# Effects of protein-free energy supplementation on blood metabolites, insulin and hepatic PEPCK gene expression in growing lambs offered rice straw-based diet

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**ABSTRACT**: This study was conducted to investigate the effects of increasing levels of protein-free energy supplementation on blood glucose, urea nitrogen, insulin and gene expression of hepatic phosphoenolpyruvate carboxykinase (PEPCK) in growing lambs offered rice straw-based diet. Thirty-six male Hu lambs (3.5 months old) were divided into four equal groups according to body weight. All animals were fed rice straw *ad libitum* and supplemented with cornstarch at levels of 0 (control), 60, 120, and 180 g/day, respectively, along with 160 g/day of rapeseed meal. The trial lasted for 60 days with the first 15 days for adaptation. Body weight change and feed intake were recorded. Blood samples were taken at different time points after feeding at the end of the trial, and analyzed for blood glucose, total protein, urea nitrogen and insulin. Liver samples were collected and analysed for the mRNA abundance of hepatic PEPCK. Increasing cornstarch showed a low effect on rice straw intake, but increased average daily gain of lambs significantly ($P < 0.05$). Blood glucose tended to increase with starch supplementation, but altered within a narrow range. Blood urea nitrogen was decreased significantly ($P < 0.05$) with increment in supplemental starch. Supplementation of starch at 120 or 180 g/day significantly increased the insulin concentration ($P < 0.05$) compared with the control. The abundance of cytosolic PEPCK (PEPCK-C) mRNA increased 2.47 times and 3.98 times with 60 and 120 g per day of starch supplementation compared with the control, respectively, while the supplementation of 180 g per day of starch showed a low effect on PEPCK-C gene expression ($P > 0.05$). Amounts of mitochondrial PEPCK (PEPCK-M) mRNA were not affected by the supplementation of starch at any level ($P > 0.05$). These results indicate that proper energy supplementation increases the expression of PEPCK-C, and consequently gluconeogenesis and blood glucose increase, while excessive energy may have an inhibitory effect on gluconeogenesis through insulin-involved mechanisms.

**Keywords**: cornstarch; glucose; insulin; PEPCK gene; lambs

Energy supplementation is important for ruminants consuming low quality forage-based diets to achieve a desired rate of gain (Bowman and Sanson, 1996). Most of the studies concerning energy supplementation to forage-based diet were focused on the associative effects in digestive tracts (Caton and Dhuyvetter, 1997; Bodine and Purvis, 2003; Lardy et al., 2004), while little attention has been paid to the effect of supplemental energy on glucose metabolism and protein status in body tissues. It is well known that the fermentation of readily fermentable energy in the rumen will result in enhanced production of volatile fatty acids and lactate (Vrzalová and Zelenka, 2001). These changes in end products of rumen fermentation would consequently influence blood glucose metabolism and endocrine (Sowinska et al., 2001; Sano and Fujita, 2006), possibly through the regulation of gene expression of key enzymes

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for gluconeogenesis in liver (Hesketh et al., 1998). Data obtained in lactating ruminants have shown that gluconeogenic substrates, glucose requirements and hormonal status all affected glucose metabolism (Danfaer et al., 1995; Overton et al., 1999; Drackley et al., 2001) while the literature on growing ruminants, which usually present lower nutritional requirements than lactating ones, has been limited, especially in an energy-supplementing system based on low-quality forage.

Dietary protein-free energy influences profoundly not only glucose metabolism in the ruminant body, but also protein metabolism via changing the metabolic fate of amino acids compared with that in non-ruminants (Vrzalová et al., 2001) because the requirement for glucose in ruminants depends mainly on gluconeogenesis (Lindsay, 1978). The relationship between protein and glucose kinetics is not well understood in ruminants. Therefore, the present experiment was designed to investigate the change in blood metabolites and insulin, and the gene expression of hepatic phosphoenolpyruvate carboxykinase (PEPCK) when growing lambs offered a rice straw-based diet were supplemented with different levels of starch, and to approach the possible regulation mechanism of glucose metabolism in energy-supplemented growing ruminants.

