

## Effects of Feed Restriction on Blood Biochemical Parameters and Lipid Metabolism in Visceral Adipose Tissue of Ewes during Mid-Gestation: Postprint

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### Abstract

This experiment aimed to investigate the effects of feed restriction on blood biochemical parameters, visceral adipose tissue (VAT) fatty acid composition, and lipid metabolism-related gene expression in mid-pregnant ewes. Sixteen pregnant Xiangdong black goats at (45±3) days of gestation, with body weight of (29.86±3.07) kg, were selected and randomly assigned to a control group (Group C, 100% of pregnancy nutritional requirements) and a feed restriction group (Group R, 60% of pregnancy nutritional requirements), with 8 goats per group. The experimental period lasted from day 45 to day 100 of gestation. On day 101 of gestation, blood biochemical parameters and the expression of genes related to fatty acid composition, lipid metabolism, and energy sensing in ruminal omentum, mesenteric, and perirenal adipose tissues were measured. The results showed that, compared with Group C, Group R exhibited: 1) significantly increased blood glucagon and free fatty acid contents ( $P<0.05$ ), and significantly decreased blood leptin, adiponectin, and high-density lipoprotein cholesterol contents ( $P<0.05$ ). 2) significantly reduced contents of arachidonic acid, palmitoleic acid, linoleic acid, eicosatrienoic acid, and polyunsaturated fatty acids in omental adipose tissue ( $P<0.05$ ); significantly reduced myristic acid content in mesenteric adipose tissue ( $P<0.05$ ); and significantly reduced linoleic acid, arachidonic acid, eicosatrienoic acid, and polyunsaturated fatty acid contents in perirenal adipose tissue ( $P<0.05$ ). 3) omental adipose tissue showed an upward trend in the expression of the catalytic subunit 2 of AMP-activated protein kinase (AMPK 2) gene (0.05

**Keywords:** feed restriction, mid-pregnancy, fatty acids, lipid metabolism, goats

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## Full Text

### Preamble

#### Effects of Feeding Restriction on Blood Biochemical Indexes and Lipid Metabolism of Visceral Adipose Tissue in Mid-Pregnancy Dams

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**Abstract:** This experiment investigated the effects of feeding restriction on blood biochemical indexes, fatty acid composition, and lipid metabolism-related gene expression in visceral adipose tissue (VAT) of mid-pregnancy ewes. Sixteen Xiangdong black goats at (45±3) days of gestation with body weight of (29.86±3.07) kg were randomly assigned to a control group (C group, 100% of pregnancy nutritional requirements) or a restricted group (R group, 60% of pregnancy nutritional requirements), with 8 ewes per group. The experimental period spanned days 45–100 of gestation. On day 101 of gestation, blood biochemical indexes and the fatty acid composition and expression of genes related to lipid metabolism and energy sensing were measured in ruminal omental, mesenteric, and perirenal adipose tissues. Compared with

the C group, the R group showed: 1) significantly elevated blood glucagon and free fatty acid concentrations ( $P < 0.05$ ), and significantly reduced blood leptin, adiponectin, and high-density lipoprotein cholesterol concentrations ( $P < 0.05$ ); 2) significantly decreased arachidonic acid, palmitoleic acid, linoleic acid, eicosatrienoic acid, and polyunsaturated fatty acid (PUFA) contents in omental adipose tissue ( $P < 0.05$ ), significantly decreased myristic acid content in mesenteric adipose tissue ( $P < 0.05$ ), and significantly decreased linoleic acid, arachidonic acid, eicosatrienoic acid, and PUFA contents in perirenal adipose tissue ( $P < 0.05$ ); 3) a trend toward upregulated expression of the adenosine monophosphate-activated protein kinase catalytic subunit 2 (AMPK 2) gene in omental adipose tissue ( $0.05 < P < 0.10$ ), trends toward downregulated expression of fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), and uncoupling protein 2 (UCP2) genes in mesenteric adipose tissue ( $0.05 < P < 0.10$ ) with a trend toward upregulated peroxisome proliferator-activated receptor (PPAR) gene expression ( $0.05 < P < 0.10$ ), and significantly downregulated FASN and UCP2 gene expression in perirenal adipose tissue ( $P < 0.05$ ) with significantly upregulated AMPK 1 gene expression ( $P < 0.05$ ) and a trend toward increased carnitine palmitoyltransferase 1A (CPT1A) gene expression ( $0.05 < P < 0.10$ ). These findings indicate that mid-gestation feeding restriction affects lipid metabolism regulatory factors (leptin and adiponectin) and lipid metabolites (free fatty acids) in maternal blood, alters fatty acid composition (PUFA) in VAT, and modifies expression of lipid metabolism-related genes (FASN, SCD1, CPT1A, and UCP2), thereby attenuating lipid synthesis while enhancing lipid mobilization in maternal VAT to regulate energy homeostasis.

