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Liver Metabolism Adaptation to High-Concentrate Diet-Induced Milk Fat Depression

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LL and PSY performed the experiments. LL drafted the manuscript. PSY analysed the data. XC, YSZ and SPY designed the experiments and revised the manuscript. YSZ conceived the idea.

Key words
Liver, Metabolism, High-concentrate diet, Milk fat, Milk fat depression

ABSTRACT

The high concentrate/low forage (HC/LF) diets cause a dramatic decline in milk fat secretion. Hepatic lipid metabolism is active when ruminant is on lactation period. The aim of this study was to evaluate liver metabolism adaptation to high-concentrate diet-induced milk fat depression (MFD). Six Saanen dairy goats were randomly divided into 2 groups and fed either a high-forage (HF) diet or a high- concentrate (HC) diet. The high-concentrate diet caused lower milk fat content and milk fat yield, namely induced MFD. While, the net hepatic TG production (portal vein - hepatic vein) significant increase in the HC group (P<0.01) and it was positive value. The net hepatic glucose, cholesterol and BHBA productions (portal vein - hepatic vein) were all negative values in the HC group. Expression of hepatic PC and PEPCK mRNA were up-regulated in HC group but had no significant effect (P>0.05). The mRNA expression of hepatic HMGCS2 significantly increased (P< 0.01) in HC group. The high concentrate feeding induced MFD, while fatty acids uptake into liver were stored as TG or incompletely oxidized to ketone body, the excessive consumption of fatty acids reduced the precursors of milk fat synthesis and finally decreased the milk fat content.

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INTRODUCTION

Milk fat is an important indicator of milk nutritional quality, and the fat content and fatty acid composition of milk can be dramatically affected by dietary manipulation in many species (Neville and Picciano, 1997; Palmquist *et al*., 1993). This has been studied extensively in ruminants; one striking example is the low fat milk syndrome, commonly referred to as milk fat depression (MFD) (Feng *et al*., 2008; Maxin *et al*., 2011). In this situation, high concentrate/low forage (HC/LF) diets cause a dramatic decline in milk fat secretion. First recognized by Boussingault in 1845 (Hartl and Hayer-Hartl, 2002), this phenomenon involves an interaction between alterations in rumen digestion and tissue metabolism. Although many theories have been pro-posed to explain the basis of MFD, its mechanism has remained elusive (Bauman and Griinari, 2000, 2003).

Hepatic lipid metabolism is active when ruminant is on lactation period. Both volatile fatty acids produced by rumen fermentation and long-chain fatty acids of dietary sources are mostly gotten into the liver from portal vein to metabolic conversion, which provides precursors for milk fat synthesis in mammary gland (Kristensen and Harmon, 2004). Pullen *et al*. (1989) have studied that it is proportional to the amount of free fatty acids in the blood and free fatty acid absorbed in liver. Kristensen *et al*. (2004) has also reported that liver is the most important place for propionic acid, branched chain-volatile fatty acid and long-chain fatty acid metabolism, besides the amount of precursors released from liver determined milk fat production. Dong H had induced Subacute Ruminal Acidosis (SARA) by high concentrate diet, and discovered this stringent state possible cause the changes of hepatic substance metabolism and nutrient substance redistribution (Dong *et al*., 2013). The objectives of the present study were to relate MFD induced by feeding a HC/LF diet to hepatic glycolipid metabolism. We use multiple vascular fistula technique to observe the changes of NEFA, BHBA, and TG in hepatic vein and portal vein when high-concentrate diet-induced MFD happened during lactation period. Then we explored the rule of fat metabolism in the liver combined with metabolism-related gene expression.

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MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, People's Republic of China) approved all of the procedures (surgical procedures and care of goats). The experiment was performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China).