## MATERIAL AND METHODS

### Animals, diets and experimental designs

This experiment was approved by the Institutional Animal Care and Use Committee at Zhejiang University and conducted in accordance with the "Regulations for Administration of Affairs Concerning Experimental Animals" (State Council of China, 1988). Thirty-six male Hu lambs (3.5 months old), with an average live weight of 21 ± 1.5 kg, were divided into four equal groups of nine each according to body weight. Lambs in each group were kept in 3 pens (three lambs in each pen) and given free access to rice straw (DM, 880 g/kg; OM, 855 g/kg; CP, 48 g per kg; NDF, 686 g/kg) and water. Supplements were designed to contain four levels of cornstarch: 0 (control), 60, 120, and 180 g/day. Each lamb was offered 160 g rapeseed meal (DM, 899 g/kg; OM, 905 g/kg; CP, 408 g/kg; NDF, 409 g per kg) together with 15 g of mineral mixture per day. The level of supplemental rapeseed was presumed, together with the protein ingestion from rice straw, to provide a crude protein allowance for 100 g of average daily gain (ADG), and supplemental cornstarch was presumed to elevate the energy level from 1 (control) to 1.9 times energy requirement for maintenance, according to the "Feeding Standard of Meat-producing Sheep and Goats" (Ministry of Agriculture of China, 2004). Supplements were given twice daily at 8:00 and 16:00.

### Samplings, recordings and blood analysis

The experiment lasted for 60 days, 15 days of which were for adaptation followed by 45 days of measurements. Rice straw offered and refused was recorded daily. Each lamb was weighed for two consecutive days at the beginning and at the end of the trial.

In the last morning of the feeding trial, blood samples were taken before morning feeding (0 h), 2, 4, 6, and 8 h after feeding by jugular venipuncture from six lambs of each group randomly, and centrifuged at 3 000 rpm for 15 min for the separation of serum. Biochemical reagent kits (Sigma, St. Louis, MO, USA) were used to determine serum glucose, total protein and urea nitrogen. Insulin concentrations in serum were measured by ELISA (ALPCO Diagnostics, Windham, NH, USA). The inter- and intra-assay coefficient of variation for the insulin assay was 4.7 and 3.4%, respectively.

Four lambs from each group were randomly selected and slaughtered on the morning following the feeding trial. Liver samples were immediately dissected and rapidly frozen in liquid nitrogen and stored at –80°C until RNA analysis.

### Quantification of PEPCK mRNA by real-time PCR

**Total RNA extraction and cDNA synthesis.** Total RNA was isolated in parallel using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After pulverisation and homogenisation of the tissues under liquid nitrogen, RNA was extracted from the homogenate with chloroform and then precipitated by isopropanol. The resulting pellets were dissolved in autoclaved water treated with diethyl pyrocarbonate (DEPC). Total RNA was reverse-transcribed into cDNA using a commercially available reverse transcription system kit (PrimeScript$^{TM}$ RT reagent,

Table 1. Primers used in real-time PCR

| Gene | Primer sequences | Length (bp) |
|---|---|---|
| PEPCK-C(F) | 5'-CTGACAGACTCGCCCTACG-3' | 104 |
| PEPCK-C(R) | 5'-TGGAGGCACTTGACGAACT-3' | |
| PEPCK-M(F) | 5'-TCTGCCACCACCAACCC-3' | 101 |
| PEPCK-M(R) | 5'-ATGCCCTCCCAATACACG-3' | |
| GAPDH(F) | 5'-AAGTTCCACGGCACAGTCA-3' | 247 |
| GAPDH(R) | 5'-GGTTCACGCCCATCACAA-3' | |

TaKaRa, Japan) according to the manufacturer's instructions. The synthesized cDNA was stored at –20°C until use.

