Effects of Dietary Energy Level on Urinary Purine Derivative Excretion and Rumen Microbial Nitrogen Production in Tibetan Sheep under Low-Nitrogen Conditions: Postprint

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Abstract

This experiment was conducted to investigate the effects of dietary energy levels on urinary purine derivative (PD) excretion and rumen microbial nitrogen (MN) production in Tibetan sheep under low-nitrogen conditions, aiming to provide theoretical basis and data support for appropriate energy and nitrogen supplementation techniques for Tibetan sheep during the cold season. Five healthy castrated male Tibetan sheep aged 1.5 years with similar body condition and body weight [(47.7±2.46) kg] were selected for a digestion and metabolism trial using a 4×4 Latin square design (with one sheep as a replicate). During the trial period, four diets with similar crude protein (CP) content [(6.97±0.05)%] but different digestible energy (DE) levels were fed, with DE levels of 8.21 (low-energy diet), 9.33 (medium-low-energy diet), 10.45 (medium-high-energy diet), and 11.57 MJ/kg (high-energy diet), respectively. The entire experiment consisted of 4 periods, each lasting 21 d, including a 15-d preliminary period and a 6-d formal trial period. The results showed: 1) With increasing dietary energy level, urinary PD excretion and duodenal PD absorption increased linearly ($P<0.05$). Among the urinary PD components, uric acid excretion increased linearly with dietary energy level ($P<0.05$), allantoin excretion showed an increasing trend (0.05 P 0.10), and the percentage of each PD component in total PD excretion did not differ significantly among groups ($P>0.10$). 2) Total digestible nutrient (TDN) intake increased linearly with dietary energy level ($P<0.05$), but nitrogen intake did not differ significantly among groups ($P>0.10$). With increasing dietary energy level, urinary nitrogen excretion decreased linearly ($P<0.05$), while urinary purine nitrogen excretion and purine nitrogen index (PNI) increased linearly ($P<0.05$). Meanwhile, rumen MN production and microbial protein synthesis efficiency (MPS) also increased linearly

with dietary energy level (P<0.05). 3) Urinary PD excretion and rumen MN production showed high linear correlations with TDN intake, with model equations as follows: Urinary PD excretion (mmol/d) = 18.09 TDN intake (kg/d) - 1.11 (R2=0.97); Rumen MN production (g/d) = 18.32 TDN intake (kg/d) - 2.51 (R2=0.97). 4) Nitrogen balance (NB) showed a high linear correlation with digestible energy intake (DEI), with the model equation: NB (g/d) = 8.38 DEI (MJ/kg BW0.75) - 3.58 (R2=0.68). The above results indicate that when dietary CP content was 6.97%, the DE requirement for Tibetan sheep to maintain nitrogen balance was 0.43 MJ/kg BW0.75; increasing dietary energy level (DE level: 8.21~11.57 MJ/kg) could increase rumen MN synthesis, improve dietary nitrogen utilization efficiency, and thereby compensate for dietary nitrogen deficiency. Therefore, during the cold season on the Qinghai-Tibet Plateau, supplementing energy nutrients can improve the adaptability of Tibetan sheep to nitrogen nutritional stress.

Full Text

Effects of Dietary Energy Level on Urinary Purine Derivatives Excretion and Ruminal Microbial Nitrogen Production of Tibetan Sheep under Low Nitrogen Conditions

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Abstract: This study investigated the effects of dietary energy level on urinary purine derivatives (PD) excretion and ruminal microbial nitrogen (MN) production in Tibetan sheep under low nitrogen conditions, aiming to provide theoretical basis and data support for appropriate energy-nitrogen supplementation strategies during the cold season. Five healthy 1.5-year-old castrated male Tibetan sheep with similar body condition [(47.7±2.46) kg body weight] were selected for a digestion and metabolism trial using a 4×4 Latin square design (with one sheep as a replicate). During the experiment, four diets with similar crude protein (CP) content [(6.97±0.05)%] but different digestible energy (DE) levels were fed: 8.21 (low-energy), 9.33 (medium-low-energy), 10.45 (medium-high-energy), and 11.57 MJ/kg (high-energy). The trial consisted of four periods, each lasting 21 days with a 15-day pre-trial and 6-day formal collection period. The results showed: 1) Urinary PD excretion and duodenal PD absorption increased linearly with dietary energy level (P<0.05). Among urinary PD components, uric acid excretion increased linearly (P<0.05), allantoin excretion showed an increasing trend (0.05 P 0.10), while the percentage of each PD component in total PD excretion did not differ significantly among groups (P>0.10).

