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Original Research

Expression Regulation of Gluconeogenesis Related Genes in Ovine Skeletal Muscle Cells

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Abstract

Background: Under fasting conditions, the pathway converting gluconeogenesis precursors into muscle glycogen becomes crucial due to reduced glycogen reserves. However, there is limited research on skeletal muscle gluconeogenesis and the impact of fasting on gluconeogenic gene expression. **Methods**: Sheep fetal skeletal muscle cells cultured *in vitro* were used to study the effects of varying lactic acid concentrations (0 to 30 mM) and 2.5 mM glucose on the expression of gluconeogenesis-related genes after 6 h of fasting. The effects on mRNA and protein expression of key genes involved in skeletal muscle gluconeogenesis were measured by quantitative real time polymerase chain reaction (qRT-PCR), immunofluorescence, and western blotting at 48 h. **Results**: Fasting increased the expression of key gluconeogenic genes, fructose-1,6-bisphosphatase 2 (*FBP2*), glucose-6-phosphatase 3 (*G6PC3*), pyruvate kinase M (*PKM*), monocarboxylate transporter1 (*MCTS1*), glucose transporter type 4 (*GLUT4*), pyruvate carboxylase (*PC*), and lactate dehydrogenase A (*LDHA*). The mRNA levels of *FBP2*, *G6PC3*, and *MCTS1* significantly decreased with glucose addition. Additionally, 10 mM lactic acid significantly promoted the expression of *FBP2*, *PC*, *MCTS1*, *LDHA*, *GLUT4*, and *PKM* while inhibiting phosphoenolpyruvate carboxykinase (*PEPCK*) expression. At the protein level, 10 mM lactic acid significantly increased FBP2 and PKM protein expression. **Conclusions**: This study shows that fasting regulates key gluconeogenic gene expression in sheep skeletal muscle cells and highlights the role of lactic acid in inducing these gene expressions.

Keywords: glucose; lactic acid; ovine skeletal muscle cells; gluconeogenesis

1. Introduction

Under normal physiological conditions, livestock muscles primarily utilize glucose as an energy substrate [1]. Sugar metabolism is crucial for maintaining normal cell function and status [2]. Unlike monogastric animals, carbohydrates in ruminant feed are mostly absorbed and fermented by microorganisms in the rumen to produce short-chain volatile fatty acids (VFAs). Consequently, glucose absorption from feed in the small intestine is minimal. Therefore, approximately 90% of glucose in ruminants is derived from endogenous glucose, synthesized through gluconeogenesis [3]. Gluconeogenesis converts various non-carbohydrate substances (pyruvate, lactic acid, glycerol, and amino acids) into glucose or glycogen [4].

While hepatic gluconeogenesis is well-studied, most research has focused on monogastric model animals such as humans and mice. Studies on gluconeogenesis in ruminants primarily focus on the lactation period to meet the increased glucose demand through nutritional supplementation, emphasizing hepatic gluconeogenesis. However, studies on gluconeogenesis in sheep skeletal muscle are limited. Skeletal muscles play a central role in controlling body metabolism and movement and are a major site for glucose uptake and utilization [5]. During exercise, skeletal muscles uptake glucose from circulation and completely oxidize it to provide energy for contraction [6]. Additionally, under low oxygen conditions, skeletal muscles can also undergo glycolysis to produce a large amount of lactic acid [7]. It is widely believed that lactic acid produced by skeletal muscles needs to be transported to the liver as a substrate for gluconeogenesis, to be resynthesized into glucose or glycogen [8]. Recent studies show that skeletal muscle also has a certain gluconeogenic capacity. Using carbon labeling, researchers found that lactic acid is a substrate for glycogen synthesis and lipid storage in human myotubes [9]. Another study found that lactic acid could promote the synthesis of glycerol and glycogen in rat skeletal muscle by reversing pyruvate kinase M (*PKM*) [10]. During glycolysis, *PKM* catalyzes the conversion of phosphoenolpyruvate (PEP) to enolpyruvate, and then to pyruvate, making the reverse reaction of *PKM* important to gluconeogenesis [11]. Previous studies proved that the *PKM* reaction is reversible under *in vivo* conditions [12]. Given that lactic acid easily produces pyruvate, Jin *et al.* [10] speculated that under high concentrations of lactic acid, the reversal of *PKM* is more powerful, but there is no concrete data to support this hypothesis.

In summary, preliminary research on gluconeogenesis in skeletal muscle has primarily focused on humans and mice. Studies on gluconeogenesis in ruminant skeletal muscle have not been reported. Our laboratory previously found that high-fiber, low-protein diets promote muscle gluconeogenesis and inhibit glycolysis through epigenetic regulation [13,14]. However, functional validation at the cellular level is lacking. The intensity of gluconeogenesis has not been determined by detecting key protein expression in muscle cells, such as glucose-6-phosphatase (G6PC), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate carboxylase (PC) [15,16]. This study investigates the regulatory mechanisms of gluconeogenesis in skeletal muscle cells *in vitro*. It examines key gene expression involved in gluconeogenesis in skeletal muscle cells under fasting conditions. The study also analyzes the regulatory effects of the gluconeogenesis precursor lactic acid on key genes (*G6PC*, *PEPCK*, *PC*, *PKM*, fructose-1,6-bisphosphatase 2 (*FBP2*), monocarboxylate transporter (*MCTS*), glucose transporter type 4 (*GLUT4*), and lactate dehydrogenase A (*LDHA*)) in skeletal muscle cells after fasting. Lactic acid is an important gluconeogenesis precursor and a main product of skeletal muscle exercise. Previous studies have shown that extracellular lactic acid concentration in skeletal muscle is closely related to exercise intensity [17]. Therefore, we evaluated the effect of different lactic acid concentrations on gluconeogenic genes to study the dose effect of lactic acid on skeletal muscle gluconeogenesis.

