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Effects of Energy Levels on Autophagy, Adipogenic Differentiation and Lipid Metabolism in Subcutaneous and Visceral Pre-Adipocytes

Jie Chen, Qinghua Qiu, Yanjiao Li, Xianghui Zhao, Lanjiao Xu, Xiaowen Xiong, Qingqi Wen, Mingren Qu and Kehui Ouyang*

Jiangxi Province Key Laboratory of Animal Nutrition/Engineering Research Center of Feed Development, Animal Nutrition and Feed Safety Innovation Team, Institute of South Grassland Research, Jiangxi Agricultural University, Nanchang, Jiangxi, 330045, China

Jie Chen and Qinghua Qiu contributed equally to this work as first authors.

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Key words
Subcutaneous pre-adipocytes, Visceral pre-adipocytes, Autophagy, Lipid accumulation, Energy levels

ABSTRACT

This study aimed to investigate the differences in energy levels on autophagy, adipogenic differentiation and fat metabolism between subcutaneous and visceral pre-adipocytes. C57BL/6J male mouse primary subcutaneous and visceral pre-adipocytes were isolated and *in-vitro* adipogenic differentiation was performed in high-glucose medium (4500 mg/L glucose, HGM) or low-glucose medium (1000 mg/L glucose, LGM). Lipid contents in adipocytes were determined by oil red-O staining. Autophagy, adipogenic differentiation and fat metabolism were evaluated by western blot. Subcutaneous pre-adipocytes proliferation rate, differentiation degree and lipid accumulation were higher than that of visceral pre-adipocytes both in HGM and LGM. In HGM group, autophagy marker genes i.e., microtubule-associated protein 1A/1B-light chain 3 (LC-3) and lipolysis protein i.e., hormone-sensitive lipase (HSL) expression in visceral pre-adipocytes were higher than that in subcutaneous pre-adipocytes, but adipogenesis transcriptional factors such as sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor-gamma (PPAR-γ) were lower than that in subcutaneous pre-adipocytes. Moreover, the LGM group increased the expressions of LC-3, reduced the p62 in both subcutaneous and visceral pre-adipocytes, and decreased the expression of the PPAR-γ, SREBP-1c, and HSL. It is shown that triglyceride accumulation of pre-adipocytes in LGM was more than that of HGM. There were differences in autophagy, adipogenesis differentiation and lipolysis between subcutaneous and visceral pre-adipocytes *in vitro*. In addition, the low energy levels on the adipogenesis differentiation and lipid metabolism of subcutaneous pre-adipocytes were greater than that of visceral pre-adipocytes, but not the responses to autophagy.

INTRODUCTION

The growth rate of livestock is fast during the fattening period and high production could be obtained by supplying high energy (Scheffler *et al*., 2014). However, feeding high-energy diets generally increases the fat deposition of the subcutaneous and visceral tissues in varying degrees, which is thought to be a waste and resultant inefficiency in production and economic benefits (Krieger *et al*., 2018; Bachmann *et al*., 2018). Numerous animal studies have demonstrated that energy restriction reduces the overall fat deposition of fattening animals and reduces the subcutaneous and visceral tissue in varying degrees (Abdullah and Musallam, 2007; Khanal *et al*., 2016). Fat deposition consists of two steps: first, pre-adipocytes differentiate into mature adipocytes, and then triglycerides are accumulated in mature adipocytes through fat synthesis and fat degradation. It has been reported in previous studies that different concentrations of glucose affect pre-adipocytes differentiation (Xie *et al*., 2008; Krishna *et al*., 2020). However, the relationship between higher fat deposition and high differentiation of pre-adipocytes is still not clear. Moreover, the difference in energy levels on pre-adipocytes differentiation of subcutaneous and visceral is still unknown.

Animal adipose deposition is generally determined by processes that regulate adipocyte number and size (Macotela *et al*., 2012). The pre-adipocytes undergo differentiation in response to adipogenic signal factors.

* Corresponding author: ouyangkehui@sina.com
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Furthermore, pre-adipocytes undergo differentiation to active major transcriptional signals, such as sterol regulatory element-binding protein 1c (*SREBP-1c*) and peroxisome proliferator-activated receptor-gamma (*PPAR-γ*), that sequentially turns on adipogenic process (Rosen and Macdougald, 2006). Moreover, increased adipogenesis increase adipocyte number, that is, proliferation and differentiation of pre-adipocytes. The size of adipocytes was due to lipogenesis and lipolysis with the help of fatty acid synthase (*FAS*) and hormone-sensitive lipase (*HSL*), respectively (Boström *et al*., 2005; Fujimoto and Parton, 2011). Previous study has reported that subcutaneous pre-adipocytes and visceral pre-adipocytes have the potential of differentiation, but there are biological differences between them because of different origin and growth microenvironment (Fraser *et al*., 2006). Therefore, clarifying the differences in fat deposition between subcutaneous and visceral pre-adipocytes differentiation could be helpful to reveal the regulation of lipogenesis and lipolysis of subcutaneous and visceral adipocytes by dietary energy in animal production.