2) Total digestible nutrient (TDN) intake increased linearly with dietary energy level ($P<0.05$), but nitrogen intake did not differ among groups ($P>0.10$). Urinary nitrogen excretion decreased linearly ($P<0.05$), whereas urinary purine nitrogen excretion and purine nitrogen index (PNI) increased linearly ($P<0.05$). Ruminal MN production and microbial protein synthesis efficiency (MPS) also increased linearly with dietary energy level ($P<0.05$). 3) Strong linear correlations existed between urinary PD excretion, ruminal MN production, and TDN intake, with model equations: urinary PD excretion (mmol/d) = 18.09×TDN intake (kg/d) - 1.11 ($R^2=0.97$); ruminal MN production (g/d) = 18.32×TDN intake (kg/d) - 2.51 ($R^2=0.97$). 4) A strong linear correlation was observed between nitrogen balance (NB) and digestible energy intake (DEI): NB (g/d) = 8.38×DEI (MJ/kg BW ·) - 3.58 ($R^2=0.68$). These findings indicate that when dietary CP content is 6.97%, Tibetan sheep require 0.43 MJ/kg BW · DE to maintain nitrogen balance. Increasing dietary energy level (DE: 8.21–11.57 MJ/kg) enhances ruminal MN synthesis and improves dietary nitrogen utilization efficiency, thereby compensating for dietary nitrogen deficiency. Therefore, energy supplementation during the cold season on the Tibetan Plateau can improve Tibetan sheep' s adaptability to nitrogen nutritional stress.

Keywords: Tibetan sheep; low nitrogen condition; dietary energy level; urinary purine derivatives; ruminal microbial nitrogen production

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Tibetan sheep, one of China' s three indigenous sheep breeds, primarily inhabit high-altitude regions of 3,000–5,000 m and represent a critical livestock resource for Tibetan communities, with a population of approximately 50 million head [1,2]. For centuries, these animals have been managed under traditional, extensive grazing systems without supplementation [3]. However, the unique geography and climate of the Qinghai-Tibetan Plateau cause severe seasonal forage imbalances. During the cold season, forage biomass declines sharply, and nutrient content deteriorates significantly, with crude protein (CP) content dropping to merely 2.96%–10.44% [4]. Despite these harsh conditions, Tibetan sheep maintain normal reproduction and provide high-quality animal products, demonstrating remarkable adaptive mechanisms to the plateau environment [5].

Ruminants possess distinct digestive characteristics due to their rumen microbiome. The abundant rumen microorganisms help degrade fibrous carbohydrates to provide volatile fatty acids as energy sources while utilizing dietary nutrients to synthesize microbial protein (MCP), which supplies the majority of amino acids required for host growth, production, and maintenance. Reportedly, over half of the amino acids absorbed by ruminants originate from MCP [6], and when fed forage-based diets, rumen MCP becomes virtually the sole protein source [7]. Microbial protein synthesis is influenced by dietary energy, protein, minerals, vitamins, and growth factors [8]. Zhou et al. [9] found that Tibetan sheep have low nitrogen maintenance requirements [0.50 g/(kg BW · · d)], only 66% of NRC (1985) recommendations for sheep of similar weight [10]. Furthermore, when consuming low-nitrogen diets, Tibetan sheep can recycle

88% of liver-produced urea back into the digestive tract [5] to provide nitrogen for rumen MCP synthesis. Therefore, investigating ruminal MN synthesis efficiency and its response to dietary energy levels under low nitrogen conditions holds significant practical importance for guiding cold-season production practices.

Traditional methods for estimating ruminal MN production rely on marker techniques [11], classified as endogenous or exogenous markers. These approaches typically require ruminal or duodenal cannulation, making them operationally inconvenient, procedurally complex, difficult to apply in practice, and detrimental to animal welfare [12]. The urinary purine derivatives (PD) method offers a non-invasive, simple, and accurate alternative for estimating MCP production [13,14]. The principle relies on the fact that urinary PD in ruminants primarily originates from purine metabolism of microbial protein absorbed from the duodenum, with contributions from body protein metabolism being negligible. Consequently, urinary PD excretion correlates strongly with ruminal MCP production. Over decades, species-specific estimation models have been developed for sheep [15], goats [16], cattle [17], beef cattle [18], and yaks [10]. However, research on urinary PD excretion in Tibetan sheep remains limited, particularly regarding how ruminal MCP synthesis responds to dietary energy regulation under low nitrogen conditions. This study simulated the CP content of cold-season pasture on the Qinghai-Tibetan Plateau to investigate the response patterns of urinary PD excretion and ruminal MN production to energy manipulation, aiming to elucidate the unique nitrogen metabolism mechanisms and provide theoretical basis and technical guidance for scientific supplementation strategies during the cold season.

