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Inhibition of preadipocyte differentiation by *Lycium barbarum* polysaccharide treatment in 3T3-L1 cultures

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Background: *Lycium barbarum* (also called wolfberry), a famous Chinese traditional medicine and food ingredient, is well recognized for its significant role in preventing obesity; however, the molecular mechanisms underlying its preventive effects on fat accumulation are not well understood yet. The aim of this study was to determine the effects and mechanism of *Lycium barbarum* polysaccharides (LBP) on the proliferation and differentiation of 3T3-L1 preadipocytes. MTT was used to detect the proliferation of 3T3-Ll preadipocytes. Oil red O staining and colorimetric analysis were used to detect cytosolic lipid accumulation during 3T3-L1 preadipocyte differentiation. Real-time fluorescent quantitative PCR (qPCR) technology was used to detect peroxisome proliferator-activated receptor γ (*PPARγ*), CCAAT/enhancer-binding protein α (*C/EBPα*), adipocyte fatty-acid-binding protein (*aP2*), fatty acid synthase (*FAS*), and lipoprotein lipase (*LPL*) expression.

Results: The concentration of LBP from 25 to 200 μg/mL showed a tendency to inhibit the growth of preadipocytes at 24 h, and it inhibited the differentiation of 3T3-L1 preadipocytes in a dose-dependent manner. In the preadipocytes treated with 200 μg/mL LBP, there were reduced lipid droplets in the cytoplasm, and its effect was opposite to that of rosiglitazone (ROS), which significantly reduced the *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL* mRNA expression of adipocytes.

Conclusions: LBP exerts inhibitive effects on the proliferation and differentiation of 3T3-L1 preadipocytes and decreases the cytoplasm accumulation of lipid droplets during induced differentiation of preadipocytes toward mature cells. Above phenomenon might link to lowered expression of *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL* after LBP treatment. Thus, LBP could serve as a potential plant extract to treat human obesity or improve farm animal carcass quality via adjusting lipid metabolism.

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1. Introduction

Adipose is an important energy metabolism and endocrine tissue in the body [1,2]. Existed studies have shown that excess lipid deposition in livestock and poultry adversely affects carcass quality and the health of consumers and do not greatly contribute to the taste and flavor of meat products [3,4,5]. For human health, excessive accumulation of lipids causes suffering diseases, such as type II diabetes, fatty liver, cancer, and hyperlipidemia [6,7,8]. The development of adipose tissue in the body is affected by genetic, environmental, and nutritional factors. Therefore, an in-depth understanding of the molecular genetic mechanism of lipid deposition will help to promote the understanding of fat development and advance in-depth research on obesity and related metabolic diseases.

The reverse process, the reduction of the differentiation and proliferation of preadipocytes, by reducing fat synthesis in the body or accelerating its decomposition, has become a difficult problem to overcome. Many scholars at home and abroad have studied whether the compounds isolated and extracted from plant raw materials can regulate blood lipid metabolism and fat differentiation [9,10].

Lycium barbarum polysaccharide (LBP) is the main reactive component of Chinese medicine *Lycium barbarum*, which has various functions such as immunoregulation, blood lipid lowering,

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anti-lipid peroxidation, anti-aging, and anti-tumor [11,12,13]. It has also been found in previous studies to help weight loss, regulate blood lipids, and have antioxidant effects [14,15]. This article discusses the effects of LBP on the proliferation and differentiation of 3T3-L preadipocytes cultured in vitro, and two factors closely related to *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL*. A preliminary discussion of their related mechanisms was provided as the basis for the development and utilization of wolfberry in weight loss activity.

2. Materials and methods

2.1. Culture and induced differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were maintained in complete medium (high-glucose DMEM containing 10% fetal bovine serum (FBS) in incubator with at 37°C, and 5% CO_2/95% air. They were inoculated on a culture plate when the cells were in good condition. After the cells grew to confluence for 2 days, complete medium containing 0.5 mmol/L isobutyl-3-methylxanthine (IBMX), 0.25 μmol/L dexamethasone (DEX), and 10 μg/mL insulin was used, and the cells were cultured for 48 h. The DMEM was replaced with 10 μg/mL insulin, and the cells were further cultured for 48 h. The cells were then cultured in high glucose DMEM containing 10% FBS. The medium was changed every 2 days to induce differentiation of more than 85% of 3T3-L1 cells for 8 to 12 days. It has a fat cell phenotype and can be used in subsequent experiments.