**Keywords:** feeding restriction; mid-gestation; fatty acids; lipid metabolism; goat

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During gestation, ewes are susceptible to nutritional restriction due to limited natural forage resources and quality, production management practices, and environmental stress. Nutritional restriction during pregnancy not only affects the development of maternal gestational tissues, subsequent reproductive performance, fetal organ development, and offspring growth performance, but also significantly influences maternal metabolism, particularly lipid metabolism. Previous studies on maternal nutritional restriction have focused primarily on the peri-implantation period and late gestation, with nutritional interventions in production systems also concentrated in late gestation and lactation stages. However, research on maternal nutritional requirements and energy metabolism during mid-gestation remains limited. Mid-gestation, defined as the period from one-third to two-thirds of pregnancy, represents a critical phase for the development of maternal gestational tissues and fetal organ morphogenesis and functional maturation. Understanding the metabolic changes and potential compensatory mechanisms during nutritional restriction in this period is essential for establishing nutritional requirements and developing appropriate feeding management strategies.

When maternal nutrition is restricted during gestation, the dam mobilizes its own nutrient reserves, particularly adipose tissue, to support embryonic development and meet energy demands, thereby buffering the adverse effects of the external environment on embryonic development. Adipose tissue serves as both an important energy storage organ and a key regulator of lipid metabolism. Based on distribution location, adipose tissue can be divided into subcutaneous adipose tissue and visceral adipose tissue (VAT). VAT is primarily distributed in the ruminal omentum, mesentery, and perirenal regions, and compared with subcutaneous adipose tissue, it exhibits stronger and faster lipid mobilization capacity. Its metabolites can directly enter the bloodstream via the portal vein, thereby influencing systemic lipid and energy metabolism. VAT mobilization and metabolism are closely associated with insulin resistance, lipid metabolism disorders, diabetes, fatty liver disease, and metabolic syndrome. Changes in VAT mobilization and metabolism serve as important indicators of nutrient sufficiency or deficiency and represent a critical response pathway to nutritional stress.

This study investigated the effects of feeding restriction on blood lipid metabolism-related biochemical indexes, VAT fatty acid composition, and lipid metabolism-related gene expression in mid-pregnancy ewes to elucidate the mechanisms of VAT lipid mobilization and its impact on systemic lipid metabolism under low nutritional status during mid-gestation.

## 1.1 Experimental Animals and Management

A single-factor randomized block design was employed. Sixteen healthy Xiangdong black goats at  $(45 \pm 3)$  days of gestation with body weight of  $(29.86 \pm 3.07)$  kg were randomly allocated to either a control group (C group, 100% of pregnancy nutritional requirements) or a restricted group (R group, 60% of pregnancy nutritional requirements), with 8 ewes per group. The experimental period covered days 45–100 of gestation, during which the ewes received 55 days of treatment. All ewes were housed individually and fed a diet with a concentrate-to-forage ratio of 50:50, offered in equal portions twice daily at 08:00 and 16:00 (concentrate first, then forage). Both groups received the same basal diet, with the R group receiving 60% of the feed amount provided to the C group and ad libitum access to water. During the restriction period, dry matter intake was  $(1.14 \pm 0.04)$  kg/d for the C group and  $(0.60 \pm 0.02)$  kg/d for the R group, representing an actual restriction level of 52% of the C group intake. Body weight gain at the end of the restriction period was  $(5.91 \pm 0.70)$  kg for the C group and  $(2.65 \pm 0.22)$  kg for the R group. The composition and nutrient levels of the basal diet are presented in Table 1.

