



Effects of Glucose Concentration on Differentiation, Adipogenic Marker Genes Expression and Glucose Transporter Distribution in Bovine Subcutaneous Preadipocytes

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ABSTRACT

Background: Fat deposition in beef cattle significantly influences both the meat yield of individual carcasses and the perceived eating quality for consumers. Glucose, as a crucial regulator, plays an essential role in the differentiation of bovine preadipocytes and lipid metabolism. This study aimed to investigate the effects of varying glucose concentrations (0, 3.0, 3.5 and 4.0 mmol/L) on the differentiation of bovine subcutaneous preadipocytes. Additionally, we explored the expression levels of key adipogenic marker genes and the distribution of glucose transporters during adipocyte differentiation.

Methods: The differentiation of preadipocytes was assessed using Oil Red O staining and triacylglycerol content was quantified using a triglyceride assay kit. To evaluate the expression of adipogenic genes included peroxisome proliferator-activated receptor- γ (PPAR γ), fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC1), quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure mRNA levels, while protein levels were determined through western blotting. Additionally, the distribution of glucose transporters GLUT1 and GLUT4 during differentiation was analyzed through immunofluorescence.

Result: The results showed that the differentiation of preadipocytes, along with the accumulation of lipid droplets, was enhanced at 2 and 4 days ($p < 0.05$). However, this effect gradually diminished by day 8 as glucose concentration increased. During the early stages of preadipocyte differentiation, the expression of adipogenic marker genes-PPAR γ , FAS and ACC1-significantly increased at both the mRNA and protein levels ($p < 0.05$, $p < 0.01$). However, by day 8, as glucose concentration increased, a decreasing trend in the expression of these genes was observed. The fluorescence intensity of glucose transporters GLUT1 and GLUT4 rose significantly with higher glucose concentrations. These findings suggested that adding glucose at 3.0 to 3.5 mmol/L to the differentiation medium is optimal for promoting differentiation and fat deposition in bovine subcutaneous preadipocytes.

Key words: Bovine subcutaneous preadipocytes, Differentiation, Gene expression, Glucose transport, Glucose.

INTRODUCTION

Beef consumption has been rapidly increasing worldwide (Sabow *et al.*, 2020). A high concentrate-based diet is commonly fed to beef cattle during the fattening phase to optimize growth performance (Grauagnard *et al.*, 2010; Li *et al.*, 2021b). Starch of dietary cereal grains is degraded in rumen to produce propionic acid, then absorbed into the liver, where it is synthesized into glucose through the gluconeogenesis pathway (Noziere *et al.*, 2010). Glucose plays a crucial role not only as an energy source but also as a precursor for fat synthesis, which promotes the accumulation of subcutaneous fat.

The deposition of subcutaneous fat occurs due to an increase in both the number (hyperplasia) and size (hypertrophy) of adipocytes (Chait and Den Hartigh 2020). Adipogenesis is the process where preadipocytes differentiate into mature adipocytes (Van Pham *et al.*, 2016). This differentiation is regulated by specific transcription factors, particularly peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer-binding protein alpha (C/EBP α). The activity and expression of these transcription factors can be enhanced by glucose metabolism and insulin signaling (Zhang *et al.*, 2020). These factors, in turn, activate genes involved in lipid

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metabolism and storage, such as acetyl coenzyme A carboxylase-1 (ACC1) and fatty acid synthase (FAS), which are essential for de novo lipogenesis and triglyceride metabolism (Janani and Kumari 2015; Yang *et al.*, 2018).

Glucose plays a crucial role in adipocyte differentiation by providing both energy and the necessary metabolic substrates for the transformation of preadipocytes into mature adipocytes. Previous research have demonstrated that glucose precisely regulates the proliferation and differentiation of preadipocytes.

Kolodziej *et al.* (2019) observed a significant increase in lipid accumulation and droplet size in human adipose-derived stem cells when exposed to high-glucose media. Similarly, high glucose concentrations were found to upregulate the expression of PPAR γ in HK-2 cells (Panchapakesan *et al.*, 2004). However, other research found that excessive glucose levels inhibited chicken preadipocyte differentiation, possibly by stimulating lipolysis (Qi RenLi *et al.*, 2012). Additionally, Rosita *et al.* (2021) reported that high glucose disrupted the differentiation process of Wistar rat preadipocytes isolated from peritoneal regions. These findings suggest that glucose's effect on adipocyte differentiation varies depending on its concentration and the type of adipocytes being studied.