2. Materials and Methods

2.1 Culture and Identification of Ovine Fetal Longissimus Dorsi Muscle Cells

Due to the rapid growth rate of fetal sheep cells, we collected the longissimus dorsi muscle of three-month-old maternal fetal sheep (Singleton, Mongolian sheep, and Small-Tailed Han sheep). After immersion in 75% ethanol for 30 s, the fascia and adipose tissue were excised, and the muscle was diced into 1 mm^3 fragments. Following three Phosphate-Buffered Saline (PBS) (Procell, Wuhan, China) washes, it was rested for 3 min. The supernatant was discarded, and the tissue blocks were placed in culture flasks at 0.5 cm intervals. The Dulbecco's Modified Eagle Medium (DMEM; Procell, Wuhan, China) containing 10% Fetal Bovine Serum (FBS) (XP Biomed, Shanghai, China) was added. The muscle tissue was discarded when the cells grew to about 60% on the 7th day. The cells were digested, purified, and passaged at 80% confluency.

Ovine muscle cells were separated and purified based on different adhesion rates between fibroblasts and muscle cells. The growth curve was plotted using the Cell Intelligence Monitoring Assistant (MN-100, Cellaview, Yuyao, China). Immunofluorescence was used to detect the muscle cell marker α-actin. Morphological analysis was used to validate the purity and proliferative potential of the muscle cells after each passage. Each generation of cells was cryopreserved.

2.2 Cell Culture and Treatment

Muscle cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Procell, Wuhan, China). The cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere until 90% confluency. The medium was then switched to FBS and glucose-free DMEM, and the cells underwent a 6 h fasting period. Various concentrations of lactic acid (0, 10, 20, and 30 mM) (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) and 2.5 mM glucose (Sangon Biotech, Shanghai, China) were introduced into the culture medium, followed by a 48 h incubation period.

2.3 Lactic Acid Dehydrogenase Activity Assay and Glucose Concentration Determination

The release of lactic acid dehydrogenase (LDH) was determined using the LDH cytotoxicity assay kit (Beyotime, Shanghai, China). Skeletal muscle cells were seeded into 96-well cell culture plates. After fasting for 6 h, skeletal muscle cells were cultured in a glucose-free medium containing 0, 10, 20, or 30 mM lactic acid for 48 h. The samples were centrifuged at 400 g for 5 min using a Perforated Plate Centrifuge (Centrifuge 5430R, Eppendorf, Hamburg, Germany), followed by the addition of diluted LDH-releasing reagent and continued incubation at 37 °C for 1 h. The samples were centrifuged again at 400 g for 5 min. A supernatant of 120 µL from each well was transferred to a new 96-well plate, and 60 µL of LDH detection working solution was added. The mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 490 nm using a Microplate Reader (Nanodrop2000, Thermo Fisher, Waltham, MA, USA); a dual-wavelength assay was performed using 620 nm as the reference wavelength.

Skeletal muscle cells were fasted for 6 h and cultured in a glucose-free medium containing 0, 10, 20, or 30 mM lactic acid for 48 h. The culture solution and cells were placed into the centrifuge tube, sonicated (ice bath, power 20%, sonication 3 s, interval 10 s, repeated 30 times), and then placed in a 95 °C water bath for 10 min. After cooling, the samples were centrifuged at 8000 g, 25 °C for 10 min. The supernatant was added to a 96-well plate, and the reagent was added according to the instructions of the glucose assay kit (Sangon Biotech, Shanghai, China). After mixing, the samples were placed in a 37 °C water bath for 15 min. A spectrophotometer was used to detect the absorbance at 505 nm.

2.4 Total RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Detection of Gluconeogenesis-Related Genes

Total RNA was extracted from cells fasted for 6 h, which were cultured with different lactic acid concentrations (0, 10, 20, or 30 mM) for 48 h. Total RNA was also extracted from cells fasted for 6 h and cultured with 2.5 mM glucose for 48 h. RNAiso (TAKARA, Kusatsu,

Table 1. Primer information.

Gene name	Accession number	Sequences (5′→3′)	Amplicon size (bp)
PC	XM_042237684	F: CCAACAGAGGCGAGATTGC R: TCTCCTTGGCGACCTTAATGA	198
FBP2	XM_027964239	F: TTATCACCGCCAAGGAGAAGA R: TAGCCAGCAGCCACAATGT	184
LDHA	XM_042238239	F: AGTGTCAGCGGTGTTCCTT R: TTCATCTGCCAAGTCCTTCATT	177
G6PC3	XM_027974447	F: TGGCTTAATAACTGGTGCTGTC R: ACTTAGAGGCTAGGTTGATGGA	179
PEPCK	XM_004014441	F: GATGCCTCCTCAGCTCTCA R: GGCGCTACTCTCCACGA	106
GLUT4	XM_027974995	F: GTCTGTCTGTCCCCTCCAG R: AGCCCACTGTCCCTTCC	149
MCTS1	XM_042242075	F: GTCAAAATAGTGCGATGCC R: CAACCTGCTGATGTGGAA	145
PKM	XM_004010279	F: GTGTTTAGCGGCAGCTTTGA R: CTGTCTGGTGATTCCGGGTC	113
β-actin	NM_001009784	F: GCAAATGCTTCTAGGCGGAC R: TGCTGTCACCTTCACCGTTC	194

PC, pyruvate carboxylase; *FBP2*, fructose-1,6-bisphosphatase 2; *LDHA*, lactate dehydrogenase A; *G6PC3*, glucose-6-phosphatase 3; *PEPCK*, phosphoenolpyruvate carboxykinase; *GLUT4*, glucose transporter type 4; *MCTS1*, monocarboxylate transporter 1; *PKM*, pyruvate kinase M.