Many reports revealed that autophagy plays a key role in differentiation of pre-adipocytes (Christian *et al*., 2013). In the process of pre-adipocyte differentiation, cytoplasm's other organelles are gradually degraded and triglycerides gradually occupy those organelles, for further mature adipocytes formation. The mechanism by which cytoplasm is removed is not elucidated; therefore, autophagy is one of the best well-characterized processes to examine degradation of intracellular proteins and organelles in cytoplasm (Mizushima *et al*., 2008). It has been reported that autophagy is a lysosome-mediated self-degradation pathway that widely exists in eukaryotic cells (Klionsky *et al*., 2016). It has also been reported that autophagy plays a key role in the process of differentiation and examining the responses of differentiation; thus, autophagy could be a possible marker (Dong and Czaja, 2011; Goldman *et al*., 2011). In a recent study, its similar impairment of adipocyte differentiation was observed in 3T3-L1 pre-adipocytes in which microtubule -associated protein 1A/1B-light chain-3 (*LC-3*) was removed (Hwang and Lee, 2020), or the autophagy process was blocked by chemical inhibitors (Park *et al*., 2017). Another study has also reported that removal of gene 5 or 7 related to autophagy in the mice reduces lipid biosynthesis and decreases overall body mass of fat tissues (Zhang *et al*., 2009). A recent experiment has proven that energy levels have a great regulatory effect on autophagy (Sugita, 2020), but energy levels in the subcutaneous and visceral pre-adipocytes differentiation and autophagy difference are still unknown. Therefore, in this study, mouse primary subcutaneous and visceral pre-adipocytes were cultured *in vitro* for adipogenesis differentiation with high-glucose or low-glucose, to explore the effect of energy levels on autophagy, adipogenesis differentiation, adipogenesis, and lipolysis between subcutaneous and visceral pre-adipocytes.

MATERIALS AND METHODS

Subcutaneous and visceral primary pre-adipocytes isolation and culture

The 5 week old C57BL/6J male mice were dislocated to death and soaked in ice-cold 1 × phosphorus buffer saline (PBS). The subcutaneous pre-adipocytes were collected from groin, and the visceral pre-adipocytes was collected from epididymis. The isolation and culture of mouse pre-adipocytes were performed as previously described as Wei *et al*. (2015) with minor modifications. Briefly, subcutaneous and visceral adipose tissue was rinsed with 1 × PBS and cut with scissors into approximately 1 mm^3 section under sterile conditions, followed by digestion with 1% type 1 collagenase (Solarbio, China) and 0.25% trypsin (Solarbio, China) at 37 ℃ for 50 min in a shaking water bath. Then, complete medium (Dulbecco's modified Eagle's high-glucose medium, HGM, 4500 mg/L glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (500 μg/mL penicillin and 500 μg/mL streptomycin) was added to stop further digestion. The mixture was filtered through a 150 μm and a 400 μm cell strainer, and centrifuged at 1600 rpm for 10 min. Then, the precipitation was re-suspended with erythrocyte lysate (Solarbio, China) and kept for 10 min. Obtained precipitation was centrifuged at 1200 rpm for 10 min. The pellet containing stromal vascular cells was washed by 1 × PBS, re-suspended in complete medium and incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2. All cell culture experiments were carried out in triplicate per mouse.

Determination of growth curve of subcutaneous and visceral pre-adipocytes

The subcutaneous and visceral primary pre-adipocytes were seeded in 96-well culture plate (n = 6) for 24 h. Then, 10 μL of cell counting kit-8 (CCK-8) (Solarbio, China) solution was added to each well and incubated for 4 h, followed by absorbance measurement at 450 nm by a microplate reader (Thermo Fisher, USA) (day 1). After then, every two days the number of pre-adipocytes was determined by CCK-8 until day 15. The growth curve of pre-adipocytes represented the total succinate dehydrogenase activity per 5 × 10^3 cells.

Differentiation of subcutaneous and visceral pre-adipocytes in HGM or LGM

The subcutaneous and visceral primary pre-adipocytes were seeded in 6-well culture plates and separated into several groups: subcutaneous pre-adipocytes in high-glucose medium (SH), subcutaneous pre-adipocytes in low-glucose medium (Dulbecco's modified Eagle's low-glucose medium (LGM, 1000 mg/L glucose) (SL), visceral pre-adipocytes in high-glucose medium (VH), visceral pre-adipocytes in low-glucose medium (VL). The differentiation method was carried out as described in previous study (Kaneko *et al*., 2018). For adipocyte differentiation, 100% confluent cells were further incubated for the complete cell cycle arrest and for the start of clonal expansion in HGM or LGM that was supplemented with 10% FBS and 1% antibiotics, which was further supplemented with 1.72 μM insulin (Solarbio, China), 0.5 mM 1-methyl-3-isobutylxanthine (Solarbio, China), and 1 μM dexamethasone (Solarbio, China) (day 0). Two days later, the cells were further incubated in HGM or LGM supplemented with 1.72 μM insulin, for two days. After that, the cells were replaced by the fresh HGM or LGM every two days until the cells were completely differentiated.

Evaluation of accumulated lipid contents in adipogenesis

Cells were washed twice with 1 × PBS solution, fixed in 4% paraformaldehyde 150 μL/well for 30 min at 4 ℃, and then fixative was discarded, washed with twice 1 × PBS. Cells were stained in the oil red-O (Solarbio, China) working solution (3:2, Saturated oil red-O solution: water) 120 μL/well protected from light for 30 min at 25 ℃ and washed twice with 1 × PBS. To quantify lipid accumulation, isopropanol/water (6:4) solution was added to the stained culture-well, and the dye was extracted with gentle shaking for 40 min at room temperature. After that, the absorbance at 510 nm was measured with a microplate reader. Accumulated lipid content in adipocytes was expressed as the amount of intracellular lipid accumulated per 5×10^3 cells.