Subcutaneous fat in beef cattle is closely linked to glucose metabolism, as glucose from feed fermentation plays a key role in fat deposition (Schumacher *et al.*, 2022). In the beef industry, managing glucose metabolism is crucial to balancing fat levels, ensuring desirable meat quality and minimizing excess fat that needs to be trimmed during processing. Zhao *et al.* (2020) demonstrated PPAR γ is an important effector for regulating fat deposition as PPAR γ gene expression was correlated with carcass lean content of pigs. However, the effects of glucose concentration and genes expression such as PPAR γ on the adipogenesis of bovine subcutaneous preadipocytes have been rarely reported. Therefore, the current experiment was conducted to investigate the effects of glucose concentration on differentiation, adipogenic marker gene expression and glucose transporter distribution in bovine subcutaneous preadipocytes.

MATERIALS AND METHODS

The experiment was conducted from January 2022 to January 2023 at Xiaoba Pan Farm and the laboratory of the College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University.

Isolation and culture of bovine subcutaneous preadipocytes

Preadipocytes were isolated from the subcutaneous adipose tissue located between the 12th and 13th ribs of four 23-month-old fattening Simmental cattle. The tissue samples were rinsed with phosphate-buffered saline (PBS) (Solarbio, Beijing, China) containing 1% antibiotics (10,000

U/mL penicillin and 10,000 μ g/mL streptomycin) (Gibco, Shanghai, China). Blood vessels and connective tissues were carefully dissected from the adipose tissue, which was then cut into small pieces approximately 1 mm³ in size. These pieces were digested with 1 mg/mL collagenase type I (Gibco, Shanghai, China) at 37°C for 1 hour in a shaking water bath. After digestion, the mixture was filtered through a 200- μ m cell strainer and centrifuged at 300 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium containing 10% FBS and 1% antibiotics (growth medium). The suspension was then refiltered through a 50- μ m sterile cell strainer. The filtered cells were washed twice with growth medium, seeded in culture plates at an optimal density and incubated at 37°C in a humidified atmosphere with 5% CO₂. The growth medium was refreshed every two days.

Adipogenic differentiation of preadipocytes

Two days after the cells reached confluence (day 0), the growth medium was removed and replaced with a differentiation medium containing growth medium, 10 μ g/mL insulin, 0.5 mM isobutyl methylxanthine (IBMX) (Sigma, Shanghai, China) and 1.0 μ M dexamethasone (DEX) (Sigma, Shanghai, China) for 48 hours. After this period, the medium was switched to a maintenance medium (growth medium +1 μ g/mL insulin), which was refreshed every two days until day 8. Additionally, glucose at different concentrations (0, 3.0, 3.5 and 4.0 mmol/L) was added to both the differentiation and maintenance medium. Preadipocytes were harvested on days 2, 4 and 8 to evaluate lipid droplet accumulation, adipogenic marker gene expression and glucose transporter distribution. Each treatment was performed in triplicate.

Oil Red O staining

Adipocytes were gently washed twice with cold PBS and then fixed in 4% paraformaldehyde at 37°C for 30 minutes. After fixation, the cells were washed twice more with PBS and stained with Oil Red O (Sigma, Shanghai, China) at room temperature for 30 minutes. The cells were then washed once with PBS. The number and size of lipid droplets were observed under a microscope (Olympus, Tokyo, Japan) within an hour. Triglyceride (TG) content was measured using a microplate reader (PerkinElmer, Shelton, USA) with commercial kits (Jiancheng, Nanjing, China).

Quantitative real-time PCR

Total RNA was extracted from the adipocytes at different stages (after 2, 4 and 8 days of induction) using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The extracted RNA was then reverse transcribed into cDNA using the GoScript Reverse Transcription System (Promega Corporation, USA) as

described. The primer sequences for the target genes were as follows Table 1.