## 1.2 Sample Collection and Analysis

**1.2.1 Blood Sample Collection and Analysis** At the conclusion of the experiment, ewes were fasted for 24 h and blood samples were collected via jugular venipuncture at 09:00 on day 101 of gestation using heparin sodium as an antico-

agulant. After standing at room temperature for 4 h, plasma was separated by centrifugation at  $1,200\times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Blood glucose (GLU), triglycerides (TG), total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were analyzed using a Hitachi 7600 automatic biochemical analyzer (reagents purchased from Beijing Leadman Biochemistry Co., Ltd.). Blood insulin (INS), glucagon, leptin (LEP), and adiponectin (ADP) concentrations were determined by enzyme-linked immunosorbent assay (kits purchased from Wuhan Huamei Bioengineering Co., Ltd.). Blood free fatty acid (FFA) concentration was measured using a commercial kit (purchased from Nanjing Jiancheng Bioengineering Institute).

**1.2.2 Adipose Tissue Sample Collection and Analysis** Following exsanguination via carotid artery and evisceration, omental adipose tissue (omental fat), mesenteric adipose tissue (mesenteric fat), and perirenal adipose tissue (perirenal fat) were isolated, snap-frozen in liquid nitrogen, and stored at  $-80\text{ }^{\circ}\text{C}$ .

**Fatty Acid Content Determination:** Freeze-dried and pulverized adipose tissue samples ( $\sim 0.5\text{ g}$ ) were placed in 50 mL centrifuge tubes and extracted with 4 mL of benzene-petroleum ether mixture (1:1, v/v) for 24 h under sealed conditions. Rapid methylation was performed by adding 4 mL of potassium hydroxide-methanol solution (0.4 mol/L), followed by vigorous mixing for 3 min and standing for 30 min. After adding ultrapure water for phase separation, the upper layer was collected by centrifugation at  $9,200\times g$  for 10 min and dehydrated with an appropriate amount of anhydrous sodium sulfate. A 200  $\mu\text{L}$  aliquot of the supernatant was diluted with 800  $\mu\text{L}$  of n-hexane, filtered through a 0.22  $\mu\text{m}$  membrane, and analyzed for fatty acid content using an Agilent 7890A gas chromatograph (Agilent Technologies, USA) according to the method described by Ichihara et al. [11]. Quantification was performed using peak area normalization, with fatty acid contents expressed as the mass percentage of individual fatty acids in total methylated fatty acids.

**Real-Time Quantitative PCR (RT-PCR):** Total RNA was extracted using RNAsiso Plus reagent (TaKaRa) according to the manufacturer's instructions. RNA concentration and purity were determined using a micro-volume UV spectrophotometer at 260 nm and 280 nm, with RNA purity considered acceptable when the OD260/OD280 ratio ranged from 1.8 to 2.1. RNA quality was assessed by 1% denaturing agarose gel electrophoresis, with a 2:1 intensity ratio of 28S rRNA to 18S rRNA indicating good quality. Reverse transcription was performed using 2  $\mu\text{g}$  of total RNA with the PrimeScript RT reagent kit (TaKaRa) in a 40  $\mu\text{L}$  reaction volume according to the manufacturer's protocol, with cDNA products stored at  $-80\text{ }^{\circ}\text{C}$ . Primers for target genes and the reference gene  $\beta$ -actin were designed using Primer Premier 5.0 software based on published goat gene sequences from GenBank and analyzed for specificity using the Primer-BLAST tool online. Primers were synthesized by Shanghai Sangon Biotech Co., Ltd.,

with sequences and parameters listed in Table 2 .