Shiga, Japan) was used for RNA extraction. After determining RNA purity (OD260/280 ratio >1.9) and concentration, cDNA was synthesized using a reverse transcription kit (TAKARA, Kusatsu, Shiga, Japan). qRT-PCR was performed on a Light Cycler (LC480, Roche, Basel, Switzerland) using the TB Green Fast qPCR Mix Kit (TAKARA, Kusatsu, Shiga, Japan). The reaction system included TB Green Fast qPCR Mix (10 µL), forward and reverse primers (0.8 µL each, 10 µmol/L), cDNA template (2 µL), and ddH_2O (6.4 µL). Reaction mixtures were incubated for 3 min at 94 °C for preincubation, followed by 40 cycles of 5 s at 94 °C, 10 s at 60 °C, and 15 s at 72 °C. Each sample had three independent biological replicates and three technical replicates. The *β-actin* gene was the internal reference gene. Primers were designed with Primer Premier 6.0 (PremierBiosoft, Palo Alto, CA, USA) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table 1). Gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ methodology.

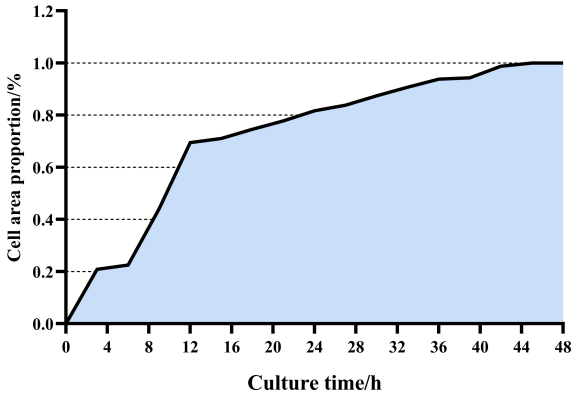

Fig. 1. Sheep skeletal muscle cell growth curve. X and Y axes are labeled in the figure.

2.5 Western Blotting

Skeletal muscle cells were cultured with different lactic acid concentrations (0, 10, 20, or 30 mM) for 48 h. After rinsing twice with pre-chilled PBS, total protein was extracted using pre-chilled RIPA (Beyotime, Shanghai, China) and Phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) premixes. Protein concentration was determined using a protein assay kit (Beyotime, Shanghai, China). Proteins were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (Mini-P4, BioRad, Hercules, CA, USA) and transferred onto polyvinylidene fluoride membranes (Solarbio, Beijing, China). After a 25-minute blocking step, membranes were incubated with primary antibodies overnight at 4 °C and washed three times. Primary antibodies included β-actin (Proteintech, Wuhan, China), FBP2 (Bioss, Beijing, China), G6PC3 (Bioss, Beijing, China), and PKM (Abcam, Shanghai, China). Membranes were incubated with a secondary goat anti-rabbit antibody (Proteintech, Wuhan, China) at room temperature for 2 h after a washing step. The ChemiDoc XRS imaging system (BioRad, Hercules, CA,

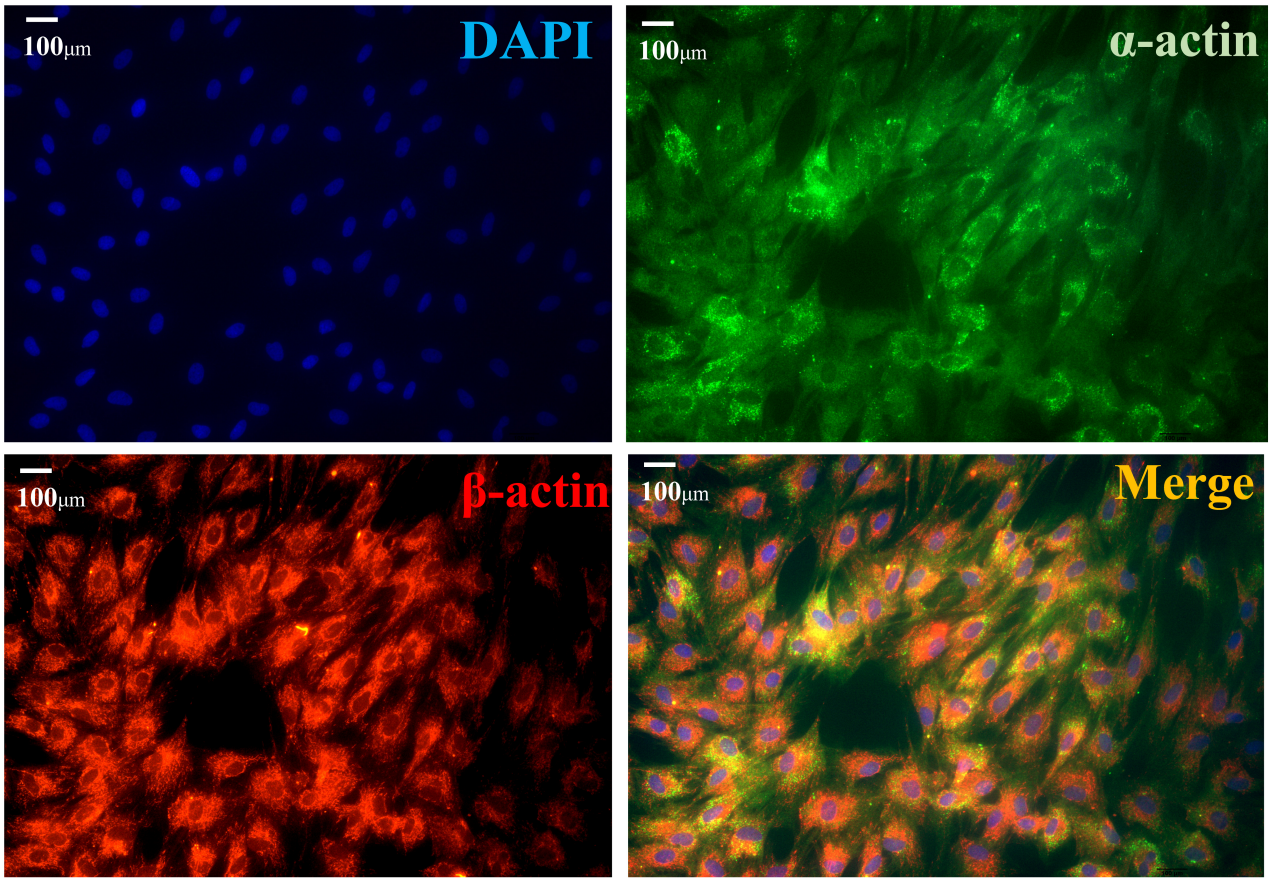

Fig. 2. Immunofluorescence detection of α-actin (10 $\times$ 20). DAPI: Nuclear dye, α-actin: selected fluorescently labeled molecules, β-actin: internal reference, Merge: merged figures. Scale bar = 100 μm.