The relative mRNA expression levels were measured using SYBR Green PCR Master Mix (Thermo Fisher, Shanghai, China) and a Roche Light Cycler 480 real-time fluorescent quantitative PCR system (Roche, Basel, Switzerland). β -actin was used as the internal control for normalizing relative gene expression. The PCR reaction conditions were as follows: an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 5 seconds, 56°C for 10 seconds and 72°C for 25 seconds. Each experiment was performed in triplicate. The relative mRNA expression levels were normalized to β -actin and analyzed using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Adipocytes were lysed in ice-cold RIPA buffer containing a protease inhibitor (Solarbio, Beijing, China) and then centrifuged at 12,000 g for 15 minutes at 4°C. Protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Thermo Fisher, Shanghai, China). Total protein samples (15 μ g per well) were separated on a 12% SDS-PAGE gel, then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with primary antibodies for PPAR γ , ACC1 and FAS (all diluted 1:1,000; Proteintech, Rosemont, USA). After three washes with TBST, the membranes were incubated with a secondary antibody (dilution 1:5,000; Jackson, West Grove, USA) for 1 hour at room temperature. Immunoreactive proteins were detected using a ChemiDoc XRS+ System (Bio-Rad, Hercules, USA) and quantified using ImageJ software. Data were expressed as integrated density values (IDVs) for statistical analysis, calculated as the ratio of the specific protein band density to the β -actin density and expressed as a percentage of the control.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 (Thermo Fisher, Shanghai, China) for 20 minutes. Afterward, the cells were blocked with 1% bovine serum albumin (BSA) (Thermo Fisher, Shanghai, China) for 30 minutes and then incubated overnight at 4°C with Anti-GLUT1 and GLUT4 antibodies (Bioss, Beijing, China). The cells were washed three times

with PBS and incubated with a secondary antibody (Bioss, Beijing, China) at 37°C for 1 hour. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Bioss, Beijing, China) for 15 minutes. All images were captured using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistics and analysis

Statistical analysis was performed using SPSS software (version 22.0, IBM, USA). Data were analyzed using one-way ANOVA. Statistical significance was defined as * $p < 0.05$ and ** $p < 0.01$ for all analyses. When a significant treatment effect was observed, differences between means were assessed using the Bonferroni multiple comparison test.

RESULTS AND DISCUSSION

Differentiation of bovine subcutaneous preadipocytes

Preadipocytes were induced with differentiation media adding different glucose concentrations (0, 3.0, 3.5 and 4.0 mmol/L). Lipid accumulation was measured using Oil Red O staining on days 2, 4 and 8 Fig 1. As shown in Fig 1, Oil Red O-stained lipid droplets became denser as glucose concentration increased on days 2 and 4. However, by day 8, the amount of stained lipid droplets decreased as glucose concentration increased. These findings were further supported by a triacylglycerol (TG) content assay Table 2. On days 2 and 4, preadipocytes had significantly higher ($p < 0.05$) TG content with increased glucose concentration, while by day 8, TG content decreased as glucose concentration increased.

Preadipocytes are precursor cells in adipose tissue, responsible for the development and maintenance of fat cells (adipocytes). They have the ability to differentiate into mature adipocytes, which store fat in the body. This process is crucial for the regulation of energy storage, metabolism and overall tissue homeostasis, continuing throughout an animal's life (Li *et al.*, 2021a; Rodriguez *et al.*, 2004).

Glucose is vital in the differentiation of preadipocytes into mature adipocytes, influencing this process through various metabolic, hormonal and molecular pathways. Previous studies have shown that high glucose conditions lead to an increase in the number and size of lipid droplets in intramuscular adipocytes of sheep, resulting in higher triglyceride (TG) levels (Yan *et al.*, 2023). Aguiar *et al.* (2008)

Table 1: The primer sequences for qRT-PCR.

Primer name	Sequences	Amplicon length (bp)
PPAR γ -F	CAGCTGCAGGCCATTCTAC	117
PPAR γ -R	GGGTAGGGTGTCAAGTCCAAG	
ACC1-F	GAGCCAGCGAAAGTGTGTGAT	110
ACC1-R	GGATCCTTGCTTGGATGCAG	
FAS-F	AAACAACTGCACCCTGACC	81
FAS-R	AGTGTTCACAGCCAGGAGAA	
β -actin-F	CCCCTCTACGAGGGCTAT	145
β -actin-R	TGTACGACGACGATTTCC	

pointed out that high glucose not only promotes the differentiation of adipose tissue-derived stem cells into adipocytes but also induces muscle-derived precursor cells to form adipose tissue. Additionally, Jackson *et al.* (2017) demonstrated that high glucose promotes adipocyte differentiation through distinct metabolic pathways, independent of fatty acids. In the present study, we observed that higher glucose concentrations increased the number and density of lipid droplets during the early stages of preadipocyte differentiation, consistent with previous research. However, in the later stages of differentiation, we noted a decrease in lipid droplet accumulation, suggesting that prolonged glucose exposure may negatively impact

fat formation and accumulation in bovine subcutaneous preadipocytes.