Western blot analysis

The cells were collected and washed with ice-cold 1 × PBS and lysed in the Radio-Immunoprecipitation Assay buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) (Solarbio, China) containing protease inhibitor cocktail (100 ×) (CWBIO, China) on ice for 10 min. Cell lysates were centrifuged at 12,000 rpm for 15 min at 4 ℃. The supernatant was then collected for SDS-PAGE analysis. Protein concentration was determined using the protein assay kit (CWBIO, China). Total proteins (20 μg) were separated on a 10% or 12% SDS-PAGE unless indicated otherwise and transferred to nitrocellulose membrane using a wet transfer system (Bio-Rad, USA) for 90 min at 200 mA. The membrane was blocked for 1.5 h at room temperature in the 5% skim milk/TBS/T (50 Mm Tris-HCL, pH 7.6, 0.1 M NaCl, and 0.1% tween-20) solution. The incubation with antibodies against LC-3 (rabbit polyclonal, 1:600, 14600-1-AP, Proteintech™), p62 (rabbit polyclonal, 1:4000, 18420-1-AP, Proteintech™), SREBP-1c (rabbit polyclonal, 1:2000, 14088-1-AP, Proteintech™), PPAR-γ (rabbit polyclonal, 1:1000, 16643-1-AP, Proteintech™), FAS (rabbit polyclonal, 1:700, 10624-1-AP, Proteintech™), HSL (rabbit polyclonal, 1:1000, 17333-1-AP, Proteintech™) and GAPDH (mouse monoclonal, 1:70000, 60004-1-Ig, Proteintech™) were dealed overnight at 4 ℃ in TBS/T. The membrane was then incubated with secondary antibodies (anti-rabbit IgG, 1:7000, zb-2305, ZSGB-BIO™) for 1.5 h at room temperature. Finally, the membrane was washed thrice in TBS/T for 10 min and analyzed with Versa Doc™ 4000 MP (Bio-Rad, USA). Semi-quantitative analysis of the data was performed using Image J (NIH). Protein band density was quantified and normalized to the GAPDH.

Statistical analysis

All data were obtained from triplicate measurements. The data are expressed as mean ± SD. The double-tailed t-test was used to analyze differences between groups after checking normal distribution. For comparisons among groups, one-way ANOVA was used, followed by the Duncan test. Statistical were calculated with SPSS 22.0 for Windows (SPSS Inc., Chicago IL, USA). Differences were considered significant at when $0.01 < p < 0.05$ (* or #) and $p < 0.01$ (** or ##).

RESULTS

Morphology and growth curve of subcutaneous and visceral primary pre-adipocytes

As shown in Figure 1A and 1B, mouse subcutaneous and visceral primary pre-adipocyte adherent cells were observed to be fusiform, polygonal, or irregular, and the primary cells of subcutaneous and visceral had no difference in morphology. As shown in Figure 1C, the growth curves of subcutaneous and visceral pre-adipocytes were S-shaped and experienced latent period (days 1-5), logarithmic growth period (days 5-11), plateau growth period (days 11-13), and aging period (days 13-15). The proliferation rate of subcutaneous pre-adipocytes was faster than that of viscera after day 7.

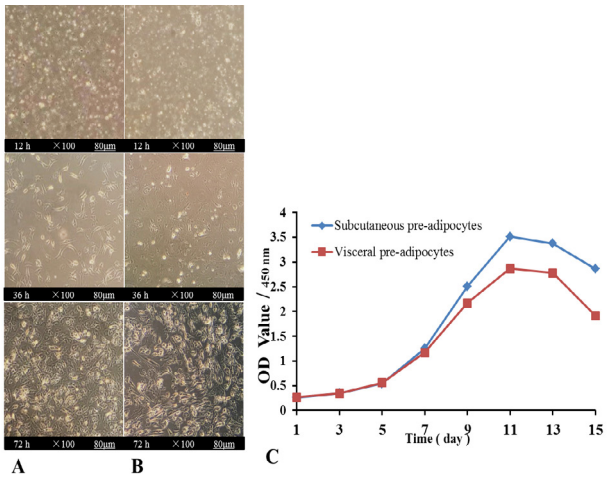

Fig. 1. Morphology of pre-adipocytes of subcutaneous and visceral extracted *in vitro*. The morphology of cells at 100 ×, A and B scalebar was 80 μm. Subcutaneous pre-adipocytes (A) and visceral pre-adipocytes (B) were photographed under the bright-field microscope at the indicated time (12 h, 36 h, 72 h). Mouse subcutaneous and visceral primary pre-adipocytes adherent cells were observed 12 h after inoculation, which were quasi-round in shape and varied in size. After 36 h of inoculation, the number of adherent cells increased greatly, and the shape was fusiform, polygonal or irregular, with a strong three-dimensional feeling. About 72 h after inoculation, the primary cells can grow to monolayer fusion, and then the primary cells become long fusiform and oval in further culture. (C) The growth curve of mouse primary subcutaneous and visceral pre-adipocytes at the indicated days (D0, D1, D3, D5, D7, D9, D11, D13, and D15). Values are presented as the mean ± SD.

Differentiation and lipid evaluation of subcutaneous and visceral pre-adipocytes

To investigate the effect of energy levels on subcutaneous and visceral pre-adipocytes differentiation, intracellular lipids were observed (Fig. 2A and 2B) and quantified (Fig. 2E) after oil red-O staining (Fig. 2C and 2D). Results showed that a large amount of reflective grease was observed on day 10. In addition, the concentration of triglyceride in subcutaneous pre-adipocytes was more than visceral in both HGM and LGM (SH > VH, $p < 0.01$; SL > VL, $p < 0.01$). Although the size of adipocytes was greater in HGM, the total amount of triglycerides was lower than LGM (SH < SL, $p < 0.01$; VH < VL, $p < 0.05$). The results showed that low glucose promotes the accumulation of triglycerides in subcutaneous and visceral pre-adipose during differentiation and has a promoting effect on visceral pre-adipocytes (VL - VH (0.039) > SL - SH (0.027), $p > 0.05$).