Animals and experimental procedures

Six Saanen dairy goats that had been lactating for 30 d with an average milk production of 1.0 kg/d were used in this experiment. Before the formal experiment, all goats were surgically prepared with cannulas in the hepatic and portal as the method of Chang (Becker and Craig, 1994). We conducted fistula care with heparin solution (300IU·mL^{-1}) twice daily to ensure the fistula unblocked. The lactating Saanen goats (n=6) were used in a single reversed design; three goats were fed the high-forage (HF) diet and the others were fed the high-concentrate (HC) diet in the first experimental period and treatments were reversed in the second period (Jiang *et al*., 2014). Treatment periods were 4 wk with an intervening 3-wk recovery interval between periods. The goats were fed daily at 8:00 and 18:00, and every goat was provided 0.9kg dry matter every time. All goats were housed in individual stalls and had free access to drinking water. Ingredients and chemical composition of experimental diets are given in Table I.

Milk composition analysis

Goats were milked twice daily and milk yield recorded. The milk samples were taken from each milking during the last 2d of the experimental periods and were analyzed for fat and protein by milk composition analyzer (Fossomatic 5000, Foss Electric, Hillerod, Denmark).

Measurement of plasma biochemical parameters

At the end of every experiment period, plasma was sampled using EDTA-containing vacuum tubes from hepatic and portal vein. Plasma glucose, cholesterol, triglyceride (TG), nonesterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) were detected by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Liver collected and analysis

After all the trail period, the goats were sacrificed and liver samples were collected. Liver tissues were snap-frozen in liquid nitrogen and transported to the laboratory where they were kept at -80°C until used for RNA extraction. Total RNA was extracted from liver tissue referring to previous methods (Jiang *et al*., 2013). Gene-specific primers were designed using the Primer 5.0 software (Table II). Quantitative RT-PCR (qRT-PCR) analysis was performed with MyiQ2 Real-time PCR system (Bio Rad, USA). PCR was performed using iTaq Universal SYBR Green Supermix (Bio Rad, USA), according to the manufacturer's protocol. Each sample was analyzed in triplicate. For qRT-PCR, the amplifications were performed with the following protocol:95°C for 1 min, followed by 40 cycles of 95°C for 15s, 58°C for 30s. β-actin was used as a housekeeping gene (Bondzio *et al*., 2011). Each sample was quantified against its β-actin transcript contents and then normalized to the control group. In order to calculate differences in the expression level of each target gene, the $2^{-\Delta\Delta CT}$ method for relative quantification was used according to the manufacturer's manual.

Table I. Composition and nutrient levels of high-forage (HF) and high-concentrate (HC) diets (%).

Diet composition	HF	HC
Feed composition (%)		
Aneurolepidium chinsnse	40.00	26.70
Alfalfa	20.00	13.30
Corn grain	22.99	23.24
Wheat bran	0.00	20.77
Soybean meal	15.00	13.66
Limestone	0.65	1.43
Calcium phosphate dibasic	0.46	0.00
*Premix	0.50	0.50
Salt	0.40	0.40
Nutrient composition (%)		
Crude protein	17.22	16.93
Ether extract	2.87	3.21
Neutral detergent fibre	36.64	34.55
Acid detergent fibre	24.74	20.35
Non-fibre carbohydrates	31.76	35.00
Calcium	0.80	0.90
Phosphorus	0.33	0.38
Lys	0.82	0.79
Met	0.18	0.20
Branched chain amino acid	2.38	2.40
Total essential amino acid	5.04	5.07
Glucogenic amino acid	6.75	7.32
Total amino acid	12.86	13.38

Note: (1) *Premix provided: Vitamin A1 600 000 IU•kg^{-1}; Vitamin D 500 000 IU•kg^{-1}; Vitamin E 16 000 mg•kg^{-1}; Cu 1 250 mg•kg^{-1}; Fe 12 500 mg•kg^{-1}; Zn 12 500 mg•kg^{-1}; Mn 10 000 mg•kg^{-1}; I 50 mg•kg^{-1}; Co 25 mg•kg^{-1}; Mo 25 mg•kg^{-1}. (2) Net energy is 5.63(HF) and 5.83(HC) in diets.

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Table II. PCR primersfor target genes and *β*-actin.