Adipogenic marker genes expression

According to the qRT-PCR results Table 3, the expression levels of key adipogenesis-related genes, including PPAR γ , ACC1 and FAS, significantly increased on days 2 and 4 in a dose-dependent manner (PPAR γ and ACC1, $p < 0.05$; FAS, $p < 0.01$). However, on day 8, the mRNA expression levels of these genes showed a decreasing trend as glucose concentration increased. Similarly, the protein expression levels of PPAR γ , ACC1 and FAS, as determined by western blot analysis Fig 2, followed the same pattern as their mRNA

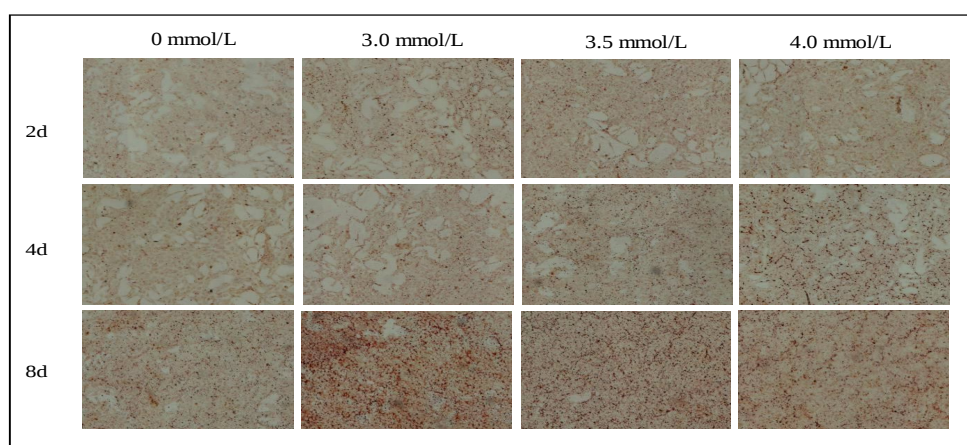


Fig 1: Different glucose concentrations on lipid accumulation of preadipocytes with oil red O staining under a microscope (40 $\times$ magnification) on day 2, 4 and 8.

Table 2: Effect of glucose concentration on triglycerides content of preadipocytes (mmol/L).

	0	3.0	3.5	4.0	SEM	P
2d	1.04 ^a	1.19 ^b	1.34 ^c	1.30 ^c	0.028	<0.01
4d	2.14 ^a	2.49 ^b	2.57 ^c	2.61 ^c	0.032	0.017
8d	3.15 ^a	4.11 ^b	3.69 ^c	3.42 ^c	0.042	<0.01

^{a b c} Mean values within a row with different superscript letter differ significantly ($p < 0.05$).

Table 3: Effects of glucose concentrations on adipogenic marker genes mRNA expression.

	0	3	3.5	4	SEM	P
2d						
PPAR γ	0.72 ^a	0.79 ^a	0.82 ^a	1.00 ^b	0.05	0.024
ACC1	1.00 ^a	1.17 ^b	1.28 ^b	1.40 ^c	0.74	0.013
FAS	1.01 ^a	1.12 ^{ab}	1.34 ^b	1.59 ^c	0.11	<0.01
4d						
PPAR γ	0.59 ^a	0.74 ^a	0.84 ^b	1.14 ^c	0.24	0.023
ACC1	0.87 ^a	0.98 ^a	1.40 ^b	1.32 ^b	0.08	0.020
FAS	1.18 ^a	1.22 ^a	1.74 ^b	1.96 ^b	0.16	0.006
8d						
PPAR γ	0.65 ^a	1.06 ^b	0.58 ^a	0.58 ^a	0.04	0.041
ACC1	1.28 ^a	1.70 ^b	1.79 ^b	1.50 ^a	0.16	0.048
FAS	1.44 ^a	2.54 ^b	2.94 ^b	2.48 ^b	0.27	<0.01

^{a b c} Mean values within a row with different superscript letter differ significantly ($p < 0.05$).

levels. These findings suggest that adipogenic marker gene expression significantly increases with higher glucose concentrations during the early stages of preadipocyte differentiation but tends to decrease in the later stages.