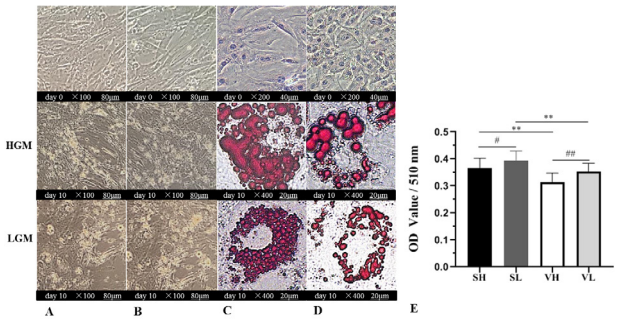

Fig. 2. Adipocyte differentiation from subcutaneous and visceral pre-adipocytes. HGM represents the differentiation of this row into high-glucose medium (supplemented 4500 mg/L glucose), and LGM represents the differentiation of this row into low-glucose medium (supplemented 1000 mg/L glucose). Subcutaneous (A) and visceral pre-adipocytes (B) were photographed under the bright-filed microscope at day 0 and day 10. Subcutaneous pre-adipocytes (C) and visceral pre-adipocytes (D) were stained with oil red-O dye was photographed under the bright-field microscope at day 0 and day 10. Cells at the indicated days were stained with Oil-Red O dyes as described in "Materials and methods". (E) Histogram of triglyceride extraction after differentiation of subcutaneous in high-glucose medium (SH), subcutaneous in low-glucose medium (SL), visceral in high-glucose medium (VH), visceral in low-glucose medium (VL). The values were expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$, comparisons were done between SH and VH, SL and VL, # $p < 0.05$, ## $p < 0.01$, comparisons were done between SH and SL, VH, and VL.

Autophagy-related protein content in pre-adipocytes during differentiation

As shown in Figure 3, our results showed that autophagy-related protein LC-3 expression increased by differentiation time. The LC-3 protein in SH was increased at day 0, 2 and 6, but reduced at day 4, 8, and 10 compared with VH. The SL was lower than VL on day 2, 8 and 10. In energy levels, the LC-3 protein in LGM was higher than the HGM both in subcutaneous and visceral pre-adipocytes during differentiation. The p62 gene is a negative regulatory gene of autophagy, and its expression is negatively correlated with autophagy to some extent. The protein expression of p62 has reached the maximum on day 6 and decreased during differentiation (Fig. 3D). The p62 protein in SH was higher than VH on day 2, 4 and 10. The SL of p62 protein was higher than VL on day 4 and 6. In energy levels, the p62 protein in HGM was higher than LGM both in subcutaneous and visceral pre-adipocytes during differentiation. Our results indicated that low energy stimulated autophagy of subcutaneous and visceral pre-adipocyte during differentiation. It was shown

that the enhancement of subcutaneous pre-adipocytes was lower than that of viscera on autophagy during differentiation (Table I).

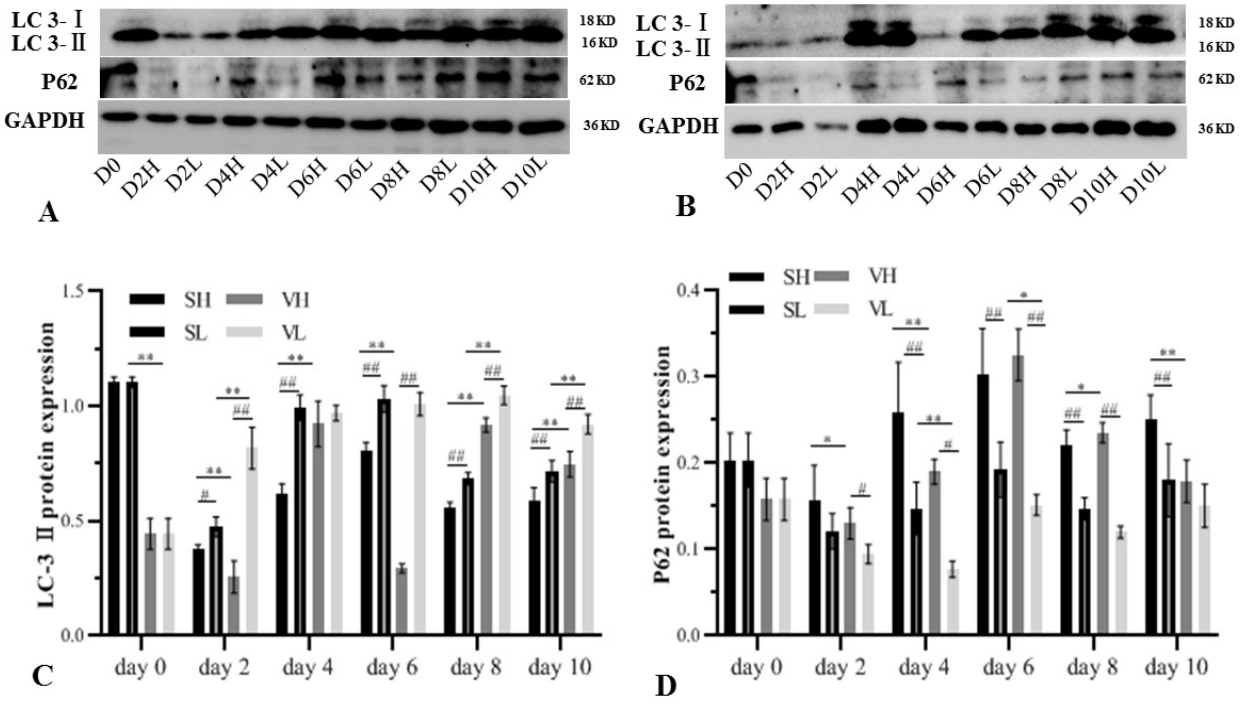

Fig. 3. Expression of autophagy-related protein during adipogenesis differentiation. Subcutaneous pre-adipocyte in high-glucose medium (SH), subcutaneous pre-adipocyte in low-glucose medium (SL), visceral pre-adipocyte in high-glucose medium (VH), visceral pre-adipocyte in low-glucose medium (VL) at the indicated days (D0, D2, D4, D6, D8, D10). High-glucose medium supplemented 4500 mg/L glucose, low-glucose medium supplemented 1000 mg/L glucose. Protein band of LC3 and p62 in subcutaneous (A) and visceral pre-adipocyte (B). The cells extracts were prepared at the indicated days, and total cell proteins (25 μg) were separated on a 12% SDS-PAGE gel and analyzed by western blotting using abti-LC3 and anti-p62 antibody. Loading control was verified by GAPDH. Gray value quantification of LC-3 (C) and P62 (D). The values were expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$ comparisons were done between SH and VH, SL and VL, # $p < 0.05$, ## $p < 0.01$ comparisons were done between SH and SL, VH, and VL.