USA) was used for exposure, and the Enhanced Chemiluminescence system (c600, Azure biosystems, Dublin, CA, USA) was used to visualize the bands. ImageJ 1.X software (National Institutes of Health, Bethesda, MD, USA) was used to estimate the grayscale density. The original images of western blotting are included in the **Supplementary Material**.

2.6 Immunofluorescence

Skeletal muscle cells were cultured with different lactic acid concentrations (0, 10, 20, or 30 mM) for 48 h. Cells were rinsed twice with PBS and incubated with 4% paraformaldehyde (Solarbio, Beijing, China) for 10 min at room temperature. After three PBS rinses, samples were incubated with 0.1% Triton X-100 (Solarbio, Beijing, China) for 10 min to permeabilize cells. Membranes were incubated with primary antibodies overnight at 4 °C, including PC (SinoBiological, Beijing, China), α-actin (Bioss, Beijing, China), β-actin (Bioss, Beijing, China), FBP2 (Bioss, Beijing, China), G6PC3 (Bioss, Beijing, China), PKM (Abcam, Shanghai, China), and PEPCK (Bioss, Beijing, China). After washing with PBS, cells were incubated with goat anti-rabbit (Bioss, Beijing, China) and goat anti-mouse (Thermo Fisher, Waltham, MA, USA) antibodies for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI (Beyotime, Shanghai, China) for 3 min after washing cells in the dark with PBS. Cells were then fixed and stored using an anti-quencher. Exposure and image capture were performed using an ECLIPSECI fluorescence microscope (Ci-S, Nikon, Tokyo, Japan).

2.7 Statistical Analysis

This experiment had three biological replicates and three technical replicates, totaling nine independent experimental units. Sheep skeletal muscle cells were the experimental unit. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA), with One-Way ANOVA to determine group differences. The results are expressed as means $\pm$ SEM. Data significance is defined as * $p < 0.05$, and ** $p < 0.01$.

3. Results

3.1 Identification of Skeletal Muscle Cells

After purification and culture, skeletal muscle cells extracted in this experiment were found consistent with known skeletal muscle cells and considered suitable for subsequent research. The cell growth curve was drawn using the cell intelligence monitoring assistant (Fig. 1). Addi-

tionally, α-actin was selected as the identification marker of skeletal muscle cells and stained by immunofluorescence. The results showed that more than 99% of the cells expressed α-actin, validating the use of purified skeletal muscle cells in this study (Fig. 2).

3.2 Expression Changes in Key Gluconeogenesis Genes in Fasted Skeletal Muscle Cells

To detect the expression of key genes during gluconeogenesis in skeletal muscle cells, we subjected skeletal muscle cells to fasting treatment. The results showed that after 6 h of fasting, the expression levels of key gluconeogenic genes such as *FBP2*, *PC*, *G6PC3*, *MCTS1*, *LDHA*, and *PKM* were significantly upregulated ($p < 0.05$). Meanwhile, there were no significant differences in the expression levels of the *PEPCK* and *GLUT4* genes (Fig. 3).

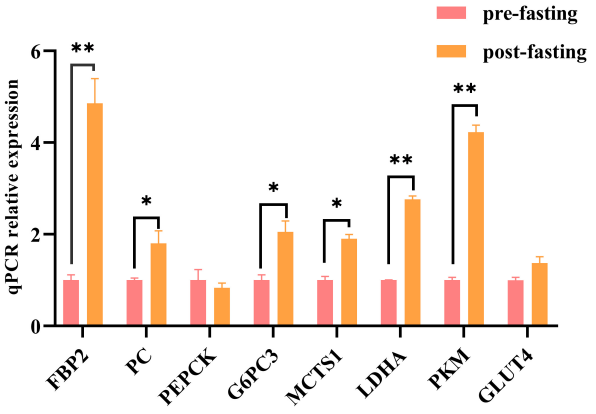

Fig. 3. Relative mRNA expression levels of key genes involved in skeletal muscle gluconeogenesis before and after 6 h of fasting. The data represent three independent cell culture experiments. * $p < 0.05$, and ** $p < 0.01$. *β-actin* was the reference gene.

3.3 Effects of Glucose on the Expression of Key Gluconeogenesis Genes

The results showed that after fasting for 6 h and incubation of skeletal muscle cells with 2.5 mM glucose for 48 h, a notable decrease in mRNA levels of *FBP2*, *G6PC3*, and *MCTS1* genes ($p < 0.05$) was observed, along with a significant rise in mRNA levels of *PC*, *PKM*, and *GLUT4* genes ($p < 0.05$) (Fig. 4). However, expression levels of the *PEPCK* and *LDHA* genes did not show any significant differences.

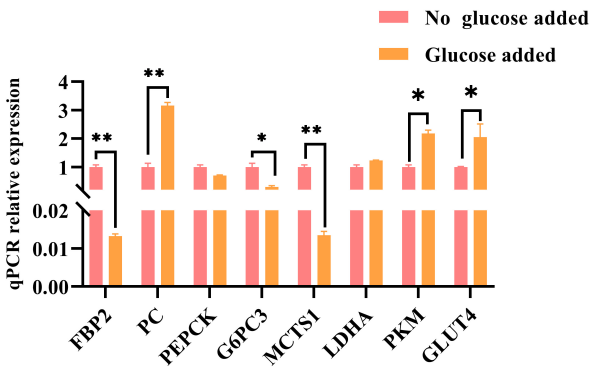

Fig. 4. After 6 h of fasting, the relative mRNA expression levels of key genes involved in gluconeogenesis in muscle cells cultured with glucose for 48 h. The data represent three independent cell culture experiments. * $p < 0.05$, and ** $p < 0.01$. *β-actin* was the reference gene.

