MICROBIAL NITROGEN PRODUCTION, NITROGEN BALANCE AND EXCRETION OF URINARY PURINE DERIVATIVES IN CORRIEDALE EWES UNDER WATER DEPRIVATION

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Abstract

The objective of this study was to determine microbial N production, and urinary excretion of PDs in Corriedale ewes under water deprivation. Nine Corriedale ewes (average BW=45±4.5 kg) were individually fed diets based on maintenance requirements in metabolic crates. Ewes were assigned to three treatment groups according to a 3×3 Latin square design for 3 periods of 21 days duration. The treatments were free access to water (FAW), 2h water deprivation (2hWD), and 3h water deprivation (3hWD) following feeding. Daily water intake decreased linearly as water deprivation time following feeding increased. Feed intake and fecal excretion were not different among the treatment groups. Urine weight and volume were higher in FAW than water deprived groups. Nitrogen balance including urinary N, retained N, urinary N/intake N, and retained N/intake N were lower in FAW group than other treatment groups, whereas no differences were observed in intake N, fecal N, digestible N, and fecal N/intake N among the treatment groups. Allantoin concentration tended to be higher in FAW group than 2hWD and 3hWD groups while no differences were observed in uric acid, xanthine+hypoxanthine and creatinine concentrations. Microbial N production per DOMR was higher in 2hWD and 3hWD groups than FAW group whereas no differences were observed between 2hWD and 3hWD groups. Index of PDC decreased linearly with water deprivation. It is concluded that water deprivation following feeding, especially 2 h water deprivation in ewes improved microbial N production.

Key words: ewes, microbial nitrogen production, purine derivatives, water deprivation

Recent studies reported that water deprivation for less than 3 h improved nutrient digestibility of feed in ewes (Ghassemi Nejad et al., 2014 b) and in lactating cows (Ghassemi Nejad et al., 2015) during heat stress; however, no influence was observed on feed intake and wool/hair cortisol (Ghassemi Nejad et al., 2014 a, b; Ghassemi Nejad et al., 2016). The authors suggested that an improvement in nutri-

ent digestibility during water deprivation is a result of an increase in retention time of digesta. This will result in altering the rate of dilution in rumen and increasing microbial enzyme attachments to feed particles by giving more time for microbial degradation (Casamassima et al., 2008; Ghassemi Nejad et al., 2014 b, 2015). Therefore, this follow-up study is designed to investigate the effect of water deprivation following feeding on microbial N production and excretion of urinary purine derivatives (PDs) in ewes.

Given the fact that rumen microbes constitute the major source of protein for ruminants, PDs as the end products are considered as important variables to measure (Chen et al., 1992). Hence, microbial protein production may be estimated from the urinary excretion of PDs including allantoin, uric acid, xanthine, and hypoxanthine which are mainly of microbial origin (Mupangwa et al., 2000; Salman et al., 2013; Ma et al., 2014). Furthermore, normal ruminal function is altered during periods of water deprivation (Fluharty et al., 1995; Ghassemi Nejad et al., 2015). Water is necessary for proper rumination and digestion (Casamassima et al., 2008). Efficient utilization of dietary carbohydrates and protein by ruminants to optimize microbial protein synthesis and reduce N output is desirable (Ma et al., 2014). Depending on the time and duration that water is deprived, microbial protein production may alter. The implications that water deprivation may change the synchronization between carbohydrate and protein in rumen of sheep needs to be investigated. Therefore, this study was conducted to investigate the proper timing of water deprivation following feeding and its influence on microbial N production, fecal and urine output, and urinary excretion of PDs in Corriedale ewes.

Material and methods

Animals, treatments and experimental design

The experimental procedure and methods were approved by the Animal Welfare and Ethics Authority of Kangwon National University, Chuncheon, Korea. Nine 3-year-old Corriedale ewes (average BW=45±4.5 kg) were individually fed diets based on maintenance requirements with an additional 15% to ensure that ewes fed at their maintenance levels (NRC, 1985). Ewes were kept in metabolic crates (0.75 m×1.45 m) and were assigned to three treatment groups according to a Latin square design (3×3) for 3 periods of 21 days each. Treatments were free access to water (FAW), 2 h water deprivation (2hWD), and 3 h water deprivation (3hWD) following feeding. Following the water deprivation time, ewes were given free access to water.