Table I. Average protein expression content of subcutaneous and visceral pre-adipocytes in high-glucose medium and low-glucose medium during differentiation.

Protein name	SH	SL	VH	VL	(SL –SH)/SH	(VL-VH)/VH
LC-3	0.59±0.15^{a}	0.78±0.21^{b}	0.63±0.31^{ab}	0.95±0.09^{c}	32.20%	50.79%
p62	0.23±0.06^{a}	0.16±0.04^{b}	0.20±0.07^{a}	0.12±0.03^{b}	-33.67%	-41.81%
SREBP-1c	1.04±0.20^{a}	0.71±0.15^{b}	0.49±0.26^{c}	0.48±0.23^{c}	-31.73%	-2.04%
PPAR-γ	0.30±0.07^{a}	0.23±0.04^{b}	0.29±0.12^{ab}	0.27±0.06^{ab}	-23.33%	-6.90%
HSL	0.13±0.06^{a}	0.09±0.04^{a}	0.29±0.17^{b}	0.23±0.17^{b}	-30.77%	-20.69%
FAS	0.40±0.23^{a}	0.39±0.18^{a}	0.43±0.21^{a}	0.47±0.20^{a}	-2.50%	9.30%

Note: The values were shown as means ± SD, different letters in the same row means significant between the treatments ($p < 0.05$), same letter in the same row means no significant between the treatments ($p > 0.05$). The data come from western blot gray value analysis, which is the relative expression analysis of differentiation day 0 to 10. Subcutaneous pre-adipocyte in high-glucose medium (SH), subcutaneous pre-adipocyte in low-glucose medium (SL), visceral pre-adipocyte in high-glucose medium (VH), visceral pre-adipocyte in low-glucose medium (VL). High-glucose medium supplemented 4500 mg/L glucose, low-glucose medium supplemented 1000 mg/L glucose. (SL-SH)/SH and (VL-VH)/VH was represents the low-glucose medium relative high-glucose medium of target expression, value > 0 represents promotion, value < 0 represents inhibition.

Protein content of adipogenic transcription factors in pre-adipocytes during differentiation

As shown in Figure 4, during the differentiation period, the SREBP-1c and PPAR-γ protein expression was increased initially and then decreased during differentiation. The SREBP-1c protein expression in SH was higher than VH during differentiation, and the SL also was higher than VL except on day 4 and 10. In energy levels, the SREBP-1c protein expression in HGM was higher than the LGM in both subcutaneous and visceral pre-adipocytes. Low glucose decreased the expression of SREBP-1c in subcutaneous and visceral pre-adipocytes during differentiation, especially in subcutaneous pre-adipose. The PPAR-γ protein expression results showed that (Fig. 4D), the protein contents of PPAR-γ were not different in subcutaneous and visceral pre-adipocytes during differentiation (only at day 6, the SL was lower than VL). But in energy levels, the PPAR-γ protein in HGM was higher than the LGM on day 4 and 6; however, no difference was observed on other days. Our results indicated that low energy inhibited adipogenic transcription factors of SREBP-1c and PPAR-γ in subcutaneous and visceral pre-adipocyte during differentiation, and the inhibition of subcutaneous pre-adipocytes was higher than that of viscera pre-adipocytes (Table I). The results showed that low glucose decreased the differentiation degree of subcutaneous and visceral pre-adipocytes, especially on subcutaneous pre-adipocytes.

Protein content of adipogenesis and lipolysis in pre-adipocytes during differentiation

The results of the pre-adipocytes differentiation period and fat metabolism-related protein expression are shown in Figure 5. The FAS protein expression was increased by differentiation time. The FAS protein expression in SH was increased at day 0, 6 and 10, but reduced at day 4 and 8 compared with VH. The SL was increased on day 8 and 10, reduced on day 2 and 4 compared with VL. In energy levels, the FAS protein in HGM was increased at day 4 and 10 of pre-adipocytes and reduced at day 2 and 8 in