1.1 Experimental Location and Duration

The experiment was conducted from November 2016 to January 2017 at the Wushaoling Experimental Station of the International Center for Tibetan Plateau Ecosystem Management, Lanzhou University, located in Anyuan Town, Tianzhu Tibetan Autonomous County, Gansu Province (37°14 20.54 N, 102°48 34.32 E, altitude 3,154 m).

1.2 Experimental Animals and Design

Five healthy 1.5-year-old castrated male Tibetan sheep with similar body condition and body weight of (47.7±2.46) kg were selected for a 4×4 Latin square design (with one sheep as a replicate). Animals were fed low-energy, medium-low-energy, medium-high-energy, and high-energy diets across four experimental periods, each lasting 21 days with a 15-day adaptation phase and 6-day sample collection period. The detailed experimental design is presented in Table 1 .

1.3 Experimental Diets

Diet formulation followed Chinese Feeding Standards for Meat Sheep (NY/T 816−2004) [19] and Chinese Feed Composition and Nutritional Value Table (26th edition, 2015) [20]. The four experimental diets contained similar CP levels [(6.97±0.05)%, simulating cold-season pasture CP content which falls below minimum maintenance requirements for sheep] with DE levels of 8.21 (low-energy), 9.33 (medium-low-energy), 10.45 (medium-high-energy), and 11.57 MJ/kg (high-energy). Diet composition and nutrient levels are shown in Table 2 .

1.4 Animal Management

Prior to the trial, sheep were dewormed with ivermectin, and pens and equipment were cleaned and disinfected. Sheep were housed individually and fed total mixed rations (TMR) twice daily (08:00 and 17:00) at 4.5% of metabolic body weight (BW ・) on a dry matter basis, with free access to water. Residual feed was collected before morning feeding to record individual daily intake. To minimize stress, a 20-day adaptation period preceded the formal experiment to acclimate animals to the housing environment and experimental diets.

1.5 Sample Collection and Analytical Methods

1.5.1 Sample Collection and Processing On day 16 of each period (the first day of the collection phase), total feces and urine were collected continuously for 5 days starting at 08:00 (pre-feeding). Daily urine volume and fecal output were recorded for each sheep. Fecal samples were collected by quartering, with 10% of total output stored in sealed bags at -20°C. Urine samples were mixed thoroughly, and 10% of total volume was acidified with 50% sulfuric acid to pH<3.0 to stabilize urinary nitrogen and inhibit microbial growth, then stored at -20°C pending analysis.

1.5.2 Analytical Methods and Calculations Urinary PD content (allantoin, uric acid, xanthine, and hypoxanthine) was determined by high-performance liquid chromatography (Agilent LC-1200) using a Phenomenex Synergi 4u Hydro-RP80A column (250×4.6 mm) at 35°C, with sample temperature at room temperature, injection volume of 10 L, detection wavelength of 220 nm, mobile phase of 30 mmol/L ammonium acetate, flow rate of 1 mL/min, and isocratic elution, following the method of Li et al. [22]. Nitrogen content in diets, urine, and feces was determined by the Kjeldahl method [23].

Nitrogen balance (NB, g/d) = nitrogen intake (g/d) - fecal nitrogen excretion (g/d) - urinary nitrogen excretion (g/d).
Urinary PD excretion (mmol/d) = allantoin excretion (mmol/d) + uric acid excretion (mmol/d) + xanthine excretion (mmol/d) + hypoxanthine excretion (mmol/d).

Ruminal MN production was calculated using the model established by Chen et al. [24], which estimates ruminal MN production based on duodenal purine absorption:

Y = 0.84X + 0.150BW · · e · ²

where X represents duodenal purine absorption and Y represents urinary PD excretion.

MN production (g/d) = (X × 70) / 0.83 × 0.116 × 1000 = 0.727X

where X is duodenal purine absorption, 70 represents nitrogen content per mmol of purine (70 mg), 0.83 is the digestibility of microbial nucleic acids (83%), and 0.116 is the proportion of purine nitrogen in total nitrogen of rumen microbes (11.6%).

Total digestible nutrient (TDN) intake, purine nitrogen index (PNI), MCP production, and microbial protein synthesis efficiency (MPS) were calculated as follows:

TDN intake = (CP intake - fecal CP) + [neutral detergent fiber (NDF) intake - fecal NDF] + [nitrogen-free extract (NFE) intake - fecal NFE] + {2.25 × [ether extract (EE) intake - fecal EE]} [25]
PNI = urinary purine nitrogen excretion / urinary nitrogen excretion [26]
MCP production = MN production × 6.25
MPS = MCP production / TDN intake [27]

1.6 Statistical Analysis

All data were preprocessed using Excel 2007. Single-variable analysis was performed using the polynomial orthogonal contrast module in the general linear model (GLM) procedure of SPSS 22.0. Differences were considered significant at P<0.05, non-significant at P>0.10, and trends were identified at 0.05 P 0.10.