RT-PCR was performed using a fluorescence quantitative PCR instrument (ABI 7900 HT, ABI, USA) with a 10  $\mu$ L reaction system containing SYBR Premix Ex Taq II (2 $\times$ ) 5.0  $\mu$ L, forward primer (10  $\mu$ mol/L) 0.4  $\mu$ L, reverse primer (10  $\mu$ mol/L) 0.4  $\mu$ L, cDNA template 1.0  $\mu$ L, and RNase-free dH<sub>2</sub>O 3.2  $\mu$ L. Cycling conditions were: 95  $^{\circ}$ C for 30 s pre-denaturation; 40 cycles of 95  $^{\circ}$ C for 5 s denaturation and 60  $^{\circ}$ C for 20 s extension; followed by melting curve analysis (95  $^{\circ}$ C for 5 s, 65  $^{\circ}$ C for 60 s) and cooling (50  $^{\circ}$ C for 30 s). Each sample was run in triplicate.  $\beta$ -actin served as the internal reference gene, with the mean Ct value of the control group used as the calibrator. Relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method.

### 1.3 Statistical Analysis

All experimental data were organized using Excel 2013 and analyzed by t-test using SPSS 19.0 software. Differences were considered significant at  $P < 0.05$  and indicative of a trend at  $0.05 < P < 0.10$ . Results are presented as means with standard errors.

## 2 Results

### 2.1 Blood Biochemical Indexes

As shown in Table 3 , compared with the C group, the R group exhibited significantly increased blood glucagon and FFA concentrations ( $P < 0.05$ ) and significantly decreased blood leptin, adiponectin, and HDL-C concentrations ( $P < 0.05$ ). No significant differences were observed in blood INS, GLU, TG, CHOL, LDL-C concentrations, or the HDL-C/CHOL ratio ( $P > 0.10$ ).

### 2.2 Fatty Acid Composition of Visceral Adipose Tissue

Table 4 shows that compared with the C group, the R group had significantly decreased contents of arachidonic acid (C20:4n6), palmitoleic acid (C16:1), linoleic acid (C18:2n6c), eicosatrienoic acid (C20:3n6), and polyunsaturated fatty acids (PUFA) in omental adipose tissue ( $P < 0.05$ ), with trends toward decreased myristic acid (C14:0) and increased palmitic acid (C16:0) contents ( $0.05 < P < 0.10$ ). No significant differences were detected in other measured fatty acids between the two groups ( $P > 0.10$ ).

As shown in Table 5 , compared with the C group, the R group exhibited significantly decreased myristic acid content in mesenteric adipose tissue ( $P < 0.05$ ), with trends toward decreased linoleic acid,  $\gamma$ -linolenic acid (C18:3n6), eicosenoic acid (C20:1), eicosatrienoic acid, and PUFA contents ( $0.05 < P < 0.10$ ), and a trend toward increased trans-oleic acid (C18:1n9t) content ( $0.05 < P < 0.10$ ). No significant differences were observed in other measured fatty acids between the two groups ( $P > 0.10$ ).

Table 6 reveals that compared with the C group, the R group had significantly decreased linoleic acid, arachidonic acid, eicosatrienoic acid, and PUFA contents in perirenal adipose tissue ( $P < 0.05$ ), with a trend toward decreased myristic acid content ( $0.05 < P < 0.10$ ). No significant differences were detected in other fatty acids between the two groups ( $P > 0.10$ ).

### 2.3 Gene Expression Related to Lipid Metabolism in Visceral Adipose Tissue

Table 7 shows that compared with the C group, the R group exhibited a trend toward upregulated AMPK 2 gene expression in omental adipose tissue ( $0.05 < P < 0.10$ ), with no significant differences in other gene expressions between the two groups ( $P > 0.10$ ).