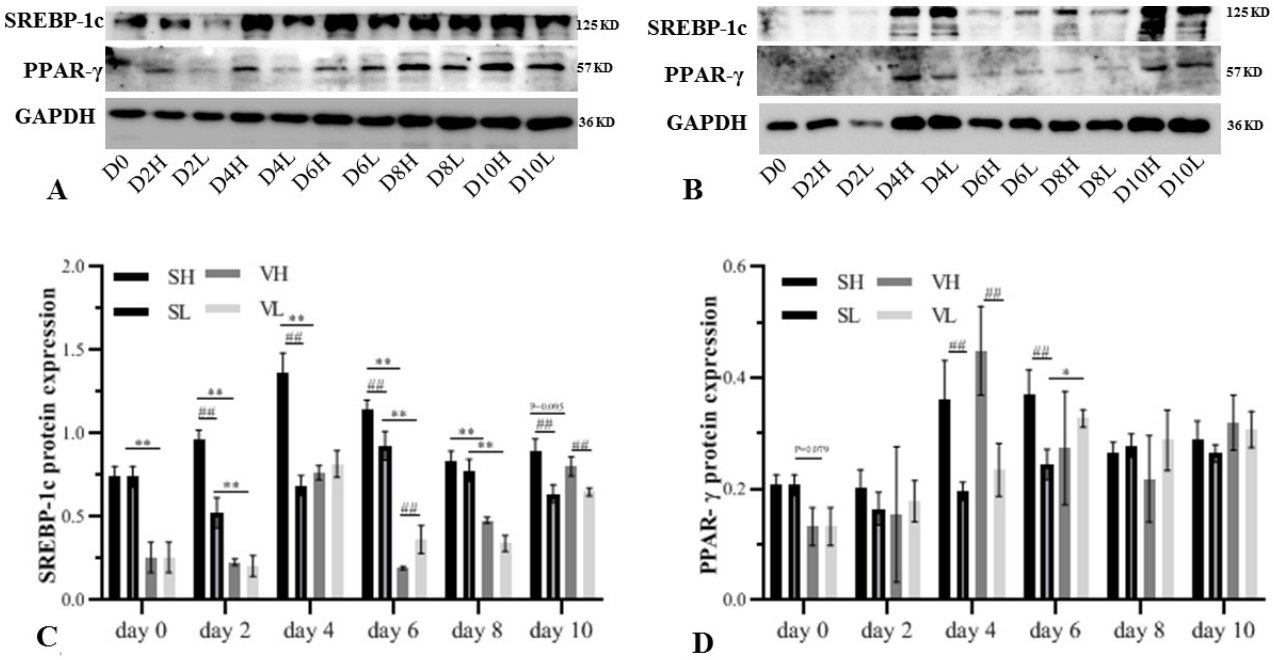

Fig. 4. Expression of adipogenic transcription factors related protein during adipogenesis differentiation. Subcutaneous pre-adipocyte in high-glucose medium (supplemented 4500 mg/L glucose) (SH), subcutaneous pre-adipocyte in low-glucose medium (supplemented 1000 mg/L glucose) (SL), visceral pre-adipocyte in high-glucose medium (VH), visceral pre-adipocyte in low-glucose medium (VL) at the indicated days (D0, D2, D4, D6, D8, D10). High-glucose medium supplemented 4500 mg/L glucose, low-glucose medium supplemented 1000 mg/L glucose. Protein band of SREBP-1c and PPAR-γ in subcutaneous pre-adipocyte (A) and visceral pre-adipocyte (B). cells extracts were prepared at the indicated days, and total cell proteins (25 g) were separated on a 10% SDS-PAGE gel and analyzed by western blotting using abti-SREBP1c and anti-PPARγ antibody. Loading control was verified by GAPDH. Gray value quantification of SREBP-1c (C) and PPAR-γ (D). The values were expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$ comparisons were done between SH and VH, SL and VL, # $p < 0.05$, ## $p < 0.01$ comparisons were done between SH and SL, VH, and VL.

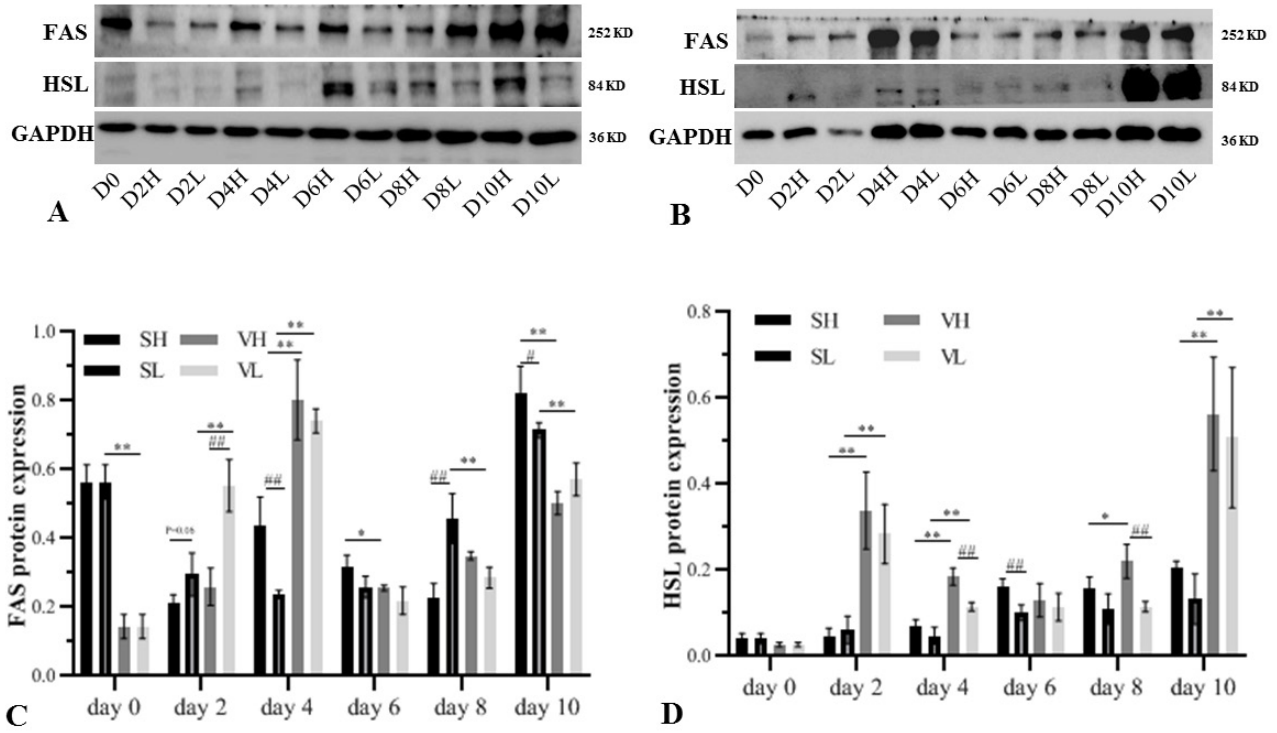

Fig. 5. Expression of lipogenesis and lipolysis-related protein during adipogenesis differentiation. Subcutaneous pre-adipocyte in high-glucose medium (SH), subcutaneous pre-adipocyte in low-glucose medium (SL), visceral pre-adipocyte in high-glucose medium (VH), visceral pre-adipocyte in low-glucose medium (VL) at the indicated days (D0, D2, D4, D6, D8, D10). High-glucose medium supplemented 4500 mg/L glucose, low-glucose medium supplemented 1000 mg/L glucose. Protein band of HSL and FAS in subcutaneous pre-adipocytes (A) and visceral pre-adipocytes (B). Cells extracts were prepared at the indicated days, and total cell proteins (25 μg) were separated on a 8% SDS-PAGE gel and analyzed by western blotting using abti-FAS and anti-HSL antibody. Loading control was verified by GAPDH. Gray value quantification of FAS (C) and HSL (D). The values were expressed as mean ± SD, * $p < 0.05$, ** $p < 0.01$ comparisons were done between SH and VH, SL and VL, # $p < 0.05$, ## $p < 0.01$ comparisons were done between SH and SL, VH, and VL.