2.2. Effect of LBP on proliferation of 3T3-L1 preadipocytes by MTT method

Cells in the logarithmic growth phase were seeded at a density of 5×10^4 cells/mL in a 96-well plate at 100 μL per well. After 48 h incubation, the medium was replaced with a complete medium containing 0, 25, 50, 100, and 200 $\mu g \cdot L^{-1}$ LBP (Provided by Ningxia Wolfberry Biological and Food Engineering Co., Ltd.), and blank zeroing wells were set. Each group had 3 replicates in parallel. After 24 h and 48 h, the cells were stained with MTT, the absorbance (A) at 492 nm was measured with a microplate reader and adjusted the zero with a blank well, and the cell proliferation rate was calculated according to the following formula: inhibition rate (%) = (1−Test group A_{492}/Control group A_{492}) × 100.

2.3. Quantitative detection of 3T3-L1 preadipocyte differentiation

3T3-L1 preadipocytes were induced to differentiate as described above. The blank control group (control, CON group), *Lycium barbarum* polysaccharide intervention group (LBP group) (LBP was purchased from Ningxia Wolfberry Biological and Food Engineering Co., Ltd) and rosiglitazone intervention group (ROS group) (ROS was purchased from Zhejiang Tianma Pharmaceutical Co., Ltd) were set, and different intervention drugs were added to the culture medium. The extraction process of LBP refers to Xu's method [16], and the concentration of LBP used in this study was 50% (the mass concentration of LBP in dried *Lycium barbarum* (w/w)). LBP was 200 μg/mL (LBP solution concentration (w/v)), ROS was 10 μmol/L, and an equal volume of normal saline was added to the CON group. When the differentiation solution is added at the same time, the drug is changed simultaneously with the culture solution. Oil red O staining was performed on the 8th day of induced differentiation, and photographs were taken. After staining, the lipids in the cells were extracted with isopropanol, and the OD_{510} value of the extracted solution was determined.

2.4. Effects of LBP on the expression of PPARγ, C/EBPα, aP2, FAS and LPL mRNA in 3T3-L1 adipocytes

In a 12-well culture plate, the differentiation of preadipocytes and drug intervention were the same as before. On the eighth day of differentiation, the culture supernatant was discarded, and the cells were collected for use. Real-time polymerase chain reaction was used to detect the expression of *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL* mRNA. The TRIzol Reagent Kit was used to extract the total RNA of each group of cells. One microlitre of RNA sample was added to 99 μL of ultrapure water, colorimetric analysis was performed on a UV spectrophotometer, and the purity of RNA was determined using the ratio of A260 and A280. A260/A280 is between 1.7 and 1.9. Primers were synthesized by Shanghai Bioengineering Technology Services Co., Ltd. Primer information is shown in Table 2. Reverse transcription into cDNA was performed using the AMV First-Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai). The reaction consisted of 1 μg of RNA from each sample and 1.0 μL of Oligo-dT, and DEPC water was added to a final volume of 12 μL. The samples were left at 70°C for 5 min, and then 4 μL of 5 × RT buffer, 1.0 μL of RNasin, and 2 μL of dNTP were added and mixed. After 5 min at ℃, 1.0 μL of M-MLV was added, for a total volume of 20 μL. After 60 min at 42°C, and then at 70°C for 10 min, the reaction was terminated on ice, and cDNA was synthesized. PCR consisted of 5.0 μL of 5 × RT-PCR buffer, 0.3 μL of 250 mmol/L $MgCl_2$, 0.75 μL of 10 mmol/L dNTP, 1.0 μL of 10 μmol/L primer, 1.0 μL of 25 × SYBR Green Ⅰ, 1.0 μL of 10^3 × Calibration, 0.25 μL of 5 U/μL HS Ex-Taq enzyme, 1.0 μL of template, and DEPC H_2O in a final volume of 25 μL. Amplification conditions included pre-denaturation at 95°C for 3 min, and then 75 cycles at 95°C for 20 s and 60°C for 20 s. After the reaction, Sequence Detection System software was used to analyze the threshold cycle (CT) value of each sample detected in the PCR process. The CT value decreased as the template concentration increased. Therefore, the real-time quantitative PCR results showed exactly the same CT value. The mRNA expression level is reversed, and the statistical data were converted into a linear form for statistical processing using the $2^{-\Delta\Delta CT}$ calculation.