Ewes were placed in individual metabolic crates with metal grated floors designed for urine and feces collection. Steel pads with small holes beneath the cages were used to collect feces with a second one under the first to collect urine. Each period consisted of an adaptation period (14 days) during which ewes were adapted to the new treatment, and a measurement period (7 days) during which samples were collected. Lighting in the experimental house was maintained between 08:00 and 21:30 h.

Feed, water and sample collection

Feed was provided as a commercial TMR (70% concentrate, 30% forage, Table 1) and was weighed and offered twice daily at 09:00 h and 18:00 h. Ewes were fed based on maintenance requirements throughout the experiment, and thus no residual feed remained to weigh each day. Body weights were measured at the beginning and at 14 d and 21 d of each period. Water was provided in plastic buckets and was available free of choice to the FAW group; however, it was provided following feeding at 11:00 h and 20:00 h for 2hWD group, and at 12:00 h and 21:00 h for 3hWD group. Water consumption was recorded two times daily for the 5 final days of each period. Thus, ewes in 2hWD and 3hWD groups were provided water at 2 and 3 h post-feeding, respectively. Urine (g and mL) and feces (g) were collected daily for the final 7 days of each period, and stored for analyses. The daily total volume of urine was measured using calibrated cylinder. Urine from each individual was collected daily in a bucket containing 100 ml of 7.2 N sulphuric acid (Chen and Gomes, 1995; Ma et al., 2013). The acid was added to the buckets prior to urine collections in order to maintain pH at less than 3 and to minimize ammonia volatilization by exposing urine to the air. The volume was measured and then diluted to 4 l with tap water. The urine samples were then diluted 1:20 with the buffer A, filtered through syringe filter (0.2µm pore size, 10 mm diameter, Whatman, Kent, UK) and a sample of 20 ml was collected, pooled across days for each animal, and stored at -20°C for analyzing total N and PD as described by El Shazly (1958). Approximately 200 g of daily fecal excretion from the each individual were sub-sampled to a plastic bag, dried (65°C for 3 days), finely ground (1 mm) and analyzed for OM (method 927.02; AOAC, 1990). The N (except for urine N) was determined by Kjeldahl procedure using Cu²⁺ as a catalyst, and CP was calculated as N×6.25 (method 984.13; AOAC, 1990).

Table 1. Chemical composition of TMR

Chemical composition	(%DM)	SEM
Dry matter	59.2	1.78
Crude protein	15.8	1.91
Crude fat	3.9	1.01
Neutral detergent fiber	37.5	1.23
Acid detergent fiber	19.7	1.19
Ash	7.3	0.45
Nitrogen free extract (NFE)	54.9	5.75
Total digestible nutrients (TDN)	67.9	3.11

NFE (%DM)=100-(CP+CF+EE+total ash). TDN (g/kg)=(Digestible Protein+Digestible Carbohydrate) g/kg+(Digestible Fat×2.25) g/kg (National Research Council, 2001).

Estimation of microbial N production

PD: Creatinine ratio (PDC index which is the ratio of PD/Creatinine along with PD and Creatinine are considered as indicators of protein status from microbial synthesis) was calculated according to the following formula (Seresinhe et al., 2004):

$$PDC\ index = ([PD]/[Creatinine]) * W^{0.75}$$

where:

W is the body weight in kg and PD and $Creatinine$ are their concentrations in mmol/l.

PD excretion (mmol/d) was calculated using the following equation:

$$PD\ excretion\ (mmol/d) = (PDC\ index) * C$$

where:

C is the daily creatinine excretion (mmol/kg $W^{0.75}$) for these ewes.

Creatinine excretion averaged 0.96 mmol/kg $W^{0.75}$ /d (Seresinhe et al., 2004).

Using the above data, absorbed exogenous purine concentration (mmol/d) was determined as follows:

$$Y = (0.385\text{kg } W^{0.75}) + 0.85X$$

where:

Y = Urinary PD excretion (mmol/d),

X = absorbed exogenous purine as mmol/d,

W = live weight (kg).