**Design of Primers for Real-Time PCR**. Primers for cytosolic PEPCK (PEPCK-C) were designed based on the mRNA sequences of ovine PEPCK-C (EF 062826). Primers for mitochondrial PEPCK (PEPCK-M) were designed based on the conservative mRNA sequences of bovine PEPCK-M (BC 102244), human PEPCK-M (NM 004563) and mouse PEPCK-M (BC 010318). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was selected as reference for the quantification of PEPCK (Pfaffl, 2001), and the primes were designed based on ovine GAPDH (AF 030943). Primers for each gene were designed to flank a region that contains at least one intron to ensure that no contaminating genomic DNA was amplified. Primer sequences and PCR product length are described in Table 1. Primers were synthesized by Invitrogen Corp. (Shanghai, China).

**Quantitative Real-Time PCR**. Real-time PCR was conducted in triplicates by amplifying 2 µl of cDNA with the SYBR® PrimeScriptTM RT-PCR Kit (Perfect Real Time, TaKaRa, Japan) on an ABI 7500 Real-Time PCR System (Applied Biosystems). PCR reactions (20 µl final volume) contained 0.4 µl of each primer (10µM) and 0.4 µl of ROX Reference Dye II, 10 µl of SYBR® Premix Ex TaqTM and 6.8 µl $ddH_2O$. Amplification conditions were 95°C for 10 s (pre-degeneration), followed by 40 cyc-les of 95°C for 5 s, and 62°C for 34 s. The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected (melting temperatures were 87.9 ± 0.05°C, 84.1 ± 0.05°C and 86.2 ± 0.05°C for PEPCK-C, PEPCK-M and GAPDH respectively). Upon 2% agarose gel electrophoresis, all PCR products moved with one single band and showed the expected size. Products were verified by sequencing as described by Hammon et al. (2003), and all showed over 99% homology with the sequences used for the primer design. After completion of the PCR amplification, data were analysed with the ABI 7500 Real-time PCR System Sequence Detection Software v 1.4 (Applied Biosystems). The Ct values obtained by amplification were used to create relative comparative $2^{-\Delta\Delta Ct}$ analysis for target and reference genes (Livak and Schmittgen, 2001).

Table 2. Effects of cornstarch supplementation on feed intake and body weight change in lambs

| Item | Supplemental cornstarch (g/day) | | | | SEM |
|---|---|---|---|---|---|
| | 0 | 60 | 120 | 180 | |
| Rice straw intake (DM, g/day) | 386.2 | 404.8 | 401.4 | 398.1 | 9.82 |
| Total intake (DM, g/day) | 560.1$^{d}$ | 632.6$^{c}$ | 683.2$^{b}$ | 733.9$^{a}$ | 9.82 |
| Initial weight (kg) | 20.7 | 21.0 | 21.2 | 21.0 | 0.43 |
| Final weight (kg) | 22.4$^{c}$ | 23.8$^{b}$ | 25.3$^{a}$ | 24.8$^{a}$ | 0.34 |
| Weight gain (g/day) | 37.2$^{c}$ | 61.2$^{b}$ | 91.6$^{a}$ | 84.4$^{a}$ | 3.58 |

$^{a,b,c,d}$means with different superscripts within the same row differ at $P < 0.05$

### Statistical analysis

Statistical analysis of the data was performed by the General Linear Model (GLM) Procedure of SAS (1996). The differences among means for treatments were tested using Duncan's new multiple range test (SAS, 1996).

## RESULTS

### Feed intake and growth performance

Effects of supplemental starch on feed intake and growth performance are shown in Table 2. Supplementing cornstarch showed a low effect on rice straw intake, while total dry matter intake was increased with cornstarch increment ($P < 0.05$). The ADG of starch-supplemented lambs was significantly higher than that of the control ($P < 0.05$), with the highest value for 120 g/day of cornstarch treatment.