2.5. Statistical analyses

Graph Pad Prism 5 was used for statistical processing and charting. The data are expressed as mean ± standard deviation (SD)s. Comparisons between multiple groups were performed using single factor analysis of variance (ANOVA). Comparisons between the two groups were performed using the t-test where $P < 0.05$ was considered as statistically significant.

3. Results

3.1. 3T3-L1 preadipocytes induced differentiation into mature adipocytes

Before inducted differentiation, 3T3-L1 preadipocytes were similar in shape with fibroblasts and were spindle-shaped with no lipid droplets in the cytoplasm. On the 8th day after induction, 90% of 3T3-L1 preadipocytes differentiated into mature adipocytes. They were characterized by abundant cytoplasm and a large quantity of large lipid droplets distributed around the nucleus, forming a "ring-like" structure, which is a typical mature adipocyte morphology (Fig. 3 CON).

3.2. Effect of LBP on the activity of 3T3-L1 adipocytes

To determine whether LBP affects the growth of mature adipocytes, cells were treated with different concentrations of LBP (0, 25,

50, 100, 200 μg/L) for 24 h, and cell viability was measured by MTT assay. Obtained results showed that the LBP with concentration ranging from 25 to 200 μg/mL showed a tendency to inhibit the growth of preadipocytes at 48 h, and it also inhibited the differentiation of 3T3-L1 preadipocytes in a dose-dependent manner (Table 1, Fig. 1).

3.3. Effect of LBP on lipid content of 3T3-L1 adipocyte

After Oil red O staining, isopropyl alcohol was used to lyse the cells. The results showed that compared to the control group, the intracellular lipid content of the LBP group was significantly lowered; however, compared to the ROS group, that was significantly increased, suggesting that LBP can inhibit intracellular lipid accumulation (Fig. 3 and Fig. 4).

3.4. Effect of LBP on PPARγ, C/EBPα, aP2, FAS and LPL mRNA expression in 3T3-L1 adipocytes

After 8 days of LBP treatment of 3T3-L1 preadipocytes, the mRNA expression levels of *PPARγ*, *C/EBPα*, and *FAS* were significantly reduced compared to the CON group ($P < 0.05$); the mRNA expression levels of *PPARγ*, *C/EBPα*, *LPL*, and *FAS* were significantly reduced compared to the ROS group ($P < 0.05$). In contrast, the mRNA expression levels of *aP2* and *LPL* were of no significant difference and did not change obviously compared to the CON group; no significant difference was observed on the expression level of *aP2* between group ($P > 0.05$) (Table 2, Fig. 2).

4. Discussion

Due to drastic changes in modern lifestyles and dietary composition, obesity, as the core metabolic syndrome, has become one of the most important non-infectious chronic diseases threatening human health [17,18]. Adipose tissue is not only the body's passive fuel depot, but also is a large endocrine system that releases a variety of adipokines into systemic circulation that participate in the regulation of the neuroendocrine-immune network [19,20]. The abnormal differentiation of adipocytes can cause an excess amount of body fat, which in turn leads to endocrine dysfunction of adipocytes, leading to insulin resistance and type Ⅱ diabetes [21,22].