Gene	Primers sequence (5'→3')	Product size (bp)
β-actin	F: GGGCAGGTCATCACCATT	160
	R: CCGTGTTGGCGTAAAGGT	
PC	F: ACTTCAAGGACTTCACTGCCACCT	385
	R: AAGGCTTTGATGTGCAGCGTCTTG	
PEPCK-C	F: GTGCTGGAGTGGATGTTC	306
	R: GTGCGTTGTATGGATTGGA	
CPT-I	F: CCCATGTCCTTGTAATGAGCCAG	158
	R: AGACTTCGCTGAGCAGTGCCA	
HMGCS2	F: GAGAGGGCTTAGAGGAACCC	182
	R: CCAGCTTGCTTCCACTGTTT	

RESULTS

The changes of milk yield and milk composition

Milk yield were not affected by dietary treatment (Table III). Similarly, the yield and content of milk protein were unaltered by dietary treatment. In contrast, the yield and percentage of fat in the milk were significantly reduced by the HC diet.

Table III. Effect of a high concentrate (HC) diet (mean± SEM) on performance of lactating dairy goats[1].

		HF	HC	P value
Milk yield (g/d)		995.25±30.35	1131.44±56.30	0.114
Milk protein				
	%	3.12±0.01	2.62±0.21	0.091
	g/d	31.10±0.84	29.44±1.89	0.513
Milk fat				
	%	3.76±0.13	3.00±0.06	0.003
	g	37.13±0.33	33.54±0.44	0.050

[1]Treatments were high-forage (HF) diet and high concentract (HC) diet designed to induce milk fat depression. Values are least squares means of the last 2d of each experimental period (n=6).

Plasma metabolites in hepatic vein and portal vein

The productions of metabolites were estimated by measurement of the hepatic vein and portal vein. The results are listed in Table IV. In the portal vein, TG content was significant up- regulated in HC group compared to HF group (P<0.05), concentrations of NEFA and BHBA increased in HC group but were not significantly different (P>0.05). Compared with HF group, in the hepatic vein, NEFA and BHBA concentration in HC group was increased significantly (P<0.05), and glucose and cholesterol concentrations also showed a tendency to increase (P>0.05).

Net rates of metabolite production or uptake were calculated from these data and the results are presented in Table V. The net hepatic TG production (portal vein - hepatic vein) significant increased in the HC group (P<0.01) and it was positive value. It means the high-concentration feeding reduced output of hepatic TG and TG was accumulated or translated in the liver. The net hepatic glucose, cholesterol and BHBA productions (portal vein - hepatic vein) were all negative values in the HC group, and it indicated that the productions of hepatic glucose, cholesterol and BHBA were increased.

Table IV. The effects of a high concentrate (HC) diet (mean± SEM) on plasma metabolite in hepatic vein and portal vein.

	Items	HF	HC
Portal vein	Glucose	3.17±0.18	3.06±0.13
	Cholesterol	2.45±0.07	2.43±0.42
	TG	0.16±0.02	0.22±0.01*
	NEFA	544.25±78.178	922.31±122.8
	BHBA(μmol/ml)	2.29±0.65	4.04±0.78
Hepatic vein	Glucose	2.87±0.04	3.33±0.33
	Cholesterol	2.01±0.11	2.27±0.12
	TG	0.18±0.02	0.15±0.01
	NEFA	283.90±38.18	470.34±49.43*
	BHBA(μmol/ml)	2.24±0.60	4.41±0.57*

TG, triglyceride; NEFA, non-esterified fatty acid; BHBA (B-hydroxybutyric acid). *P<0.05 vs. HF.
For other abbreviations, see Table III.

Table V. Net metabolite production (portal vein-hepatic vein).

Items	HF	HC
Glucose	0.33±0.17	-0.27±0.29
Cholesterol	-0.18±0.68	-0.23±0.43
TG	-0.0367±0.008	0.067±0.008**
NEFA	272.13±31.58	498.12±62.67
BHBA(μmol/ml)	0.050±0.049	-0.382±0.171

**P<0.01 vs. HF.
For abbreviations, see Table IV.

QRT-PCR analysis involved in glyconeogenesis and BHBA synthesis

Expression of hepatic pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA were up-regulated in HC group compared to HF group

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but had no significant effect (P>0.05) (Fig. 1). The mRNA expression of hepatic HMGCS2 significantly increased (P< 0.01) in HC group compared to HF group.