### Blood metabolites and insulin

Effects of supplemental cornstarch on postprandial blood glucose and insulin concentrations are shown in Figure 1. Serum glucose concentrations in four groups all reached their peaks within 4 hours after feeding and then fell down, and mostly ranged between 3.0 and 3.5 mmol/l. The increment in starch supplementation tended to increase glucose concentration with significantly higher glucose at 180 g/kg than in other groups at 2 h, but it was maintained within a relatively stable range. Overall, the serum insulin concentration in lambs supplemented with 120 and 180 g/day starch was increased significantly ($P < 0.05$) vs. the control and 60 g/day treatment (0.53, 0.64 vs. 0.29, 0.33 ng/ml). Feeding and sampling time had a small influence on insulin concentration in lambs supplemented with 0 and 60 g/day of cornstarch.

Effects of supplemental cornstarch on postprandial blood total protein and urea nitrogen concentrations are shown in Figure 2. Total protein

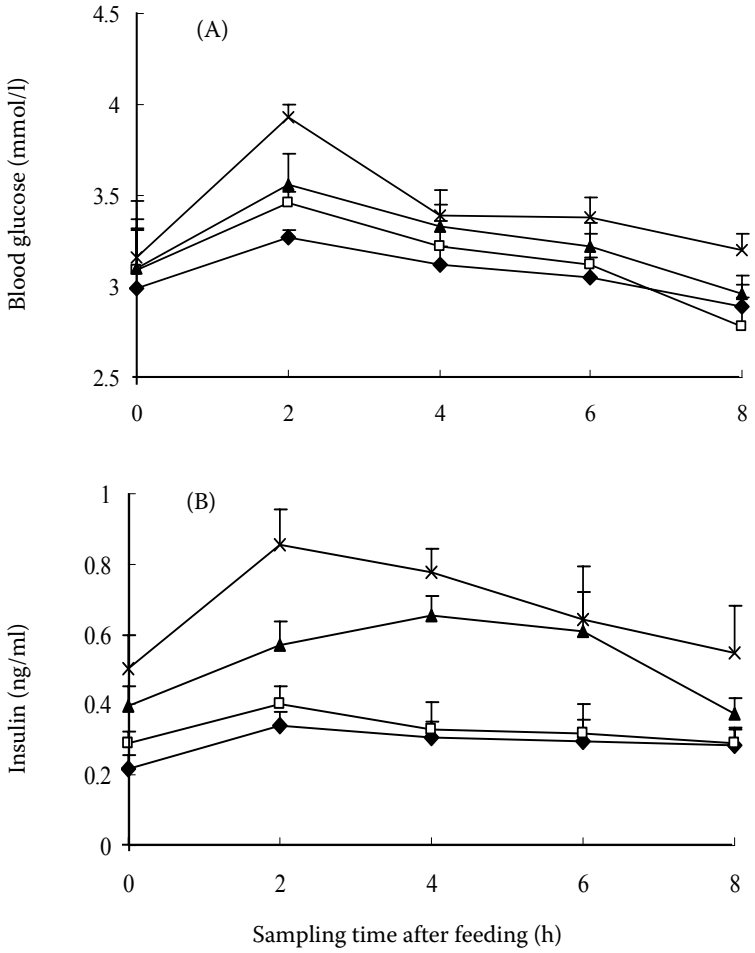


Figure 1. Concentrations of blood glucose (A) and insulin (B) at different sampling times after feeding in Hu lambs offered rice straw-based diets supplemented with cornstarch at levels of 0 (◆), 60 (□), 120 (▲) and 180 (×) g/kg ($n = 6$); data are expressed as means ± SD; A: Concentration of blood glucose in 180 g/kg was significantly ($P < 0.05$) higher than that of the others at 2 h; B: concentrations of insulin in 120 and 180 g/kg were significantly ($P < 0.05$) higher than those of the control and 60 g/kg