The following formula (Chen and Gomes, 1995) was applied to estimate the amount of microbial purine absorbed (X , mmol/d) corresponding to urinary PD (Y , mmol/d), as:

$$Y = 0.84 \times X + (0.150 \times BW^{0.75} e^{-0.25X})$$

and the production of microbial N was calculated as follows:

$$\text{Microbial } N = (X \times 70) / (0.116 \times 0.83 \times 1,000) = 0.727 \times X$$

where:

0.83 is the assumed digestibility of microbial purine,

70 is the N content of purines (mg/mmol),

0.116 is the ratio of purine- N : total N in mixed microbial biomass measured (Chen, 1989).

Urinary PDs analysis

Urinary purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) were determined as previously described by Balcells et al. (1992). The analyses of purine derivatives (PDs) were performed using a Waters 2690 HPLC system, equipped with Waters 996 photodiode array detector and autosampler (Milford, MA, USA).

Separations were conducted by using two reversed phase Nucleosil C18 columns (250 mm×4 mm, 5 µm, Agilent, CA, USA) connected in series. The buffer A was 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (pH adjusted to 6 with 10% NH_4OH). The buffer B consisted of the mixture of 150 mL of acetonitrile and 600 mL of 12.5 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (pH adjusted to 6 with 10% NH_4OH). The column was equilibrated by 100% of buffer A. The ratio of the buffer B increased in a linear gradient to 100% in 30 min and this ratio was maintained for 10 min. Then, the column was re-equilibrated by increasing the solvent A to 100% in 5 min and maintaining this ratio for 15 min before injecting the next sample. The absorbance was adjusted at 205 nm. The kit allantoin, uric acid, xanthine and hypoxanthine were purchased from Sigma Chemical Co. (USA). Creatinine was measured using Creatinine Colorimetric Assay Kit (Cayman Chemical Co.Ltd. no. 500701, Ann Arbor, MI, USA).

Statistical analysis

Statistical analysis was carried out using the GLM procedure of SAS (version 9.0; SAS Institute Inc., Cary, NC) for a Latin square (3×3) design. The model included effects of sheep, treatment, and period as follows:

$$Y_{ijkl} = m + a_i + S_j + P_k + e_{ijkl}$$

where:

Y_{ijkl} = each observation,
 μ = total mean,
 a_i = effect of treatment,
 S_j = effects of sheep,
 P_k = effects of period,
 e_{ijkl} = error.

Effects of sheep and period were not significant. Duncan's multiple range test was used for ranking treatment means within a significant F test and means were considered significantly different at $P < 0.05$. Means with probabilities between $0.05 < P < 0.10$ reflected a tendency to difference.

Results

Water and feed intake, feces and urine output, and nitrogen balance

Daily water intake (mL/d) decreased as water deprivation time following feeding increased ($P < 0.05$, Table 2). Feed intake was not different among the treatment groups ($P > 0.05$, Table 2). Fecal excretion (g/d, DM basis) showed no difference ($P > 0.05$) among the treatment groups (Table 2). Both urine weight (g/d) and volume (mL/d) decreased ($P < 0.05$) when water deprivation following feeding increased (Table 2). Water deprivation following feeding decreased the urinary N excretion

(g/d) and urinary N as a percentage of N intake ($P<0.05$), however, fecal N excretion (g/d) and fecal N as a percentage of N intake were not affected by water deprivation ($P>0.05$). With water deprivation, retained N (g/d) and retained N as a percentage of N intake increased ($P<0.05$) whereas no difference was observed for digestible N (g/d) among the treatment groups ($P>0.05$). However, N balance remained positive in this experiment in all of treatment groups. No differences were observed in BW of ewes among treatment groups ($P>0.05$).

Table 2. Water consumption, feed intake and N balance in ewes under water deprivation

Item	Treatments			SEM	P-value
	FAW	2hWD	3hWD		
Water consumption					
average water consumption (mL/d)	4547.7 a	4114.1 b	3565.1 c	198.3	0.03
Feed intake (g/d)					
average feed consumption (% of DM)	750.5	763.5	739.1	35.3	0.89
Fecal excretion (g/d)	221.6	198.9	211.5	8.7	0.17
Urine output					
urine weight (g/d)	3065.7 a	2550.7 b	2302.3 b	112.3	0.001
urine volume (mL/d)	2898.1 a	2420.3 b	2304.3 b	125.7	0.001
Nitrogen balance					
intake N (g/d)	18.76	18.76	18.76	0.06	0.55
fecal N (g/d)	4.63	4.50	4.34	0.30	0.43
digestible N (g/d)	14.05	14.22	14.40	0.22	0.53
urinary N (g/d)	12.01 a	9.01 b	8.19 b	0.32	0.01
retained N (g/d)	2.05 b	5.25 a	6.23 a	1.23	0.01
fecal N/intake N (%)	25.01	25.33	26.33	2.05	0.34
urinary N/intake N (%)	64.15 a	48.03 b	43.66 b	4.01	0.01
retained N/intake N (%)	10.06 b	27.48 a	33.20 a	4.13	0.01

Treatments included free access to water (FAW), 2 (2hWD) and 3 h (3hWD) water deprivation following feeding.

a, b – values within a row with different letters differ significantly at $P<0.05$.