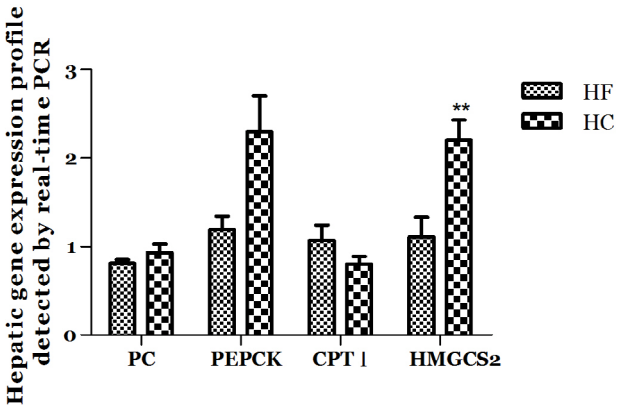

Fig. 1. Hepatic gene expression profile detected by real-time PCR. β-actin was used as the reference gene for gene expression. Values are mean± SEM. **P<0.01 vs. HF. PC, pyrivate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; CPT1, carnitine palmitoyltransfease 1; HMGCS2, 3 hydroxy-3-glutarineacyl-Coenzyme A synthase 2.

DISCUSSION

Milk fat depression

Milk fat depression in dairy cows occurs when rumen fermentation is altered. One type of diet that causes MFD is a diet that is high in starchy concentrates such as corn and marginal in effective fiber. In the present study, we fed a HC diet that reduced milk fat content and yield by 20 and 10%, respectively, without any effect on milk yield or milk protein. The other type of diet caused MFD is the diet contained a certain amount of high unsaturated fatty acid (HUFA). The forage level in diet can affect the rumen fermentation properties and the composition of volatile fatty acids. A high level of concentrate in diet results in low rumen PH, which is negative for the fermentation of acetic acid, while acetic acid is the main resource of carbon for milk fat synthesis. Jenkins *et al* found the composition of diet had great effects on milk production and milk fat yield, high-concentrate diet increased the milk production but decreased the milk fat content (Jenkins and McGuire, 2006). Baumgard *et al* also found high-concentrate diet decreased milk fat content about 60%, they explained high-concentrate diet had higher content of non-structural carbohydrate (NSC) which increased milk production but decreased the milk fat content (Baumgard *et al*., 2000). Our study showed high-concentrate caused lower milk fat content and milk fat yield, namely induced MFD phenomena.

Hepatic substance distribution and MFD

Liver is the metabolic center of nutrition substance. Nutrition substance absorbed by alimentary canal and collected in portal vein via mesentery and then flow to liver, subsequently entered postcaval vein after hepatic metabolism and transformation, transported to peripheral tissue for utilizing by heart. Therefore, liver plays an important role in the regulation of the composition and metabolism of nutrition substance in peripheral blood. The degree of hepatic metabolism and transformation of the nutrition absorbed by alimentary canal have a directly effect on the quality and quantity of precursors of milk in lactating ruminants. Different types of diets contain alterable content of nutrition substances, which affect the category and quantity of nutrition substances flowed into liver, meanwhile, the self-metabolism and the function condition of the liver affected the output of nutrition substances as well. We determined the concentration of TG, NEFA, BHBA and TC in the blood collected from hepatic portal vein and hepatic vein, the results showed high-concentrate group had higher levels of TG and NEFA in hepatic portal vein blood but lower levels in hepatic vein blood, while BHBA was opposite, more in hepatic portal vein blood and less in hepatic vein blood, the content of TC remained no obvious change. The results explained liver produced BHBA and consumed TG and NEFA, excessive consumption of NEFA may resulted in MFD.