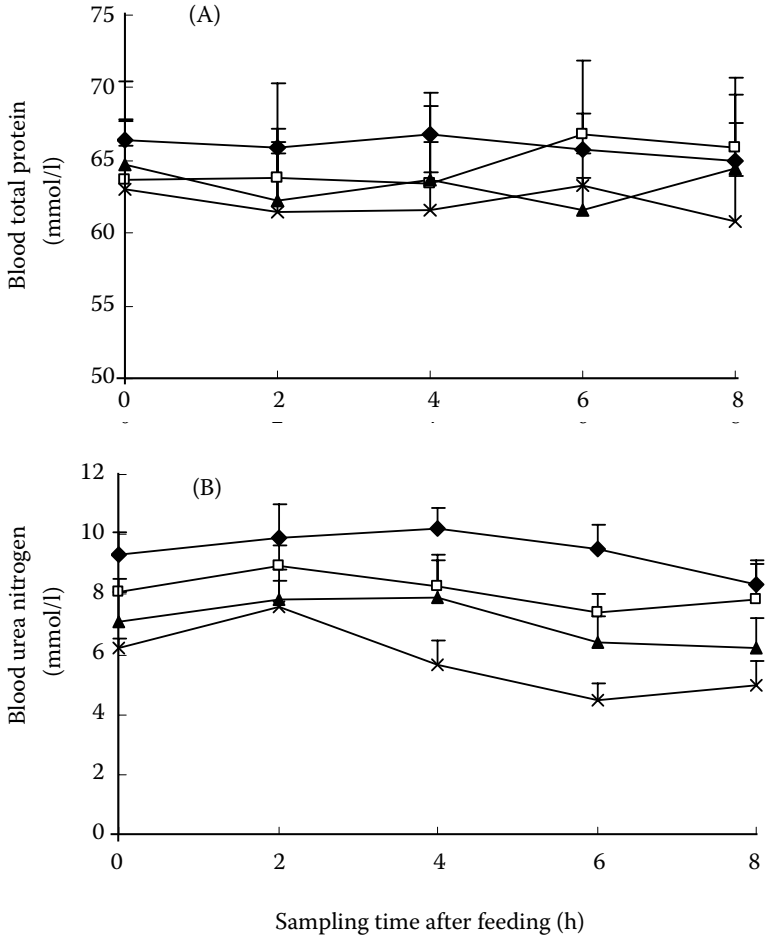


Figure 2. Concentrations of blood total protein (A) and blood urea nitrogen (B) at different sampling times after feeding in Hu lambs offered rice straw-based diets supplemented with cornstarch at levels of 0 (◆), 60 (□), 120 (▲) and 180 (×) g/kg ($n$ = 6); data are expressed as means ± SD; A: no significant difference was found in blood total protein among the groups; B: concentration of blood urea nitrogen was significantly ($P$ < 0.05) lower in 120 and 180 g/kg groups than that of the control at 4 and 6 h

concentrations for all treatments were relatively stable across the feeding cycle. Overall, an increment in supplemental starch tended to decrease total protein concentration, but the difference between treatments was not significant ($P$ > 0.05) whereas the serum urea nitrogen concentration was reduced by 120 and 180 g/day starch supplementation significantly ($P$ < 0.05) vs. the control (7.07, 5.79 vs. 9.42 mmol/l). The time course of changes in serum urea nitrogen over the 8 h period of measuring was similar for different treatments.

### PEPCK mRNA expression

Effects of supplemental cornstarch on the abundance of hepatic PEPCK-C and PEPCK-M mRNA are shown in Figure 3. The abundance of PEPCK-C mRNA increased 2.47 times and 3.98 times with 60 and 120 g/day of starch supplementation compared with the control, respectively ($P$ < 0.05), whereas the supplementation of 180 g/day starch showed a low impact on PEPCK-C mRNA expression ($P$ > 0.05). The amounts of PEPCK-M mRNA were not affected by supplementation of starch at any level ($P$ > 0.05).