PDs, CP and OM digestibility and microbial N production

Urinary allantoin concentration tended to be higher ($P=0.07$) in the FAW group than for 2hWD and 3hWD groups while no differences ($P>0.05$) were observed for uric acid, xanthine+hypoxanthine and creatinine concentrations (Table 3). Digestibility of CP, OM and consequently DOMI was higher ($P<0.05$) in 2hWD group among the treatment groups. Microbial N production (g of N/d) tended to be higher ($P=0.08$) in 2hWD group and it was higher ($P<0.05$) in 2hWD group than other treatment groups when reported per DOMI (Table 3). Index of PDC decreased ($P<0.01$) linearly when water deprivation time following feeding increased (Table 3).

Linear correlation ($R^2=0.81$) was found between daily PDs excretion and PDC index in treatment groups (Figure 1).

Table 3. Urinary PDs concentration and microbial N production in ewes under water deprivation

Item	Treatments			SEM	P-value
	FAW	2hWD	3hWD		
PDs (mmol/d)					
allantoin	4.03	3.05	2.89	0.43	0.07
uric acid	0.93	0.82	0.73	0.47	0.53
X+H	0.51	0.42	0.86	0.29	0.27
creatinine (mmol/d)	6.10	5.82	5.63	1.02	0.46
CP digestibility (g/kg)	698.53 b	735.57 a	713.39 b	8.27	0.02
OM digestibility (g/kg)	711.33 b	742.33 a	717.73 b	8.82	0.04
DOMI (g DM/d)	485.00 b	510.13 a	489.03 b	10.57	0.03
Microbial N production					
g of N/d	6.10	7.53	6.87	0.63	0.08
g of N/kg of DOMR	16.01 b	21.02 a	23.08 a	1.75	0.04
PDC index	38.58 a	30.05 b	27.52 b	3.01	0.01

Treatments included free access to water (FAW), 2 (2hWD) and 3 h (3hWD) water deprivation following feeding. X+H=xanthine plus hypoxanthine. OM=Organic matter. DOMI=Digestible organic matter intake. Microbial N was calculated according to Chen and Gomes (1995). DOMR=digestible OM in rumen calculated as $0.65 \times \text{DOMI}$ (g/d), Chen et al. (1992). PDC index = $\text{PD} / \text{Creatinine} \times \text{BW}^{0.75}$.

a, b – values within a row with different letters differ significantly at $P < 0.05$.

Figure 1. Correlation between PD excretion mmol/d and PDC index in total urine of ewes

Discussion

Water is a critical nutrient and should be available to livestock in adequate amounts (Ghassemi Nejad et al., 2014 a, b). Average water consumption was higher in FAW group which is in agreement with Casamassima et al. (2008) who reported higher water consumption for sheep with FAW compared with sheep that were restricted to 60% and 80% of total water consumption. Mengistu et al. (2004) suggested that ruminants are able to conserve water with a reservoir system for use in periods of reduced water supply. Water deprivation did not affect feed intake (Singh