PPAR γ plays an important role in glucose metabolism and adipogenesis. It controls the final differentiation of adipocytes and is essential for maintaining their differentiated state (Lee *et al.*, 2019). The expression of PPAR γ is a key factor in the development of varying levels of marbling in Wagyu, Angus and Nellore cattle, playing a vital role in promoting fat deposition in beef cattle (Liu *et al.*, 2020). ACC1 and FAS are two key enzymes essential for the differentiation of preadipocytes into mature adipocytes. ACC1 plays a crucial role early in adipogenesis by

increasing its activity to produce malonyl-CoA, which is necessary for fatty acid biosynthesis (Ito *et al.*, 2021). FAS is vital for generating the fatty acids required during this process (Rowland *et al.*, 2023). As preadipocytes differentiate, the need for fatty acid synthesis rises to produce the lipids stored in mature adipocytes. Furthermore, the expression of both ACC1 and FAS is regulated by PPAR γ , which is activated during adipogenesis.

In the present study, we observed that the mRNA and protein levels of PPAR γ , ACC1 and FAS significantly increased in bovine subcutaneous preadipocytes on days 2 and 4 as glucose concentration rose. This finding aligns with previous research showing that high glucose

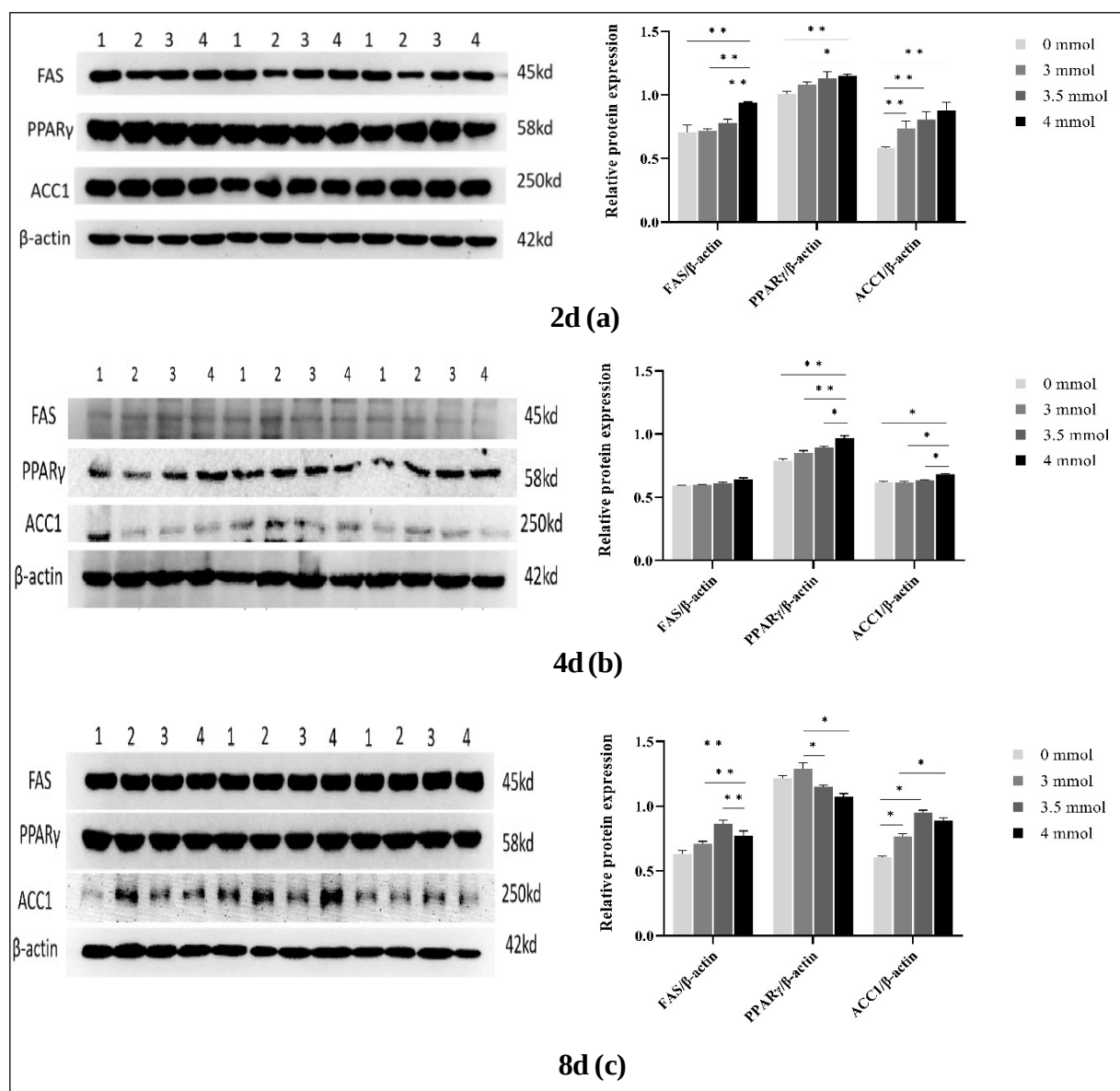


Fig 2: (a-c) Adipogenic marker genes protein expression levels during preadipocyte differentiation on different days.

availability increases mRNA expression levels of PPAR γ and FAS and promotes adipocyte differentiation in mouse 3T3-L1 cells (Jackson *et al.*, 2017). Similarly, Yan *et al.