3.4 Effects of Lactic Acid on Skeletal Muscle Cells and Glucose Concentration

Fasting and glucose addition significantly affected the expression of key genes of gluconeogenesis in skeletal muscle cells. To determine the effect of the gluconeogenic precursor lactic acid on skeletal muscle cell metabolism and glucose concentration, different concentrations of lactic acid (0 to 30 mM) were added to the culture medium. The activity of lactate dehydrogenase (LDH) and glucose concentration in cells were estimated. The results showed that the addition of 10 and 20 mM lactic acid resulted in slightly higher glucose concentration than 0 and 30 mM (Fig. 5A). Additionally, 10 and 20 mM lactic acid resulted in slightly lower lactate dehydrogenase (LDH) release than 0 and 30 mM (Fig. 5B), but there was no significant difference between the groups ($p > 0.05$).

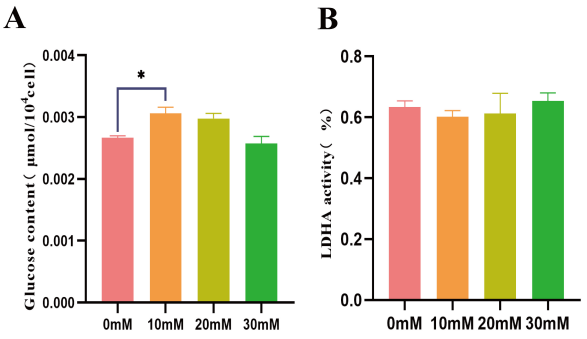

Fig. 5. Effects of lactic acid on skeletal muscle cells and glucose concentration. (A) Glucose content detection. (B) Lactate dehydrogenase activity. * $p < 0.05$.

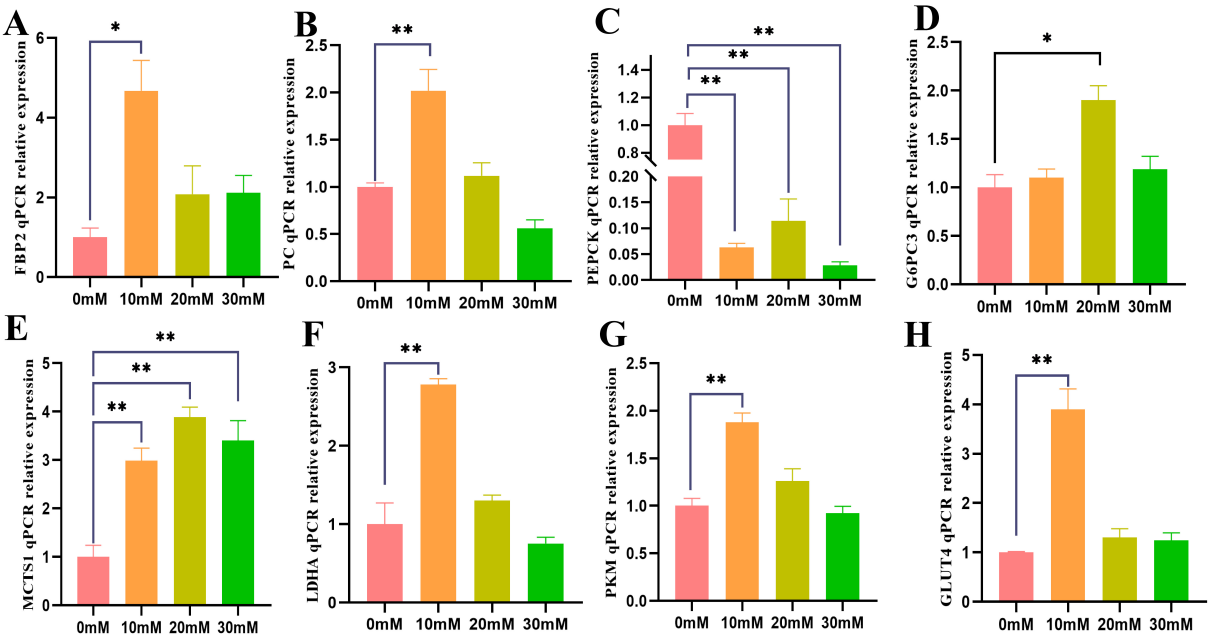

Fig. 6. After 6 h of fasting, skeletal muscle cells were cultured with 0, 10, 20, and 30 mM lactic acid for 48 h, and the relative mRNA expression levels of key genes involved in gluconeogenesis were measured. The qRT-PCR relative expressions of (A) *FBP2,* (B) *PC*, (C) *PEPCK*, (D) *G6PC3*, (E) *MCTS1*, (F) *LDHA*, (G) *PKM*, and (H) *GLUT4* are shown. The data represent three independent cell culture experiments. * $p < 0.05$, and ** $p < 0.01$. *β-actin* was the reference gene.

3.5 Effect of Lactic Acid on mRNA Expression Levels of Key Gluconeogenesis Genes

To explore the regulatory role of lactic acid in the gluconeogenesis pathway of skeletal muscle cells after fasting, changes in mRNA expression levels of key genes were analyzed using qRT-PCR technology. The results showed that adding 10 and 20 mM lactic acid promoted the expression of *FBP2*, *G6PC3*, *PKM*, *MCTS1*, *GLUT4*, *PC*, and *LDHA* in skeletal muscle cells cultured for 48 h after fasting for 6 h. Among them, 10 mM lactic acid significantly increased mRNA expression levels of *FBP2*, *PKM*, *MCTS1*, *GLUT4*, *PC*, and *LDHA* ($p < 0.05$). The 20 mM lactic acid significantly upregulated mRNA expression of *G6PC3* and *MCTS1* ($p < 0.05$). Additionally, 30 mM lactic acid significantly increased MCTS1 expression and significantly decreased PEPCK expression ($p < 0.05$) (Fig. 6). This indicates that lactic acid addition effectively promoted the expression of gluconeogenesis-related genes. Genes such as *G6PC3*, *FBP2*, *PKM*, *PEPCK*, and *PC* are key rate-limiting enzymes in gluconeogenesis, and their expression levels changed significantly. Based on these findings, this study focused on these five genes for further experimental studies to understand how lactic acid regulates the gluconeogenic pathway in skeletal muscle cells.