2 Results and Analysis

2.1 Effects of Dietary Energy Level on Urinary PD Excretion and Duodenal Purine Absorption in Tibetan Sheep under Low Nitrogen Conditions

As shown in Table 3 , urinary PD excretion and duodenal PD absorption increased linearly with dietary energy level (P<0.05). Among urinary PD components, uric acid excretion increased linearly (P<0.05), allantoin excretion showed an increasing trend (0.05 P 0.10), hypoxanthine excretion followed a cubic pattern (P<0.05), while xanthine excretion was not significantly affected by dietary energy level (P>0.10). Additionally, the percentage contribution of each PD component to total PD excretion did not differ among groups (P>0.10).

2.2 Effects of Dietary Energy Level on Ruminal MN Production and PNI in Tibetan Sheep under Low Nitrogen Conditions

Table 4 shows that TDN intake increased linearly with dietary energy level ($P<0.05$), while nitrogen intake did not differ among groups ($P>0.10$). Urinary nitrogen excretion decreased linearly ($P<0.05$), whereas urinary purine nitrogen excretion and PNI increased linearly ($P<0.05$) with increasing dietary energy level. Both ruminal MN production and MPS increased linearly with dietary energy level ($P<0.05$).

2.3 Correlation Analysis Between TDN Intake and Urinary PD Excretion or Ruminal MN Production

Strong linear relationships were observed between TDN intake (kg/d) and urinary PD excretion (mmol/d) or ruminal MN production (g/d) (Table 5). Linear regression analysis established the following mathematical models (Figure 1 [Figure 1: see original paper]):

Urinary PD excretion = 18.09 × TDN intake - 1.11 (n=20, R^2=0.97)
Ruminal MN production = 18.32 × TDN intake - 2.51 (n=20, R^2=0.97)

2.4 Linear Relationship Between Nitrogen Balance and Digestible Energy Intake

A strong linear correlation was observed between NB (g/d) and DEI (MJ/kg BW ·). Linear regression analysis established the following mathematical model (Figure 2 [Figure 2: see original paper]):

NB = 8.38 × DEI - 3.58 (n=20, R^2=0.68)

3 Discussion

3.1 Response Pattern of Urinary PD Excretion to Dietary Energy Level in Tibetan Sheep under Low Nitrogen Conditions

Urinary PD represent the end products of purine metabolism in ruminants, influenced by multiple factors including nutrient intake (dry matter, protein, energy), feed additives, body weight, and animal species [16]. In ruminants, urinary PD primarily originate from nucleic acid purines absorbed from the duodenum (mostly from microbial nucleic acids), establishing a strong positive correlation between urinary PD excretion and ruminal MCP synthesis. Allantoin and uric acid constitute the major proportion of urinary PD components [27], while xanthine and hypoxanthine contribute minimally [28,29]. In this study, allantoin, uric acid, and xanthine+hypoxanthine accounted for 67%–76%, 12%–15%, and 10%–17% of total urinary PD excretion, respectively. The proportions of allantoin and uric acid were slightly lower than those reported by Chen et al. [30] in sheep, while xanthine+hypoxanthine proportions were slightly higher. Research indicates that cattle [31] and yaks [32] exhibit very low or negligible xanthine

and hypoxanthine in urinary PD (<1%) due to high xanthine oxidase activity in blood, liver, and intestinal mucosal cells, which readily oxidizes these compounds to uric acid or allantoin. Therefore, the relatively higher xanthine and hypoxanthine content in Tibetan sheep urinary PD compared to other sheep may reflect lower xanthine oxidase activity.