* (2023) reported higher levels of lipids and triglycerides in intramuscular adipocytes of sheep, along with increased mRNA expression of ACC and FAS under high glucose conditions. Other studies also demonstrated upregulation of PPAR α in various cell types, such as HK-2 and nucleus pulposus cells, under high-glucose conditions (Jiang *et al.*, 2018; Panchapakesan *et al.*, 2004). Despite these findings, gene expression levels decreased under high glucose conditions by day 8 in our study. Previous research has shown that PPAR γ , ACC and FASN expressions increase gradually, peaking around days 3 and 5 of differentiation before declining (Tokach *et al.*, 2015; Wang *et al.*, 2013). This pattern of gene expression mirrored the changes in the number of lipid droplets observed in bovine subcutaneous preadipocytes. These results suggest that

glucose initially promotes PPAR γ expression and regulates genes involved in fat storage, including those responsible for fatty acid uptake and triglyceride formation.

GLUT1 and GLUT4 protein distribution

Fluorescence images of differentiated preadipocytes on day 8 showed immunofluorescence staining Fig 3. The cell nuclei were stained with DAPI (blue), while GLUT1 and GLUT4 proteins (green) were primarily expressed in the cytoplasm. The fluorescence intensity of GLUT1 and GLUT4 increased with higher glucose concentrations.

GLUT1 and GLUT4 are glucose transporters responsible for facilitating the movement of glucose across cell membranes, a crucial step in cellular glucose uptake and metabolism. GLUT1 and GLUT4 play significant roles in adipogenesis. GLUT1 supports glucose uptake during the early stages of adipogenesis and provides a basal level of glucose supply in adipocytes (Chadt and Al-Hasani 2020). As preadipocytes mature into fully functional