subcutaneous compared with the LGM. There was no difference between HGM and the LGM in visceral pre-adipocytes except on day 2. These results indicated that low glucose inhibited adipogenesis of FAS in subcutaneous expression but stimulated visceral pre-adipocyte during differentiation (Table I). The lipolysis protein expression of HSL was increased during differentiation (Fig. 5D). The HSL protein expression in SH was lower than VH except on day 6, and the SL was also lower than VL except day 6 and 8. In energy levels, the HSL protein expression in LGM was lower than HGM, indicating that low energy was inhibiting lipolysis both in subcutaneous and visceral pre-adipocyte during differentiation, and inhibition of subcutaneous pre-adipocytes was higher than that of viscera pre-adipocytes (Table I). These results showed that low glucose significantly decreased the expression of lipolysis in subcutaneous and visceral pre-adipocytes during differentiation, and weakened the process of fat degradation, especially on subcutaneous pre-adipocytes.

DISCUSSION

As a mature cell line, 3T3-L1 has been well studied in adipocytes (Fan *et al*., 2019), but its properties and characteristics are far from those of primary adipocytes. They do not represent the function of subcutaneous and visceral pre-adipocytes. In the present study, we extracted mouse subcutaneous and visceral pre-adipocytes culture *in vitro*. Subcutaneous and visceral pre-adipocytes were not different in cell morphology as Toyoda *et al*. (2009) have described, no significant difference in morphology was observed between subcutaneous and visceral. In terms of cell proliferation, subcutaneous and visceral pre-adipocytes were different in the logarithmic growth and decline phases. This may explain why the number of subcutaneous adipocytes was greater than viscera. In addition, subcutaneous pre-adipocytes lipid accumulation was more than viscera, which was the same as that of HGM and LGM differentiation as previous study in 3T3-

L1 was described (Lustig *et al*., 2017). From the stained with oil red-O dyes photographed, the lipid droplets formed by HGM differentiation were more extensive than that by LGM, but the LGM differentiation of triglycerides accumulation was more than HGM differentiation, in both subcutaneous and visceral. These results were not consistent with cattle and cows, feeding high concentrate diet could significantly increase fat deposition in Hanwoo Cattle muscle (Reddy *et al*., 2017), and high energy group increased total visceral adipose and abdominal adipose of cow (Drackley *et al*., 2014). The lipid droplet volume of pre-adipocyte fusion induced by high glucose is larger than that induced by low glucose. We speculate that the rate of β-oxidation may be more intense in high glucose environment, although we did not detect the index of β-oxidation. This may be a possible explanation for the contradiction between the volume of lipid droplets and total triglycerides induced by different energy levels.

Many reports have revealed that the autophagic process is required for pre-adipocytes adipogenesis (Dong and Czaja, 2011; Armi *et al*., 2013). Autophagy plays important role in recycling intracellular energy resources in response to nutrient-depleted conditions and removing cytotoxic proteins and organelles under stressful conditions (Fujimoto and Parton, 2011). Our results showed that the expression of autophagy-related genes fluctuated with the induction of differentiation time, and the expression of autophagy protein LC-3 increased gradually. Our result was consistent with that of Hahm *et al*. (2014), the protein of LC-3 was gradually increased by differentiation day. Our results showed that during differentiation of LGM, the protein expression of LC-3 in subcutaneous and visceral pre-adipocytes was higher than that in HGM, which was consistent with previous results that alleged that low energy could induce autophagy (Yoshizaki *et al*., 2012). The low glucose (1000 mg/L) used in this study was aimed to fulfill minimum requirement for cell growth; thus, it can be assumed that autophagy occurs due to lack of energy for cell differentiation or pre-adipocytes faced survival stress (Mizushima *et al*., 2004). Furthermore, the LGM differentiation of lipolysis and adipogenesis transcriptional factors was lower than HGM, which may be caused by autophagy. The LGM was used in this study not only enhanced the degree of autophagy of pre-adipocytes, but also increased the accumulation of triglycerides, indicating that increasing autophagy in the process of differentiation can increase the differentiation of pre-adipocytes. Moreover, we found that visceral autophagy was higher than subcutaneous pre-adipocyte during differentiation, with Choi *et al*. (2020) in mouse result was similar, reduced adipose tissue mass was more prominent in visceral compared to subcutaneous white adipose tissue by activating autophagy. Although we did not detect the expression of Beclin1, another important regulatory pathway of autophagy, we speculate that the expression of Beclin1 gene in visceral pre-adipocytes may also be higher than that in subcutaneous pre-adipocytes (Yang *et al*., 2014). Results represent that visceral pre-adipocytes may be has more sensitivity to autophagy. Moreover, our study found that the protein expressions of LC3 in subcutaneous was higher than visceral at day 0. These findings have already been explained in the 3T3 pre-adipocytes expression with differentiation time (Hahm *et al*., 2018; Zutphen *et al*., 2014), who partially explained that the subcutaneous pre-adipocytes proliferation is more than that of viscera, and after receiving the differentiation signal, the cell state changes lead to subcutaneous pre-adipocytes cycle arrest (Wei *et al*.,2018). We hypothesized that the higher lipolysis of visceral pre-adipocytes during differentiation might be due to their higher degree of autophagy, that is, autophagy enhances the role of visceral lipolysis. It may also inhibit the expression of SREBP-1c (Zhang *et al*., 2013), leading to the difference in triglycerides accumulation between subcutaneous and visceral pre-adipocytes differentiation.