## DISCUSSION

### Growth performance

Data available on energy supplementation in growing animals mostly showed a positive effect (Gerrits et al., 1996; Demeterová and Vajda, 2000; Schoonmaker et al., 2003). The growth performance of this trial clearly demonstrated that feeding only rice straw and rapeseed meal was inadequate (37.2 g/day live weight gain). Supplemental cornstarch increased ADG drastically, by 64.5, 146.2, and 126% in 60, 120, and 180 g/day of cornstarch treatments compared to the control, respectively. These results suggested that adequate energy supplementation to an energy-deficient diet may effec-

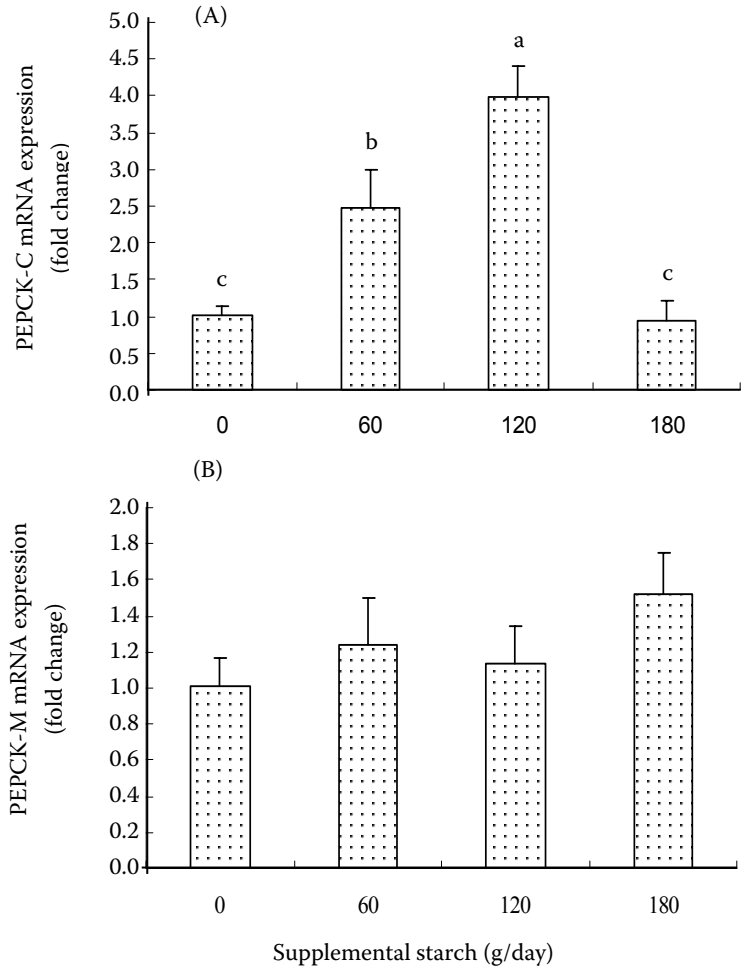


Figure 3. Effect of starch supplementation on the hepatic PEPCK-C (A) and PEPCK-M (B) mRNA expression in Hu lambs offered rice straw-based diets; results are expressed as means ± SD folds changes relative to the control ($n$ = 4). a, b, c: figures with different superscripts differ at $P < 0.05$

tively improve the growth performance of animals, while too much readily fermentable energy would not be beneficial, probably due to the negative associative effect on digestion.

## Blood metabolites and insulin

It was not surprising that the blood glucose concentration tended to increase with supplementing cornstarch, but the differences between treatments were not significant, indicating that there exist mechanisms to regulate the blood glucose concentration within a normal physiological range (Devaskar et al., 1984; Apatu and Barnes, 1991). Serum insulin is highly sensitive to blood glucose, and was increased by supplemental cornstarch in the present study (Figure 1). This result was in agreement with Barton et al. (1992) and Yelich et al. (1995, 1996). The response of insulin to supplemental cornstarch may result from a higher supply of gluconeogenic precursors and insulinotropic factors (Trenkle, 1978; Harmon, 1992; Marston et al., 1995), especially when the supply of energy was very high (e.g. 180 g/day of starch in this study). This increased insulin may help regulate gluconeogenesis in liver and glucose utilization in body tissues, hence it maintains the blood glucose concentration within the normal physiological level.