The occurrence of obesity is affected by many factors. Excessive nutrition and lack of exercise are the most important environmental factors, which lead to the imbalance of energy intake and consumption by human body [23]. Excessive energy can cause pathological growth of adipocytes, such as the proliferation and differentiation of preadipocytes and excessive hypertrophy of mature adipocytes [24]. The regulation of adipocyte proliferation and differentiation is a major factor affecting insulin resistance caused by obesity, and it is also one of the ways to improve insulin sensitivity and insulin resistance [25,26]. Therefore, inhibiting the formation of mature adipocytes and the formation of lipid droplets

Table 1
Effects of LBP on proliferation of 3T3-L1 preadipocytes.

Concentration (μg/ml)	A492
0	0.842 ± 0.024
25	0.774 ± 0.037*
50	0.758 ± 0.029*
100	0.761 ± 0.047*
200	0.696 ± 0.034*#

*, # indicate significant difference ($P < 0.05$) between tested different treatment group according to *t*-test.

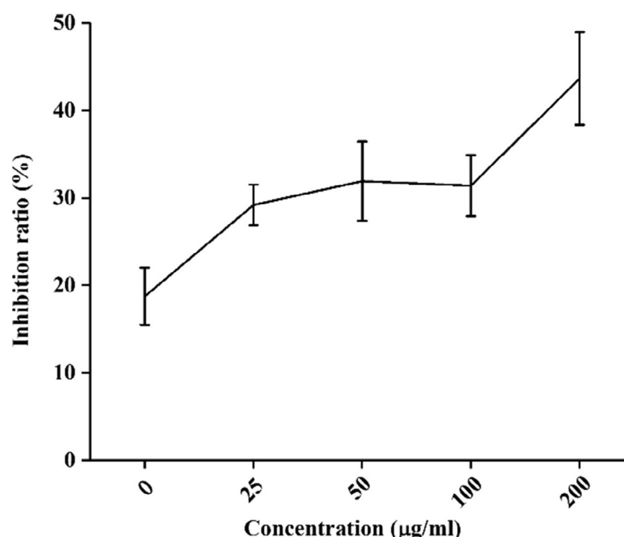

Fig. 1. Inhibition rate of LBP on 3T3-L1 of preadipocytes.

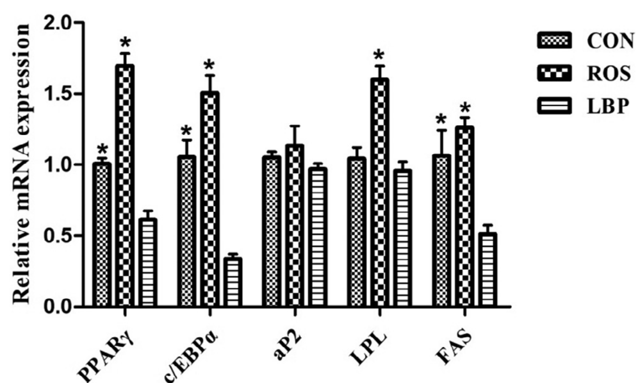

Fig. 2. Effects of LBP on PPARγ, C/EBPα, aP2, FAS, and LPL mRNA expression in 3T3-L1 adipocytes. * indicate significant difference ($P < 0.05$) between tested different treatment group according to *t*-test.

are two important ways to prevent obesity. In recent years, the regulation of adipocyte differentiation and its relationship with the pathogenesis of obesity and insulin resistance has been a hot topic at home and abroad [27,28]. Therefore, the regulation of the proliferation and differentiation of adipocytes and their relationship with the pathogenesis of obesity and insulin resistance have become global hotspots.