Pre-adipocytes differentiation was a crucial and strictly regulated process that occurs principally in adipose tissue, in which key transcription factors, such as PPAR-γ and SREBP-1c, sequentially turn on adipogenic process (Ameer *et al*., 2014). Our study results show that the protein expression of SREBP-1c in subcutaneous pre-adipocyte was higher than that in viscera, and there was no difference in the expression of PPAR-γ protein between subcutaneous and visceral. We speculate that it is precisely because PPAR-γ is an important transcription factor of adipogenesis and a decisive switch that determines the differentiation of pre-adipocytes into mature adipocytes, so its role in the process of adipogenic differentiation of pre-adipocytes in different parts should be consistent, so there is no significant difference in PPAR - γ protein expression between subcutaneous and visceral pre-adipocytes (Wajchenberg *et al*., 2002). D-allulose (a rare sugar with almost zero energy) could decrease intracellular lipid accumulation during 3T3-L1 adipocyte differentiation, and along with reducing PPAR-γ mRNA expression, but the effect on SREBP gene was not significant (Moon *et al*., 2020). The subcutaneous and visceral pre-adipocyte SREBP-1c gene in LGM was lower than that of HGM as previously described in offspring mouse (Vithayathil *et al*., 2018), and it may be regulated by autophagy. This indicated that low glucose reduced the adipogenic differentiation of subcutaneous pre-adipocytes, but this effect was minor in viscera. However, LGM differentiation accumulated lipid more than HGM while

reduced adipogenesis transcriptional factors and inhibited the lipolysis. These results explored that the adipocytes differentiation was different with triglyceride accumulation (Thounaojam *et al*., 2011). Pre-adipocyte differentiation was regulated by many genes, and triglyceride accumulation is the result of the dynamic action of lipogenesis and lipolysis. Although the degree of adipogenic differentiation can affect the triglyceride accumulation of cells, but the accumulation of more triglycerides does not mean that the degree of adipogenic differentiation is very high.

According to previous reports, lipid accumulation was caused by subtle differences between lipogenesis and lipolysis (Shuster *et al*., 2012; Poussin *et al*., 2008). In the process of pre-adipocyte adipogenic differentiation, day 0-2 was considered a determinant of cellular behaviors at the late stage, and day 2-10 can be regarded as the process of pre-adipocyte accumulation of triglyceride. Our results showed that FAS expression in subcutaneous and visceral was not different in HGM and LGM. An interesting phenomenon is that the expression of FAS in subcutaneous pre-adipocyte was higher than that of visceral on day 0. This may be an explanation that the adipogenic differentiation potential of subcutaneous pre-adipocytes is higher than that of visceral pre-adipocytes. In addition, a previous study found that primary pre-adipocytes have the potential to differentiate automatically even without the addition of inducers consistent (Abuna *et al*., 2016). Although FAS protein expression in viscera was higher than subcutaneous in the early stage (day 2 and 4) of differentiation time, but the early stage is not the peak of triglyceride accumulation, and pre-adipocytes must be degraded other organelles such as mitochondria before they began to accumulate triglycerides (Drackley *et al*., 2014). Adipogenic differentiation is a continuous process, and the accumulation of triglycerides is affected by adipogenesis and lipolysis, which is not determined by the difference in gene expression at every moment. Lipolysis is a catabolic pathway that promotes triglycerides mobilization in a step-wise fashion by increasing HSL (Wang *et al*., 2013). Our results showed that the expression of HSL in visceral was higher (day 2 and 10) than subcutaneous at HGM differentiation, indicating that visceral pre-adipocyte lipolysis maybe related to autophagy (Harada *et al*., 2003). Although lipolysis is not the main metabolic pathway in pre-adipocyte adipogenic differentiation stage, fat deposition is the result of the combined action of adipogenesis and lipolysis.

Besides, our results found that LGM differentiation of pre-adipocytes reduces lipolysis along with induced autophagy, which is inconsistent with previous results that autophagy enhancement was along with an increase in lipolysis (Shin *et al*., 2014; Leu *et al*., 2018). We hypothesized that in the LGM differentiation process, both subcutaneous and visceral pre-adipocytes produce a high level of autophagy due to the lack of energy needed for cell differentiation (Sathyanarayan *et al*., 2017). This autophagy can degrade other organelles, but it may also indirectly inhibit the occurrence of lipolysis, indicating that the lipolysis gene of HSL in LGM was lower than HGM in subcutaneous and visceral pre-adipocytes differentiated. Moreover, our findings represent that the subcutaneous is more sensitive to changes in energy levels. Our study suggests that energy supply should not be restricted during the early cell development stage to ensure the normal biological development of cells. This study may also provide information to reduce targeted fat accumulation *via* improving certain lipolysis. Adipose differentiation is a complex process, but the detailed functional connection between autophagy and adipogenesis or lipolysis remains further investigated.

CONCLUSION

Current results showed differences in autophagy, adipogenic differentiation, and lipolysis between subcutaneous and visceral pre-adipocytes *in vitro*. At the stage of adipose differentiation, visceral pre-adipocytes attenuate lipid accumulation *via* stimulating lipolysis and inhibiting adipogenic transcriptional factors during adipogenesis as compared to subcutaneous pre-adipocytes. Low-energy enhanced the autophagy of pre-adipocytes and decreased the differentiation of subcutaneous pre-adipocytes and inhibited the lipolysis of subcutaneous and visceral pre-adipocytes. The effect of decreasing the energy levels on the adipogenic differentiation and lipid metabolism of subcutaneous pre-adipocytes was greater than that of visceral pre-adipocytes, except for autophagy. However, the relationships among energy levels induced autophagy, lipolysis, and adipose transcription factors need to be further studied.

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Institutional review board statement

The experimental protocol was approved by the animal ethics committee of Jiangxi Agricultural University

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Statement of conflicts of interest

The authors have declared no conflicts of interest.

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