Blood total protein is a major index for the protein status. In the present study, protein intake and blood total protein were at the same level in all lambs, indicating that the amount of absorbable protein was not influenced by supplemental energy. However, blood urea nitrogen, the most useful indicator for protein utilization in ruminants (Kohn et al., 2005), was significantly reduced by cornstarch increment. These results indicated that the efficiency of protein utilisation was elevated by supplemental energy. A similar result was also reported by Dror et al. (1969) and Chase et al. (1993). The elevated efficiency of protein utilization with energy increment may be related to changes in the available glucose supply and insulin secretion,

which would positively affect protein utilization (Fujita et al., 2006).

### PEPCK mRNA expression and regulation of gluconeogenesis

Glucose metabolism in ruminants is characterized by gluconeogenesis, which takes place mainly in liver. The efficiency of gluconeogenesis is regulated mainly by two factors: activities of key enzymes for gluconeogenesis and amounts of gluconeogenic precursors. The other factors, such as endocrine and glucose requirement, may exert their influences through these two approaches indirectly.

Brockman (1993) reported that as much as 85% of glucose was synthesised de novo in liver from a range of precursors: mainly propionate, L-lactate, gluconeogenic amino acid, and glycerol, contributing 50–70, 10–20, 10–30 and 5% to hepatic gluconeogenesis, respectively (Majdoub et al., 2003). In the present study, when lambs were fed rice straw and rapeseed meal without supplementation, the gluconeogenic amino acid would account for a large proportion in all the precursors because of deficiency in the carbohydrate supply. When the basal diet was supplemented with starch, more gluconeogenic precursors would result from enhanced production of propionate and lactate in the rumen. They would perhaps change the metabolic fate of parts of the amino acid from gluconeogenesis into body deposition (Lobley, 1992). This deduction was consistent with the serum urea nitrogen concentration, which was lower in the higher starch treatment.

PEPCK is one of the key enzymes for gluconeogenesis in liver. Its cytosolic and mitochondrial forms are products of two separate genes and are equally distributed between intracellular compartments (Hod et al., 1986). In non-ruminants, the activity of PEPCK is directly regulated at the transcriptional level of the gene (O'Brien and Granner, 1990) and is markedly increased in fasted or diabetic animals and is reduced by high carbohydrate-feeding (Lemaigre and Rousseau, 1994) through nutrient-mediated and hormonally induced changes in specific regions of the PEPCK gene promoter (Pilkis and Granner, 1992). Likewise, studies in ruminants (Hartwel et al., 1999; Greenfield et al., 2000; Agca et al., 2002) indicated a close relationship between total PEPCK activity and its mRNA level, but the effects of gluconeogenic precursors on PEPCK mRNA expression are not clear (Greenfield et al., 2000). In the present study, the abundance of PEPCK-C mRNA was significantly higher in starch treatment with 60 and 120 g/day, indicating a positive effect of gluconeogenic precursors on PEPCK-C gene expression. The increased mRNA abundance for PEPCK may also reflect an adaptation to the increased glucose requirement as the growth performance improves (Greenfield et al., 2000). However, when lambs were supplemented with 180 g/day of starch, the mRNA expression of PEPCK-C was depressed to the control level. This would mainly be due to the effect of insulin, which was regarded as the primary regulator of gluconeogenesis in ruminants (Brockman, 1986). The finding of O'Brien and Granner (1990) suggested that insulin decreased the activity of PEPCK enzyme by directly regulating the gene expression. A small difference was found in PEPCK-M mRNA abundance among the four groups. Similar results were reported by Weldon et al. (1990), Savon et al. (1993) and Agca et al. (2002), where PEPCK-M showed no response to hormones or physiological state.

## CONCLUSIONS

Energy supplementation to growing lambs offered low-quality forage can increase their growth perfor-mance and protein utilization. The blood glucose concentration may be increased by energy supplementation, but it is also self-regulated through hormone and enzyme intermediated systems. When the energy supply is within the physiological requirement, adequate energy supplementation may increase the PEPCK-C gene expression. However, when supplemental energy was excessive, there would be hormone-mediated regulation (mainly insulin) to repress the PEPCK gene expression and gluconeogenesis in liver to maintain the blood glucose within a normal level.

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