3.6 Effect of Lactic Acid on Key Proteins of Gluconeogenesis

Western blotting and immunofluorescence techniques were used to analyze the expression and intracellular localization of key proteins in gluconeogenesis. The results in-

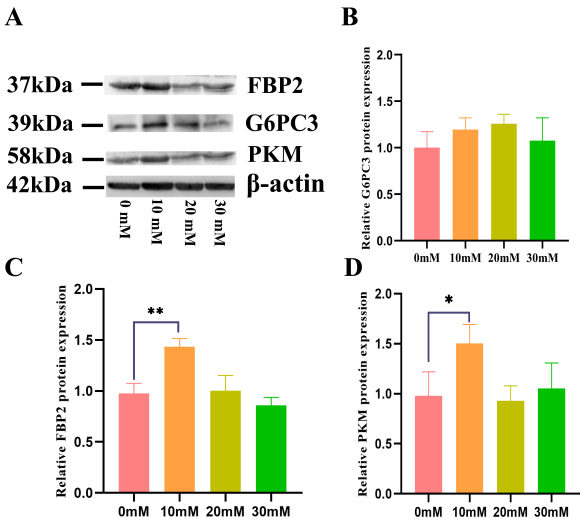

Fig. 7. After 6 h of fasting, skeletal muscle cells were cultured with 0, 10, 20, and 30 mM lactic acid for 48 h to determine the protein levels of FBP2, G6PC3, and PKM in skeletal muscle cells. Quantitative analysis of FBP2, G6PC3, and PKM protein levels by western blotting. (A) The blotting results of key gluconeogenesis proteins. The relative protein levels of (B) G6PC3, (C) FBP2, and (D) PKM. The bar graphs depict the normalized expression of respective proteins relative to β-actin (loading control) across various experimental conditions. * $p < 0.05$, and ** $p < 0.01$.

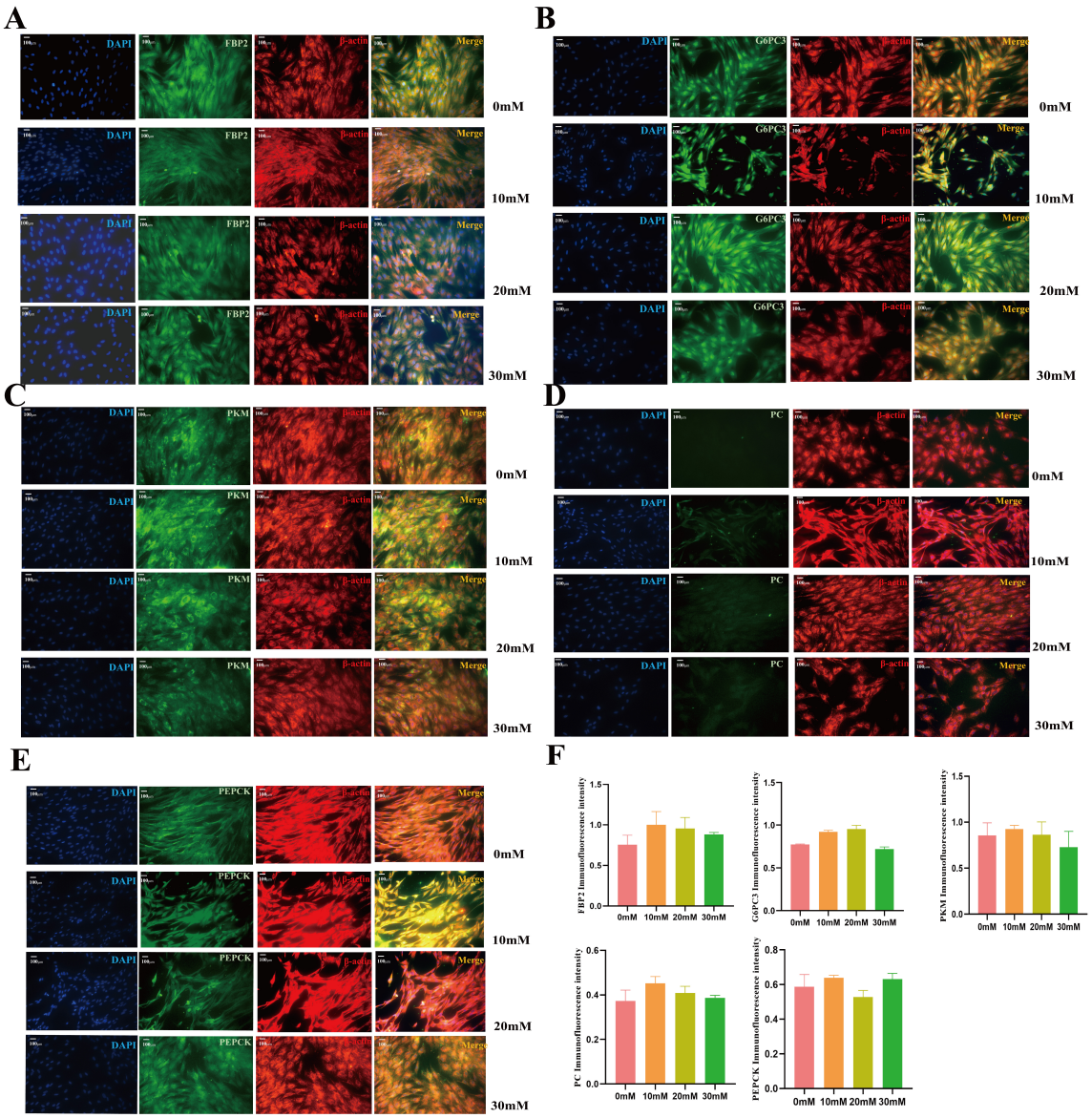

Fig. 8. After 6 h of fasting, skeletal muscle cells were cultured with lactic acid (0 to 30 mM) for 48 h. Immunofluorescence validation of key gluconeogenic protein levels in muscle cells (10 × 20). Selected fluorescently labeled molecules for (A) FBP2, (B) G6PC3, (C) PKM, (D) PC, and (E) PEPCK; DAPI: Nuclear dye, Merge: Merged figures, β-actin: internal reference. (F) Bar graphs showing immunofluorescence intensity analysis. Scale bar = 100 µm.