Allantoin excretion increased linearly with dietary energy level, while uric acid excretion increased linearly, indicating that changes in urinary PD excretion were primarily driven by variations in allantoin and uric acid output. The increase in urinary PD excretion with dietary energy level aligns with reports in sheep by Deshpande et al. [33], Fujihara et al. [34], and Chen et al. [35]. This response may be attributed to increased fermentable carbohydrates in the rumen promoting microbial growth and MCP synthesis, ultimately elevating urinary PD excretion [36]. Additionally, higher dietary energy levels increase concentrate proportion, accelerating rumen digesta passage rate and reducing protozoal predation on bacteria, thereby enhancing microbial flow to the abomasum and small intestine and increasing MCP production and urinary PD excretion [30]. Previous studies demonstrated that urinary PD in sheep [15], cattle [17], and yaks [10] increase with TDN intake, consistent with our findings in Tibetan sheep. The linear model derived: PD excretion = 18.09 × TDN intake - 1.11 (R^2=0.97). When TDN intake = 0, PD excretion = -1.11 mmol/d [-0.06 mmol/(kg BW · · d)], which can be used to estimate endogenous PD excretion. This value is lower than the endogenous PD excretion [0.09 mmol/(kg BW · · d)] reported by Liu et al. [37] in sheep but higher than the value [-0.14 mmol/(kg BW · · d)] reported by Ma et al. [38]. However, Chen et al. [24] established a sheep model with endogenous PD excretion of 0.150 mmol/(kg BW · · d), which may overestimate endogenous PD excretion and underestimate MN production in Tibetan sheep. For sheep, endogenous PD excretion estimated by extrapolating the regression line between urinary PD excretion and TDN intake may not represent true endogenous excretion, as endogenous PD decreases with increasing TDN intake and urinary PD excretion. When urinary PD excretion exceeds 0.6 mmol/(kg BW · · d) [15], endogenous PD becomes negligible. Therefore, endogenous PD excretion in sheep is not constant but varies with dietary nutrient intake.

3.2 Response Patterns of Ruminal MN Production and PNI to Dietary Energy Level under Low Nitrogen Conditions

Ruminants utilize anaerobic rumen microbes to synthesize MCP using ammonia, peptides, and amino acids from dietary protein degradation as nitrogen sources, and volatile fatty acids and ATP from organic matter fermentation as carbon skeletons and energy [10]. Microbial crude protein serves as a crucial nitrogen source, providing 40%–80% of the host' s protein requirements [7], with particular importance for nitrogen balance when dietary nitrogen intake is low. Microbial protein synthesis is influenced by dietary carbohydrate content, nitrogen form, vitamin and mineral levels, feeding frequency, additive types, and

fiber composition [9]. This study demonstrated that ruminal MN production increased with dietary energy level under low nitrogen conditions, likely due to enhanced fermentable carbohydrates promoting microbial growth and MN synthesis, consistent with Fujihara et al. [34] in sheep but contrary to Chen et al. [24]. NRC (2001) [39] uses TDN intake to estimate MCP production. Both MPS and MN production/nitrogen intake reflect the efficiency of converting dietary nitrogen to MN. The linear model MN production (g/d) = 18.32 × TDN intake (kg/d) - 2.51 (R^2=0.97) indicates that the slope representing MN synthesis per unit TDN intake is twice the value reported for beef cattle in NRC (1996) [40], reflecting high MN synthesis efficiency in Tibetan sheep. The linear increase in both MPS and MN production/nitrogen intake with dietary energy level indicates that elevated energy enhances dietary nitrogen conversion to MN under low nitrogen conditions. Insufficient energy supply suppresses microbial activity, reducing nitrogen conversion efficiency. This confirms that efficient nitrogen utilization in Tibetan sheep requires adequate energy supply. Furthermore, the linear model between NB and DEI estimates that when NB = 0, DEI = 0.43 MJ/kg BW · , representing the DE requirement for nitrogen balance maintenance when dietary CP content is 6.97% (simulating cold-season pasture CP content).

PNI, influenced by animal breed and dietary composition, serves as a simple and effective indicator of the efficiency of converting degradable dietary protein to MCP. Higher PNI values indicate greater efficiency of rumen-degradable nitrogen conversion to MCP [41]. PNI application can improve ruminant management, enhance nitrogen utilization, and reduce nitrogen excretion [25,42]. This study showed PNI ranged from 0.09 to 0.17 and increased linearly with dietary energy level under low nitrogen conditions, similar to Wang et al. [43] who reported increased PNI with increasing hay intake in yaks. The elevated PNI under high-energy, low-nitrogen conditions suggests that rumen microbial efficiency in utilizing degradable protein increases with dietary energy level to compensate for nitrogen scarcity, thereby providing more nitrogen sources for Tibetan sheep.

4 Conclusion

When dietary CP content is 6.97%, Tibetan sheep require 0.43 MJ/kg BW · DE to maintain nitrogen balance. Increasing dietary energy level (DE: 8.21–11.57 MJ/kg) enhances ruminal MN synthesis and improves dietary nitrogen utilization efficiency, thereby compensating for dietary nitrogen deficiency. Therefore, energy supplementation during the cold season on the Qinghai-Tibetan Plateau can effectively improve Tibetan sheep' s adaptability to nitrogen nutritional stress.

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