Lycium barbarum polysaccharide (LBP) is an important active ingredient in wolfberry, which is a traditional medicine and food ingredients [29]. Modern researches prove that the polysaccharides in wolfberry are one main reactive component for its outstanding efficacy in various fields. It is one of the hotspots in the research field of wolfberry and polysaccharides [30,31]. Verifying the effects of LBP on the proliferation and differentiation of adipocytes is of great significance for the prevention and reduction of metabolic diseases such as type II diabetes, hypertension, dyslipidemia, and atherosclerosis, which are closely related to obesity and insulin resistance. Past study found that activin a plays a critical role in proliferation and differentiation of human adipose progenitors [32]. Bai (2008) found that modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes [33]. Therefore, it is of great significance to research and develop natural products that regulate lipid

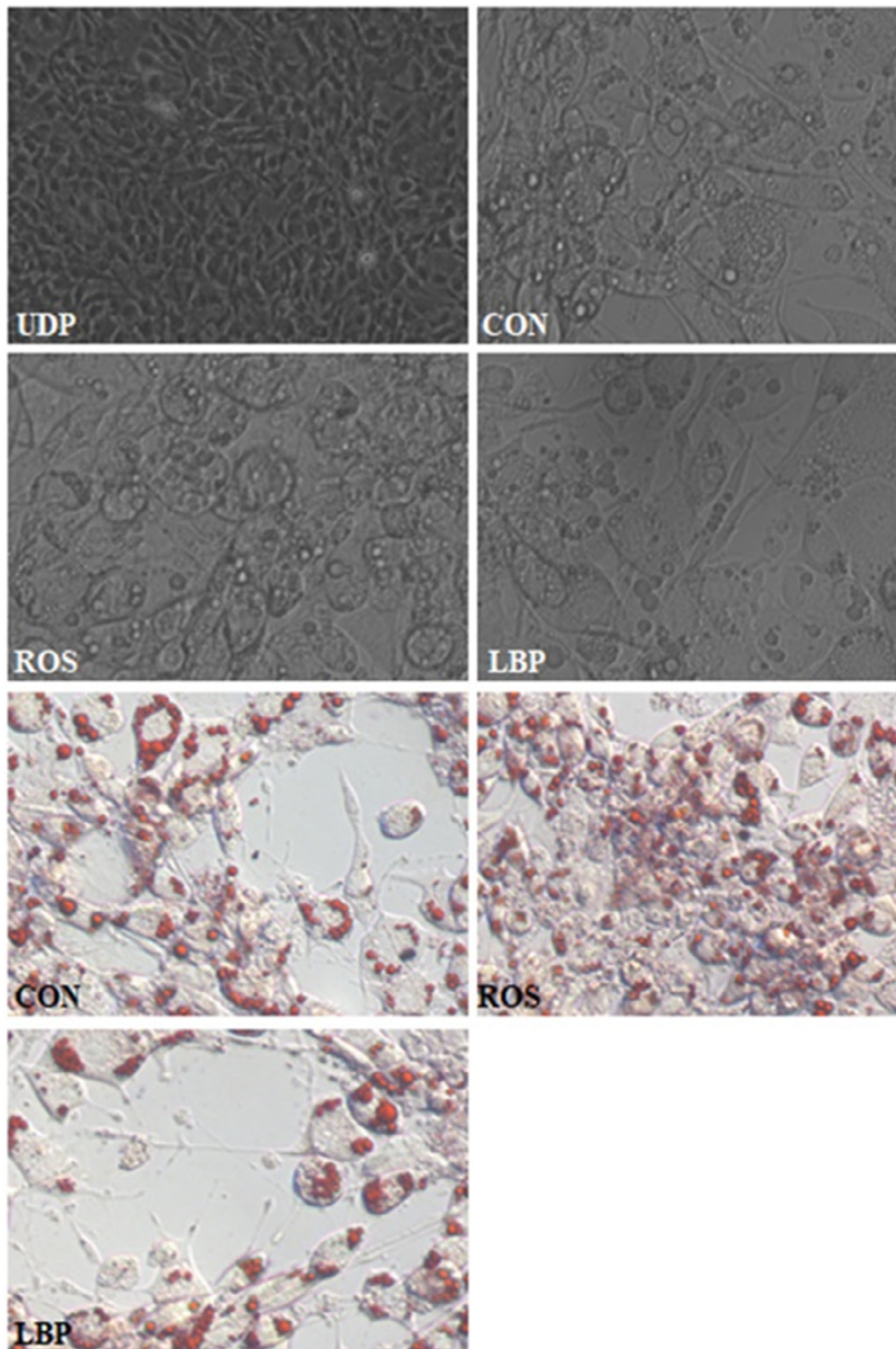

Fig. 3. Effects of LBP on differentiation of 3T3-L1 preadipocytes (red O staining, ×200). 3T3-L1 preadipocytes were treated with LBP (200 μg/mL) and ROS (10 μmol/L), respectively, during cell differentiation. Pictures were taken at the 8th day. Vacuolus in the pictures represented lipid droplet and the degree of differentiation. UDP: undifferentiated preadipocytes; CON: normal control group; LBP: Lycium barbarum polysaccharide-treated group; ROS: rosiglitazone-treated group.

metabolism safely and effectively. Further research on its physiological activity is beneficial to its development and utilization.