dicated a significant increase in levels of FBP2 and PKM proteins with the addition of 10 mM lactic acid ($p < 0.05$), while the levels of G6PC3 protein did not change significantly ($p > 0.05$) (Fig. 7). Immunofluorescence results showed that the FBP2 protein was specifically expressed in the cell membrane and cytoplasm (Fig. 8A). G6PC3 was specifically expressed in the nucleus periphery (Fig. 8B). PKM was specifically expressed in the cytoplasm (Fig. 8C), and PC and PEPCK were specifically expressed in the cytoplasm (Fig. 8D,E). Immunofluorescence results showed stronger fluorescence intensity of FBP2, PC, PEPCK, and PKM when 10 mM lactic acid was added, while G6PC3 fluorescence intensity was stronger with 20 mM lactic acid (Fig. 8F).

4. Discussion

The liver and kidneys are traditionally seen as the main sites of gluconeogenesis. However, recent studies indicate that gluconeogenesis-related genes are also expressed in the skeletal muscles of ruminants. This study found that after fasting, skeletal muscle cells expressed key gluconeogenic genes except *PEPCK*. This suggests that during prolonged fasting or hypoglycemia, muscles may provide an energy source and regulate blood glucose metabolism through gluconeogenesis and glycolysis [18]. Additionally, lactic acid, a main metabolic product in skeletal muscle, is an important precursor of gluconeogenesis, which can regulate the expression of gluconeogenesis genes.

4.1 Effects of Fasting on Gluconeogenesis and Related Genes

Glucose is the most important carbohydrate in the body. In the fed state, most circulating glucose comes from the diet, while gluconeogenesis and glycogenolysis maintain physiological glucose concentration in the fasting state. After a night of fasting, gluconeogenesis provides 25%–50% of total glucose production in the human body, while the rest is produced by glycogenolysis [19]. With prolonged fasting and depletion of glycogen reserves, total glucose production relies more on gluconeogenesis [19]. This research observed that the expression levels of gluconeogenesis-related genes, including *PKM*, *PC*, *LDHA*, *MCT1*, *FBP2*, and *G6PC3*, notably increased after fasting treatment. A previous study showed that PKM expression was highest in fasted fish and decreased with feeding [20]. This change in *PKM* may respond to the decreased glycolytic pathway flux, increasing glucose levels. Glucose effectively stimulates *PKM* [20]. Pyruvate produced by glycolysis and glycogenolysis in skeletal muscle during exercise or fasting has two fates. Pyruvate can convert to oxaloacetic acid by *PC* and enter the citric acid cycle or convert to lactic acid under the catalysis of *LDHA*, producing glucose through the gluconeogenesis pathway [21]. These studies suggest that decreased glycolytic flux and upregulated *PKM*, *PC*, and *LDHA* gene expression after fasting may improve pyruvate conversion and utilization efficiency.

The main physiological role of MCT1 is to promote lactic acid entry or exit from cells. Hoshino *et al*. [22] observed that MCT1 protein levels and muscle glycogen concentration significantly increased in the tibialis anterior muscle of mice after exercise. MCT1 protein expression positively correlated with muscle glycogen concentration ($r = 0.969$). Studies also show that *FBPase* and *G6PC*, key gluconeogenesis genes, are significantly upregulated during fasting [23]. This indicates that skeletal muscle gluconeogenesis activation after fasting or exercise may relate to muscle glycogen recovery [18].

4.2 Effect of Glucose on the Expression of Key Gluconeogenesis Genes in Skeletal Muscle Cells

Our results showed that adding glucose to skeletal muscle significantly inhibited the expression of gluconeogenesis-related genes such as *FBP2*, *MCTS*1, and *G6PC3*. In addition, glucose promoted the expression of *PKM*, *PC*, and *GLUT4*. Since *GLUT4*, *PKM*, and *PC* genes are involved in glucose transport and the synthesis of pyruvate and oxaloacetate, respectively [24–26], oxaloacetate eventually enters the tricarboxylic acid cycle. Previous research showed that continuous glucose addition to cultured mouse hepatocytes led to the inhibition of *PEPCK* expression [27], consistent with our results. Therefore, an increase in intracellular glucose concentration will change gene expression levels to adapt to metabolic changes, effectively using high-concentration glucose for energy production and metabolic regulation.

After fasting, glucose supply can affect the metabolic pathways and gene expression of muscle cells, thereby regulating muscle energy metabolism. Glucose supply can promote the glycolytic pathway, activate energy metabolism-related genes, increase ATP synthesis, and adjust muscle energy metabolism [28,29].

4.3 Effects of Lactic Acid Concentration on Metabolism and Growth Activity of Skeletal Muscle Cells

The results showed that adding 10 and 20 mM lactic acid promoted skeletal muscle cells to produce a small amount of glucose, with relatively low LDH activity. However, at 30 mM lactic acid concentration, intracellular glucose concentration decreased, and LDH activity increased. Reduced cell activity usually increases LDH release. Previous studies found that lactic acid concentrations exceeding 20 mM (22 mM, 27 mM) significantly inhibited the growth activity of osteoblast-like cells [30]; the maximum tolerated concentration was 30 mM [31]. This indicates a limit in the utilization of lactic acid by the body. Excessive lactic acid harms cells, inhibiting their growth activity. These results are significant for understanding the biological role of lactic acid in cells and its regulatory mechanisms for cell growth.

