beverly, MA), β-actin (Santa Cruz, CA), HRP Goat anti-Rabbit Antibody and HRP Goat anti-Mouse Antibody (Abgent, China). All other chemicals are reagent grade.

4.2. Preparation of extracts and the purified compounds

The isolation and purification of oligostilbenes from seeds kernel of *Iris lactea* Pall. var. *chinensis* (Fisch.) Koidz was carried out as described by Lv et. al. (2015). The extracts of seeds kernel of *Iris lactea* and its five isolated oligostilbenes (vitisin A, vitisin B, vitisin C, vitisin D and cis-vitisin A) were prepared in DMSO and stored at -20 °C.

4.3. Cell culture

3T3-L1 mouse fibroblast cells were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The pre-adipocytes were grown in DMEM containing 4.5 g/L $_D$-glucose with 10% FBS and 1% penicillin at 37 °C in a humidified incubator of 5% CO_2. The differentiation of the confluent pre-adipocytes was promoted by culturing for 2 days in DMEM medium containing 10% FBS, 10 μg/ml INS, 1μM DEX and 0.5 mM IBMX (induction medium). Then, the cells were incubated for 2 days with 10% FBS in DMEM plus 10 μg/ml INS. After 2 days (defined as day 4), the medium was replaced again with fresh DMEM every 2 days were continued until day 8. To examine the effect of compounds on 3T3-L1 pre-adipocyte differentiation, the cells were treated with five oligostilbenes (0-100 μM) at indicated concentrations. The undifferentiated cells cultures were treated in basal medium (DMEM with 10% FBS and 0.1% DMSO). The concentrations of compounds used in this assay were determined to be non-cytotoxic to the 3T3-L1 cells, as examined in the MTT viability assay. The differentiated 3T3-L1 cells were used for further analysis.

4.4. MTT viability assay

For cell viability was assessed by the MTT (3,4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium) viability assay. 3T3-L1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM containing 10% FBS. When grown to a 90% confluence, the cells were treated with five compounds (0-100 μM) for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. After 48 h, the MTT solution (5 mg/ml) was added to each well and the cells were incubated in the dark for 4 h. The resultant formazan product was dissolved by the addition of 100 μL of DMSO. Finally, absorbance was measured using a microplate reader (Molecular Devices, USA) at 490 nm.

4.5. Oil-red O staining

After differentiation in 24-well plates in day 8, the cells were washed twice with phosphate buffer saline (PBS) and fixed with 4.0% formaldehyde for 30 min at room temperature. Each well was washed with PBS three times and stained with 3.6 mg/ml Oil-red O dye/60% isopropanol solution for 1 h. Next, the staining solution was removed and the cells were washed with PBS for 3 times. The stained lipid droplets in 3T3-L1 cells were photographed by light microscopy (Olympus, Japan).

4.6. Triglyceride contents assay

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS). The total triglyceride contents in cells were determined using a commercial triglyceride assay kit. The protein concentration was determined by using Pierce BCA Protein Assay Kit.

4.7. Cell cycle analysis

3T3-L1 adipocytes were harvested and fixed with 70% ethanol at 4 °C for 12 h. After removing of ethanol and washing the cells twice with pre-cold phosphate buffered saline (PBS), cells were stained with propidium iodide solution (Beyotime, Jiangsu, China) for 30 min at 37 °C. For each measurement, date from 100000 single cell events was collected using a NovoCyte 2040R flow cytometer (San Diego, CA 92121, USA), setting at an excitation wavelength of 488 nm and emission wavelength of 572 nm.

4.8. Western blotting analysis

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells in 6-well plates were washed twice with PBS to remove the excess of culture medium, then cells were lysed for 30 min in ice-cold RIPA buffer containing 50 mM Tris-HCl (PH 7.6), 150 mM NaCl, 1% NP-40, 0.1% SDS, 100 mg/ml phenylmethyl sulphonyl fluoride (PMSF). The cell lysates were centrifuged at 12,000 g for 20 min at 4 °C to remove insoluble materials and supernatants were collected for analysis. The protein concentrations were measured using a BCA assay Reagent Kit. Protein extracts (30μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane at 250 mA for 1 h. The membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20 for 1 h at room temperature, after washing for 10 min three times with PBS, then incubated with primary antibodies (diluted 1:1000 in the blocking solution) overnight at 4 °C. Subsequently, the blots were incubated with peroxidase-conjugated antibodies for 1 h at room temperature. An β-actin was used as an endogenous control. Western blot bands were visualized using a 5200 Multi Luminescent image analyzer, according to the manufactures's protocol (Tanon Science & Technology Co., Ltd. Shanghai, China).

4.9. Relative quantitative real time RT-PCR

Cells were collected and total RNAs were isolated using TRizol reagent (Invitrogen), in accordance with the manufacturers's instructions. First-strand cDNA was generated using oligo (dT) primer. The gene expression levels were analyzed by quantitative real-time RT-PCR conducted using 7500 fast Real-Time PCR System (Applied Biosystems, Singapore). Reactions were performed with 10 μL SYBR Green Master Mix, 1 μL of each primer (10 mM) and 2000 ng template (cDNA), with RNase-free water being added to a final volume of 20 1μL .The cDNA products were denatured at 95 °C for 5 min to activate the polymerase, followed by the following 40 cycles: denaturation: 95 °C for 10 s, annealing: 60 °C for 15 s, extension: 72 °C for 20 s. Results were analyzed using the 7500 fast system software and the results were shown as levels of expression relative to those of control after normalization to β-actin using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: β-actin: CCTCTATGCCAACACAGTGC (forward), and GTACCCTGCTTGCTGATCCT (reverse); PPARγ: CCTGGCAAAGCATTTCTATG (forward), and TGGTGATTTG
TGTCT (reverse); C/EBPα: GCTGTTGGCATCCTGCTATC (forward), and TAGCTGGAAGTCACGGT (reverse); FABP4: TCACCTGGAAGACAGCTCCT (forward), and AATCCCCATTTACGCTGAT (reverse).

4.10. Statistical analysis

All of the data were expressed as the mean±SD from three independent experiments. Statistical significance was considered at $P < 0.05$ and determined by Independent Sample t-Test using SPSS 17.0 software (SPSS, Chicago, IL, U.S.A.).

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