In vitro models of adipocytes are invaluable in determining the mechanism of their proliferation, differentiation, secretion of adipokines, and gene/protein expression [34]. The differentiation of 3T3-L1 preadipocytes allows the preadipocytes to proliferate. When the cells are confluent and enter the contact inhibition stage, the cells stop dividing and proliferation; then, under the stimulation of the inducer, the preadipocytes enter a specific cell division phase and begin clonal expansion; finally, the cells enter the terminal differentiation stage and differentiate into mature adipocytes, and cloned proliferation ceases [35,36].

In the present study, we used the MTT method to detect the proliferative activity of 3T3-L1 cells when they differentiate into mature adipocytes. Our results showed that normal 3T3-L1 cells had a vigorous growth state, good extensibility, and long

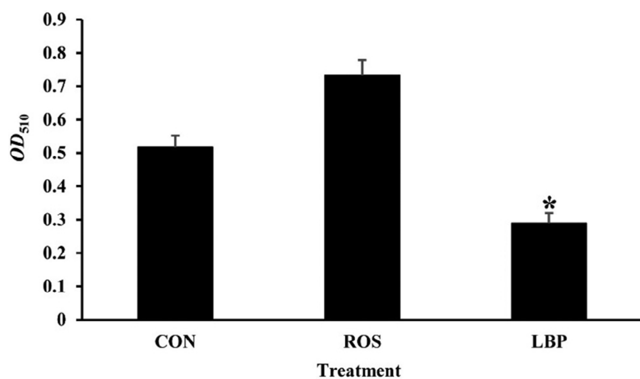

Fig. 4. Changes of lipid droplets during mature adipocytes lipolysis. * indicate significant difference (P < 0.05) between tested different treatment groups according to *t*-test.

Table 2
Primer sequences used for RT-PCR.

Gene name	Primer sequence (5′→3′)	Accession	Product size (bp)
PPARγ	F: GCCAAGGTGCTCCAGAAGATGAC R: GGTGAAGGCTCATGTCTGTCTCTG	U01664.1	103
C/EBPα	F: TGCCCCTCAGTCCCTGTCTTTAG R: GCCCTCCACCTCCCTGTAGC	BC058161.1	92
aP2	F: AGCCAGGAGAACTTTGAGCCATTC R: CCCTTGATGTCCTTCCCTTTCTGG	NC000069.6	80
FAS	F: TGCCCGAGTCAGAGAACCTACAG R: TGCCCGAGTCAGAGAACCTACAG	NM007988.3	101
LPL	F: CGCTCTCAGATGCCCTACAAAGTG R: TTGTGTTGCTTGCCATCCTCAGTC	NM008509.2	80
GAPDH	F: GGCAAATTCAACGGCACAGTCAAG R: TCGCTCCTGGAAGATGGTGATGG	GU214026.1	81

spindle-shaped growth. The cells still have proliferative activity during the terminal differentiation stage. Compared with the CON and ROS groups, LBP can inhibit the proliferation of 3T3-L1 preadipocytes, and show a dose-responsive relationship, which suggested that the administration of LBP regulates the proliferation of preadipocytes.