et al., 1976) but slowed the movement of digesta from the reticulo-rumen to the intestine (Brosh et al., 1986) which should increase apparent digestibility of nutrients by allocating more time for microbial enzymes to attach to the feed particles. Due to limited availability of water, ruminants have developed high water efficiency with improved utilization of body water resulting in the maintenance of DM intake and performance (Silanikove, 1994). Since ewes in this study were fed maintenance diets body weight remained unchanged. Higher urine excretion (volume and weight) in FAW group in this experiment is in agreement with the previous studies (Ghassemi Nejad et al., 2014 b). Ewes in FAW group had free access to water at all times. Therefore, it is assumed that they may consume more water than water deprived groups. However, there is a possibility of compensating water consumption while having access to water following deprivation time. However, in contrast, ewes in water deprived groups consumed less water than FAW group. This might be explained due to development of water reservoir system in ruminants while having access to water following the deprivation time as stated by Mengistu et al. (2004). Higher urine excretion in FAW reflects greater water consumption for this treatment. Reduced N excretion in urine with water deprivation (Table 2) reflects greater retained N resulting in positive N retention in related treatment groups. Reduced filtration in the kidney glomeruli with water deprivation (Wittemberg et al., 1986) could be the reason for decreasing urinary N loss as reported by Brosh et al. (1986). The decrease in urine excretion observed in the present experiment may have reduced N loss through urine. Increased retained N in ewes with restricted water was confirmed by lower urinary N in these two groups compared with low N retention in FAW group (Table 2). No differences were observed in retained N, urinary N of N intake and retained N of N intake between the 2hWD and 3hWD groups, suggesting that 2-3 h water deprivation following feeding did not affect N digestibility. The fact that 3hWD showed the same retained N with FAW suggests that increasing deprivation time for over 2 hrs may not necessarily lead to improve nutrient digestibility of feed. Positive N retention in all groups could be explained by higher digestible N. It may be hypothesized that a slow gastrointestinal transit time due to water deprivation may increase the bio-availability of nutrients and lead to improved efficiency of digestion (Casamassima et al., 2008) as exhibited in the present study. Higher N retention in water deprived groups compared with FAW group in ewes may reflect recycling of N through the ruminal wall and saliva for microbial synthesis (McMannus, 1962; Pursor and Moir, 1966; Ghassemi Nejad et al., 2015).

Generally water deprivation in any form should improve nutrient digestibility by increasing retention time of the digesta giving more time for microbial degradation and synthesis (Balch and Campling, 1965; Ahmed Munna and El Shafei Ammar, 2001; Casamassima et al., 2008). With water deprivation, CP and OM digestibility were significantly increased in the present study (Table 3). However, CP and OM digestibility were higher in 2hWD group compared with 3hWD. The reason why CP and OM digestibility were higher in 2hWD than 3hWD group is unknown. This might be related to the decreasing rate of passage of digesta (Balch et al., 1953) and increasing retention time of digesta (Casamassima et al., 2008; Ghassemi Nejad et al., 2015) in the rumen.

There have been studies which show microbial N production when estimated by both PD methods (Salman et al., 2013; Ma et al., 2014) and conventional method to have a slope correlation close to 1, using direct measurement of microbial marker. The marker used in the conventional method was: 2, 6-diaminopimelic acid (DAPA) (Oosting et al., 1995), amino acid profile (Oosting et al., 1995), RNA (Puchala and Kulasek, 1992), and ^{15}N (Perez et al., 1996). Greater microbial N production is supported by both higher OM and CP digestibilities in groups which were deprived from water following feeding. This contrast could be explained by higher urine output when the FAW group is compared with water deprived groups followed by lower OM digestibility that resulted in lower DOMI in the FAW group. For PD index calculation, multiple samples are required to obtain a measurement representative of the dietary regime when spot sampling is used (Chen et al., 2004; Salman et al., 2013; Ma et al., 2014). In the current study the authors observed a linear relationship between water deprivation time and decrease in PD index.

Water deprivation following feeding changes the urine output of ewes (Ghassemi Nejad et al., 2014 b) which could alter the amount of PDs excreted via urine. In the present study ewes that had free access to water exhibited higher urine output (Table 2). Higher urine output (Table 2) may be related to higher water consumption in FAW group (Table 2), as also reported by Ghassemi Nejad et al. (2014 a). Chen et al. (1995) found the correlation between PDs excretion and PDC index ($R^2=0.81$) when spot urine was collected at hourly intervals. The present study confirmed the correlation when the entire urine output was collected and the values for X+H were not considered because of diurnal fluctuations. Therefore, it appears that urine collected at hourly intervals, has a similar correlation between PDs excretion and PDC index as if the total urine output is collected. However, under practical circumstances, it is inconvenient to collect spot urine samples intensively.

Conclusions

Water deprivation for 2 and 3 h following feeding increased N retention and microbial N production in ewes, and 2 h water deprivation improved OM digestibility and digestible organic matter intake more than 3 h. Water deprivation following feeding for less than 3 h improves microbial N production in ewes.

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