4.4 Effect of Lactic Acid on the Expression of Key Gluconeogenesis Genes in Skeletal Muscle Cells

The past gluconeogenesis research has focused mainly on liver metabolic pathways, but the impact of lactic acid on key genes involved in gluconeogenesis in ruminant skeletal muscle remains unclear. The hepatic gluconeogenesis pathway has four key rate-limiting enzymes: *PC*, *PEPCK*, *FBPase*, and *G6PC*. *PC* and *PEPCK* catalyze the first and second steps, respectively. *PC* converts PEP into oxaloacetic acid (OAA), while *PEPCK* catalyzes the synthesis of phosphoenolpyruvate from OAA. This study found that in skeletal muscle cells exposed to varying lactic acid concentrations, *PC* expression initially rose and then declined, whereas *PEPCK* expression notably dropped. Adding a low dose of lactic acid (10 mM) caused these changes. The increased *PC* expression in skeletal muscle cells may enhance the carboxylation ability of pyruvate, promoting its conversion into OAA and its entry into the tricarboxylic acid cycle to generate energy for increased intracellular lactic acid metabolism [10,32]. The decreased *PEPCK* expression suggests OAA mainly entered the tricarboxylic acid cycle after lactic acid addition rather than converting to PEP. This may be due to the increased pyruvate supply from lactic acid metabolism, reducing the need for skeletal muscle cells to synthesize PEP. This study also observed that the expression of the *PKM* gene and protein in cells increased after adding different lactic acid concentrations. *PKM* is the main subtype of pyruvate kinase (*PK*) expressed in muscles [33], catalyzing the transfer of phosphate groups from PEP to ADP to produce ATP and pyruvate. It is a key

rate-limiting enzyme in the final step of glycolysis, which is generally considered irreversible [26]. However, many studies have confirmed the existence of a reverse flux of *PKM* in skeletal muscle [10,12,34]. Jin *et al*. [34] found through ^{13}C labeling that pyruvate/lactic acid in skeletal muscle does not generate PEP through the *PEPCK* pathway but directly converts pyruvate to PEP through *PKM* reverse flux, entering the gluconeogenesis pathway. Another study by Jin *et al*. [10] showed that lactic acid can promote glycerol neogenesis and glycogen synthesis in skeletal muscle by reversing *PKM*. However, under low lactic acid concentrations, reverse flux does not seem effective [34]. When lactic acid concentration increases to 10–12 mM, the reverse flux may be related to glycogen supplementation after strenuous exercise [35,36]. This study found that the relative expression of *PKM* significantly increased after adding lactic acid to skeletal muscle cells, similar to previous studies [10,12,34].

FBP2, an enzyme in muscles, hydrolyzes 1.6-diphosphofructose to 6-phosphofructose and is crucial in skeletal muscle gluconeogenesis [37]. Lactic acid addition increases *FBP2* gene expression in mouse skeletal muscle, enhancing glycogen synthesis [18]. Previous studies found that overexpressing the *FBP2* gene in mouse right tibial muscles increased glucose uptake and gluconeogenic flux [38]. *FBP2*-knockout mice exhibited severe intolerance to cold when fasted. Cold-induced conversion of lactic acid to glycogen (glycogenesis) was absent in *FBP2*-knockout muscles, causing a lack of glycogen for thermogenesis [39]. These findings underscore the role of FBP2 in skeletal muscle gluconeogenesis. This study showed that lactic acid addition increased FBP2 expression, promoting intramuscular glycogen synthesis, consistent with previous studies [18,38,39].

Recent research identified G6PC3, an enzyme in the endoplasmic reticulum that generates glucose. Unlike *G6PC1*, which is in the liver, kidneys, and intestines, *G6PC3* is commonly expressed in tissues [24]. However, G6PC activity in muscles is low and its physiological effects are often overlooked [40]. This study found that lactic acid did not significantly affect G6PC3 protein expression. Although 10 mM lactic acid increased cell glucose synthesis, it was limited and lacked physiological significance. This suggests that glucose-6-phosphate synthesized by skeletal muscle gluconeogenesis is converted to glycogen rather than released into the circulatory system [40].

Lactic acid crosses the plasma membrane via the *MCTS* of the *SLC16* gene family, promoting the transmembrane transport of lactic acid and pyruvate [41]. This study showed that varying lactic acid concentrations significantly improved *MCTS1* gene expression. *GLUT4* facilitates glucose passage across cell membranes and is the bottleneck in peripheral glucose utilization [42]. At 10 mM concentration, *GLUT4* gene mRNA expression increased significantly, consistent with the increase in glucose concentration found at 10 mM. *LDHA* converts lactic acid to pyruvate [43]. The *LDHA* gene mRNA expression also increased after adding 10 and 20 mM lactic acid.

Skeletal muscle cell growth and proliferation rates differ between fetal and postpartum sheep, but cell morphology and function are similar. Studying gluconeogenesis in fetal sheep skeletal muscle cells may help understand postpartum muscle metabolism. Sheep skeletal muscle cell gluconeogenesis is regulated by multiple factors including nutrition, hormones, and the environment. Studying fasting and lactic acid effects on key gluconeogenic genes in sheep skeletal muscle cells helps reveal energy metabolism regulation in ruminants and provides energy supply strategies for sheep during winter hay shortages.

5. Conclusions

This research discovered that fasting regulates the expression of crucial gluconeogenic genes (*FBP2*, *G6PC3*, *PKM*, *MCTS1*, *GLUT4*, *PC*, and *LDHA*) in sheep skeletal muscle cells and further revealed the role of lactic acid in inducing these key gluconeogenic genes, providing new insights and methods for related research.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

JW, YP and SH designed the research study. YP, BW and DW performed the research. FS and DW provided help and advice on the cell experiments. YP, BW, FS and JY analyzed the data and drew figures. YP wrote the manuscript. JW, SH, JY and YP reviewed and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The Experimental Animal Welfare and Ethics Committee of the College of Animal Science and Technology, Inner Mongolia Minzu University has reviewed and approved (Approval No. 2022015) for studies involving animals.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2906237.

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