As presented in Table 8, compared with the C group, the R group showed trends toward downregulated FASN, SCD1, and UCP2 gene expressions in mesenteric adipose tissue ( $0.05 < P < 0.10$ ), with a trend toward upregulated PPAR gene expression ( $0.05 < P < 0.10$ ). No significant differences were observed in other gene expressions between the two groups ( $P > 0.10$ ).

Table 9 demonstrates that compared with the C group, the R group exhibited significantly downregulated FASN and UCP2 gene expressions in perirenal adipose tissue ( $P < 0.05$ ), significantly upregulated AMPK 1 gene expression ( $P < 0.05$ ), and a trend toward increased CPT1A gene expression ( $0.05 < P < 0.10$ ). No significant differences were detected in other gene expressions between the two groups ( $P > 0.10$ ).

## 3 Discussion

When dietary nutrient supply is insufficient, pregnant dams mobilize their energy reserves—adipose tissue—to sustain their own needs and those of the pregnancy. As a vital component of the body's energy storage and lipid metabolism system, VAT can influence blood lipid metabolite concentrations through its own anabolic and catabolic regulation, thereby modulating systemic lipid and energy metabolism to maintain relative metabolic balance.

### 3.1 Effects of Feeding Restriction on Blood Biochemical Indexes in Ewes

Glucagon is a crucial hormone regulating energy metabolism, maintaining blood glucose and energy homeostasis by promoting gluconeogenesis and glycogenolysis, reducing fatty acid synthesis, and enhancing fatty acid mobilization into circulation for oxidative energy production by peripheral tissues. Elevated glucagon increases VAT lipolysis, releasing more FFAs into the bloodstream to compensate for negative energy balance. Leptin and adiponectin are important regulatory factors secreted by adipose tissue that control lipid metabolism, lipid homeostasis, and energy metabolism. Low nutritional status

and increased blood FFA concentrations inhibit leptin secretion. In this study, the R group exhibited increased blood glucagon and FFA concentrations, decreased blood leptin and adiponectin concentrations, and relatively stable blood glucose levels. These findings indicate that under feeding restriction, dams adjust lipid metabolism by increasing blood glucagon while decreasing leptin and adiponectin, thereby enhancing lipid mobilization and utilization of circulating FFA to compensate for energy deficiency. This adaptive response maintains relatively stable blood glucose and ensures pregnancy progression under nutritional limitation, consistent with the findings of Haghiaci et al. [19].

### **3.2 Effects of Feeding Restriction on VAT Fatty Acid Composition in Ewes**

VAT fatty acid composition is primarily influenced by lipogenesis and lipolysis, which are critical for reproductive performance, milk fat content, and energy metabolism in pregnant animals. This study demonstrated that feeding restriction induced significant changes in VAT fatty acid composition, with decreased saturated fatty acids (myristic acid) and unsaturated fatty acids (primarily C18 and C20) across different VAT depots. In ruminants, medium- and short-chain fatty acids are mainly synthesized *de novo* from acetate and butyrate, while PUFAs primarily originate from rumen microbial transformation and dietary deposition. The observed changes in VAT fatty acids in the R group may be attributed to: (1) reduced availability of fatty acid precursors (acetate and butyrate) due to feeding restriction, leading to decreased synthesis by rumen microbes and reduced dietary PUFA intake; and (2) increased demand due to energy deficit, resulting in enhanced long-chain fatty acid mobilization and oxidation. The combined effect of reduced supply and increased utilization led to the altered VAT fatty acid composition.

### **3.3 Effects of Feeding Restriction on VAT Lipid Metabolism-Related Gene Expression**

Nutritional status regulates adipose tissue function in lipid synthesis, mobilization, and energy sensing by modulating expression of lipid metabolism-related genes, thereby controlling systemic lipid and energy metabolism. This study focused on key genes involved in these processes.