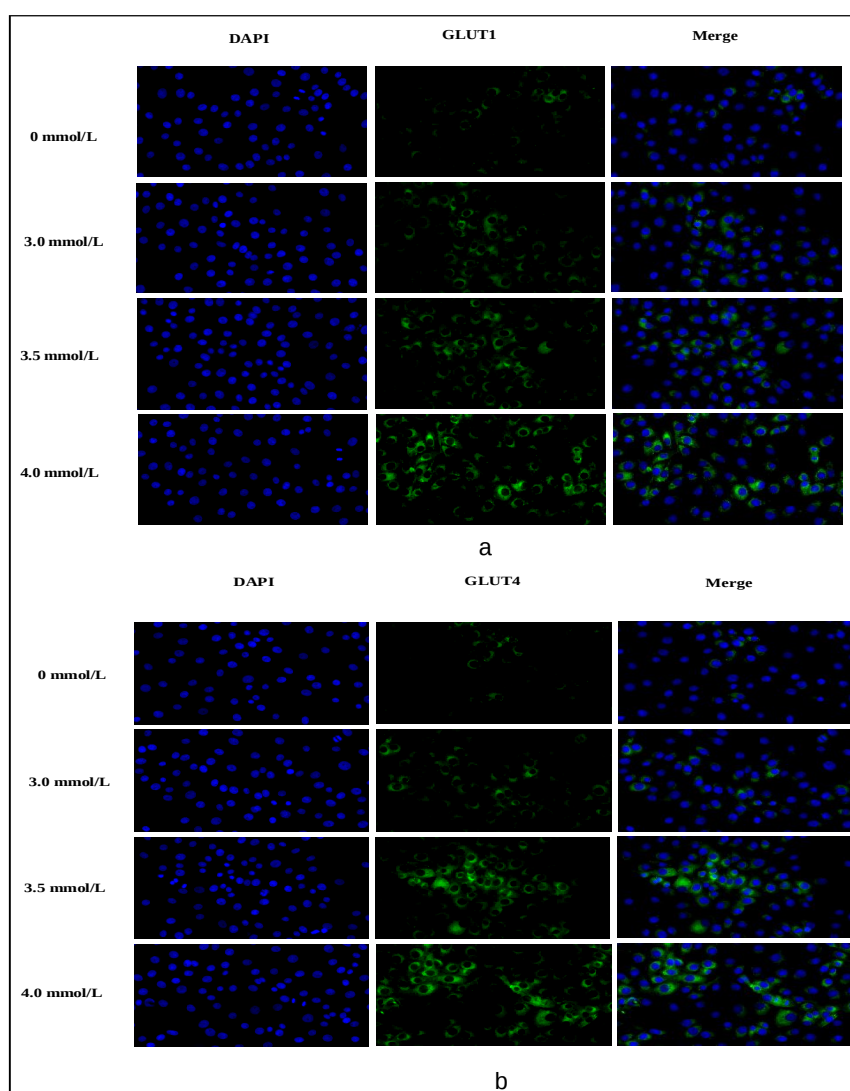


Fig 3: GLUT-1 (a) and GLUT-4 (b) distribution in differentiated preadipocytes on day 8 (200 $\times$ magnification).

adipocytes, GLUT4 becomes more important, regulating insulin-mediated glucose uptake and contributing to fat storage (Wang *et al.*, 2020).

Our study demonstrated that the distribution of GLUT1 and GLUT4 in preadipocytes becomes denser with increasing glucose concentrations, indicating an active response to higher glucose levels by enhancing glucose uptake. This finding aligns with Griesel *et al.* (2021) who reported increased glucose uptake due to heightened GLUT4 translocation to the plasma membrane. Similarly, Wang *et al.* (2013) observed increased GLUT1 and GLUT4 mRNA expression in both intramuscular and subcutaneous preadipocytes of pigs. Jackson *et al.* (2017) found that GLUT4 was first expressed at low levels on day 5, reaching peak expression by day 9 under high glucose conditions. However, Rosita *et al.* (2021) noted a significant reduction in total GLUT4 levels after 7 days of rat primary preadipocyte differentiation under high-glucose conditions. These differences may be attributed to variations in cell types, glucose concentrations and the duration of treatment.

CONCLUSION

In summary, our study revealed that during the early stages of bovine subcutaneous preadipocyte differentiation, lipid droplet accumulation and the expression of adipogenic marker genes (PPAR γ , FAS and ACC1) increased steadily with rising glucose concentrations. However, in the later stages, these effects tended to decrease under high glucose conditions. Additionally, GLUT1 and GLUT4 fluorescence intensity in preadipocytes became denser as glucose concentrations increased. These findings suggest that adding glucose at 3.0 to 3.5 mmol/L to the differentiation medium is optimal for promoting differentiation and fat deposition in bovine subcutaneous preadipocytes. Furthermore, this knowledge will be important to achieve desirable meat quality while minimizing excess fat for beef cattle.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the

information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The experimental protocol was approved by the Animal Care and Use Committee of Heilongjiang Bayi Agriculture University (permit number DWKJXY2023007).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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