Liver is the main metabolic organ of NEFA. NEFA will be absorbed by intestinal canal and be taken up to liver via hepatic portal vein. Blood that flow out from liver in hepatic vein, can reflect the metabolism condition of NEFA to a certain extent by detecting the NEFA concentration in hepatic vein blood. There are two mainly pathway of NEFA in liver tissue:1) transformed into acetyl-COA via β-oxidation, partial entered citric acid cycle for complete oxidative metabolism and the other incompletely oxidized to ketone body; 2) re-esterificated and stored as TG in the liver or secreted out of liver in the form of VLDL (van Dorland *et al*., 2012). Our study showed the output of BHBA in the liver is more than that of the input, and BHBA is the main ketone body produced by the liver. Schugar reported three aspects can control ketoplasia: (1) The amount of NEFA uptake into liver; (2) the activity of CPT-1; (3) the activity of 3-hydroxy-3-glutaricacyl-coenzyme A synthase 2 (HMGCS2), which is the key enzyme of ketoplasia (Schugar *et al*., 2014; Steele *et al*., 2011). We found a highly significant increased expression of HMGCS2 gene in high concentrate group, showed enhanced synthesis of ketone body in liver. Thus, we speculate that high concentrate feeding resulted in incomplete oxidation of acetyl-COA

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generated by β-oxidation of fatty acids, acetyl-COA prefer transformed into ketone body, and NEFA consumed excessively, resulted in a reduction of the raw material for milk fat synthesis.

The other pathway of NEFA metabolism is esterificated to TG. NEFA in blood is the main resource of TG synthesis in ruminants. Therefore, TG synthesis is related to the blood NEFA level produced by lipid mobilization. The limited capacities of hepatic fatty acid oxidative metabolism and secretion out of liver in the form of VLDL lead to superfluous NEFA re-esterificated and stored as TG in the liver tissue.

Hepatic metabolism changes

The fatty acid oxidation in mitochondria plays an important role in the body energy balance. CPT-1 is the first rate-limiting enzyme involved in β-oxidation after long-chain fatty acid transform into mitochondria. Our study found when the MFD occurred, the expression of CPT-1 gene and β-oxidation decreased, the energy supply decreased. In order to maintain the energy required for lactation, it need provide energy from glucose oxidation. Gluconeogenesis is the key link to maintain energy balance in ruminants. Propionate produced by rumen fermentation, is metabolized to pyruvic acid in liver, later catalyzed to oxaloacetic acid via pyruvate carboxylase (PC), further catalyzed to phosphoenolpyruvic acid in the role of PEPCK, and then transform to glucose. In ruminants, 90% of glucose is supplied by gluconeogenesis. The diets were generated to volatile fatty acids (acetic acid, propionic acid and butyric acid) by microbial fermentation in the rumen. And 90% propionic acid, that absorbed into the portal vein, was uptaken by the liver and transformed to glucose via gluconeogenesis pathway. Evans found the increased concentrate content in diet could improve hepatic glucose production in goat (Evans *et al*., 1975). PEPCK and PC, rate-limiting enzymes of gluconeogenesis, can regulate the rate of gluconeogenesis, and are mainly expressed in liver tissue. Our results showed that the genes encoding two key enzymes acting in hepatic glyconeogenesis, PC and PEPCK had a trend of increase in the liver of HC goats. It promoted the effect of gluconeogenesis and this was consistent with the changes of plasma glucose. The increase of blood glucose was related to the increase of lactose production, and on the other hand, supplied energy for the body to make up the shortage of fatty acid β-oxidation caused by high concentrate feeding.

Our previous analysis of liver proteomics showed, the expression of enzymes involved in lipid metabolism were down regulated, such as enoyl-CoA hydratase, and fatty acid synthesis was inhibited; the expression of cytochrome b5 and acetyl CoA acetyl transferase were up-regulated, the decomposition of acetyl CoA was blocked, and preferred to generate ketone body. Thus we concluded that high concentrate feeding induced MFD, while fatty acids uptake into liver were stored as TG or incompletely oxidized to ketone body, the excessive consumption of fatty acids reduced the precursors of milk fat synthesis and finally decreased the milk fat content. At the same time, the oxidation of fatty acids was insufficient, in order to maintain the energy balance, the effect of the hepatic gluconeogenesis was strengthened to ensure the energy requirement of the peripheral tissues and milk synthesis.

CONCLUSIONS

This study suggested that high concentrate feeding could induce MFD, while fatty acids uptake into liver were stored as TG or incompletely oxidized to ketone body (BHBA), the excessive consumption of fatty acids reduced the precursors of milk fat synthesis and finally decreased the milk fat content.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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