FASN, SCD1, ACC, liver X receptor (LXR), and PPAR are critical regulators of *de novo* fatty acid synthesis, lipid storage, fatty acid homeostasis, and adipocyte differentiation. FASN primarily controls *de novo* fatty acid synthesis and affects lipid deposition, while SCD1 catalyzes unsaturated fatty acid synthesis and desaturation of saturated fatty acids. In this study, downregulated expression of FASN in perirenal and mesenteric adipose tissues and SCD1 in mesenteric adipose tissue in the R group indicated reduced capacity for long-chain fatty acid synthesis due to nutritional insufficiency. The decreased contents of linoleic acid,  $\alpha$ -linolenic acid, and eicosenoic acid in VAT corroborated these gene expression results.

Hormone-sensitive lipase (HSL), CPT1A, acyl-CoA oxidase 1 (ACOX1), and peroxisome proliferator-activated receptor coactivator 1 (PGC-1) are key regulators of lipolysis, fatty acid oxidation, cellular lipid uptake and breakdown, and mitochondrial biogenesis and energy production. CPT1A, in particular, is a critical regulator of the initial step of long-chain fatty acid -oxidation and modulates fatty acid oxidation rates in tissues. The trend toward upregulated CPT1A expression in perirenal adipose tissue of the R group suggested accelerated long-chain fatty acid oxidation, consistent with the reduced long-chain fatty acid content in perirenal adipose tissue and indicating that dams may compensate for energy shortage through this pathway.

The AMPK pathway senses cellular energy status and regulates energy-producing and consuming metabolism, earning it the designation as a “metabolic sensor” and “master energy switch.” It is a central regulator of glucose metabolism, lipid metabolism, and fatty acid composition, and interacts with liver kinase B1 (LKB1) to maintain lipid and energy balance. AMPK 2 and AMPK 1 encode the 2 and 1 catalytic subunits, respectively. In this study, upregulated AMPK 1 expression in perirenal adipose tissue and a trend toward increased AMPK 2 expression in omental adipose tissue in the R group indicated that feeding restriction enhanced expression of energy-sensing switch genes in these depots.

The uncoupling protein (UCP) family is closely associated with energy metabolism balance, fat deposition, and thermogenesis. UCP1 primarily regulates non-shivering thermogenesis in brown adipose tissue, while UCP2 modulates basal energy metabolism through uncoupling activity and can regulate lipid synthesis by influencing FASN gene expression. In this study, downregulated UCP2 gene expression in perirenal adipose tissue and a trend toward decreased expression in mesenteric adipose tissue in the R group suggested that feeding restriction reduced basal energy metabolism, which may represent a protective adaptation to energy deficiency. This is consistent with studies showing that UCP2 expression correlates positively with fatty acid content in adipose tissue and that changes in FASN and UCP2 gene expression are synchronized. Collectively, feeding restriction upregulated the energy-sensing switch in maternal VAT while tending to reduce basal energy metabolism levels.

Furthermore, this study found that omental and perirenal adipose tissues were more affected by feeding restriction than mesenteric adipose tissue in terms of fatty acid composition, and perirenal adipose tissue showed more pronounced changes in lipid metabolism gene expression than omental and mesenteric depots. These findings suggest differential responsiveness among VAT depots to feeding restriction, with sensitivity decreasing in the order: perirenal > omental > mesenteric adipose tissue. This may be related to rapid perception and response pathways involving the starvation-hypothalamic-adrenal axis, though the underlying mechanisms require further investigation.

## 4 Conclusion

Feeding restriction during mid-gestation decreased blood concentrations of lipid metabolism regulatory factors (leptin and adiponectin) and increased lipid metabolite (FFA) concentrations in ewes. It altered VAT fatty acid composition (decreased PUFA) and modulated expression of lipid synthesis genes (FASN and SCD1), lipid mobilization genes (CPT1A), and basal energy metabolism genes (UCP2), thereby attenuating lipid synthesis while enhancing lipid mobilization and fatty acid oxidation in maternal VAT to regulate energy homeostasis.

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*Note: Figure translations are in progress. See original paper for figures.*

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