3T3-L1 preadipocytes are triangular or polygonal. After induction, the cells gradually become round, and lipid droplets are formed within cellular cytoplasm. Over the course of differentiation, the lipid droplets gradually increase in size, and the cell body gradually becomes larger. After the preadipocytes differentiated into adipocytes, the small lipid droplets inside the cells converged into large lipid droplets, and the nucleus was located at the edge of the cell. After the cells were stained with oil red O, the cell outline was clearly visible under the microscope [37]. We observed that 200 μg/mL LBP reduces the synthesis of triglycerides in mature adipocytes, decreases the accumulation of lipids, and increases the hydrolysis of intracellular triglycerides, further preventing the accumulation of lipids in adipocytes, thereby inhibiting fat cell hypertrophy. After 8 days of differentiation of 3T3-L1 preadipocytes, 200 μg/mL LBP showed a certain inhibitory effect on cell differentiation. In addition, 25~200 μg/mL LBP can significantly inhibit cell differentiation. At various time points of differentiation, LBP had an inhibitory effect on the formation of cell lipid droplets and is concentration-dependent and time-dependent. It can be concluded that the lipid-lowering effect of LBP is based on the truth of affecting the proliferation of cells by effectively inhibiting the differentiation of cells and reducing the amount of lipid droplets.

At the same time, adipocyte differentiation is carried out under the regulation of differentiation-related transcription factors. We found that with the inducting of maturation of 3T3-L1 preadipocytes, the expression levels of *PPARγ*, *C/EBPα*, *LPL*, *FAS*, and *aP2* mRNAs gradually decreased, which were contrary to the role of rosiglitazone (ROS). In turn, it initiates the downstream transcription of a series of genes related to lipid metabolism enzymes, accelerates the oxidation and decomposition of fat, and thereby improves the disorder of lipid metabolism. This may be the mechanism of LBP to improve the disorder of lipid metabolism [38]. This suggests that LBP may inhibit adipocyte differentiation and maturation by inhibiting the expression of key factors of adipocyte differentiation *PPARγ*, *C/EBPα LPL*, *FAS*, and *aP2* mRNA, which may be one of its mechanisms to improve insulin sensitivity [39].

However, the synthesis and breakdown of lipids in normal adipocytes are maintained in a dynamic balance, and imbalances in any aspect can cause related diseases [40]. It is speculated that LBP inhibits the differentiation of adipocytes by reducing the expression of *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL* mRNA, then inhibits the accumulation of lipids in adipocytes and the differentiation of preadipocytes into mature adipocytes, and increases the uptake and utilization of glucose. The accumulation of lipids eventually delays the increase in body weight, thereby improving its lipid and carbohydrate metabolism. It shows that LBP can affect fatty acid synthesis and metabolism in 3T3-L1 cells by regulating the expression of *FAS*. The expression of the *LPL* gene does not play a key role in the differentiation and lipid metabolism of 3T3-L1 cells by LBP.

LBP can reduce fatty acid synthesis, increase decomposition, and increase free fatty acids in the body's adipocytes, which may cause ectopic deposition of fat, thereby reducing insulin sensitivity in metabolism-related organs. Because metabolic syndrome is a complex disease, its formation includes the interaction of environmental and genetic factors. However, the mechanism linking LBP and metabolic syndrome cannot be fully explained by an experimental model. This result can only explain a portion of the effect of LBP on adipocytes in adipogenesis and breakdown. The role of LBP in obesity and metabolic syndrome needs further study. Therefore, in the next experiment, we will use western blot technology to further study the expression of major transcription factors that control adipocyte differentiation to explore whether LBP can regulate the expression of transcription factors related to adipocyte differentiation.

5. Conclusions

In summary, LBP can inhibit 3T3-L1 preadipocyte proliferation and decrease the expression of *PPARγ*, *C/EBPα*, *aP2*, *FAS*, and *LPL* mRNA expression, thereby inhibiting the conversion of preadipocytes to mature adipocytes and reducing their lipid production, suggesting that LBP has potential pharmacological effects on regulating lipid metabolism and treating obesity.

Conflict of interest

The authors declare that they have no competing interests.

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