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Research Article

IS THERE A RELATIONSHIP BETWEEN NITROGEN INTAKE AND TESTICULAR UREA CONCENTRATION?

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ABSTRACT
An experiment was conducted to determine the relationship between nitrogen intake and testicular urea content. Twenty ram lambs of fat-tailed Iranian native breed were used in this study. The mean (±SD) age and body weight of the lambs were 180±20 days and 36.7±6.3 kg, respectively. The lambs were divided in to five groups of equal size, and received a ration containing a single protein source; including: 1) urea as a control, 2) barley grain, 3) cotton seed meal, 4) gluten meal, and 5) canola meal. Crude protein content of the diet was adjusted at 13.6 percent, but with different ruminal degradability. During the experiment, the lambs were kept in isolation to avoid exposure to the sight and smell of female sheep. Rams were kept under natural day light conditions and ambient temperature (latitude 51◦27_N) for 11 weeks. On day 75, ruminal fluid samples were collected at 2, 4, 6, and 10 hours after morning feeding. On day 76, jugular blood samples were collected in vacuum tubes containing heparin as the anti-coagulant, and slaughtered subsequently. The right testis was removed, weighed, homogenized in phosphate buffer solution, and centrifuged to collect the supernatant for future analysis. The testicular extract and plasma were analyzed for urea nitrogen, and the ruminal fluid sample and testicular extract were analyzed for ammonia concentration. All samples were assayed in duplicate. The level of nitrogen intake significantly affected the testicular urea concentration ($P<0.05$). Plasma urea concentration was significantly correlated with rumen ammonia concentration at 4 and 6 hours, but not at 2 and 10 hours, after feeding. Testicular urea and ammonia concentrations were highly correlated. In conclusion, plasma urea concentration did not impact on testicular urea and ammonia concentrations but, nitrogen intake had a significant effect on testicular urea concentration.

Keywords: *Urea, Testis, Blood Urea, Protein Sources, Sheep*

INTRODUCTION
Protein is a key nutrient in ruminant nutrition, not only by providing amino acids to the animal, but also as a source of nitrogen (N) for microbial protein synthesis (NRC, 2007). Proteins differ in their amino acid profile, and the availability of crude protein (CP) in the rumen and at post-ruminal digestive tract (Gleghorn *et al.,* 2004; Bateman *et al.,* 2005). It is accepted that dietary protein requirements and supply are best expressed in terms of ruminal degradable (RDP) and undegradable protein. Because Nutilization efficiency inruminants is lower than in non-ruminants, it is necessary to feed large amounts of protein which increases the production cost and N loss to the environment. This may also exert detrimental effects on reproduction and cause lower production, especially during hot weather (West, 2003). Although, numerous studies have shownthat overfeeding protein negatively influences the male reproduction (Oldham *et al.,* 1978; Cameron *et al.,* 1988; Abi Saab *et al.,* 1997; Al-Haboby *et al.,* 1999), the mechanism by which excess protein intake influences reproduction is partly unknown. The relationship between protein nutrition and reproduction, usually recorded in rams, probably follows a non-linear model and hence, the magnitude of the response can vary when different levels of protein intake are compared (Fernandez *et al.,* 2004). Fernandez *et al.,* (2004) reported that above a certain level of protein intake there will not be any additional benefit on testicular growth. A number of studies have demonstrated that the spermatogenesis in rams is sensitive to increases in protein intake (Oldham *et al.,* 1978; Cameron *et al.,* 1988; Abi Saab *et al.,* 1997). This effect has been related to an increase in testicular size increase as a result of increased volume of seminiferous epithelium and greater diameter of the

seminiferous tubules (Oldham *et al.*, 1978; Martin *et al.*, 1987; Pomares *et al.*, 1991; Abi Saab *et al.*, 1997; Hotzel *et al.*, 1998). Fernandez *et al.*, (2004) also showed that sperm production could be affected by protein intake. They noted that this effect did not seem to be related to changes in testosterone secretion, On the other hands, several studies reported that semen traits were not influenced by protein intake at levels above maintenance requirement (Nsahlai *et al.*, 2000). Al-Haboby *et al*., (1999) reported a negative effect of protein supplementation on semen quality, but this effect was presumably related to an excess of RDP consumption.

Urea, a small highly polar molecule (MW ≈ 60 Da), is the major end product of nitrogen metabolism in mammals. Increased concentration of urea and other unidentified nitrogenous compounds in the blood increase the level of urea and ammonia in the reproductive tissues and fluids (Jordan *et al.*, 1983; Carroll *et al.*, 1988).

Plasmatic urea can reach the seminiferous tubules (Mann and Lutwak-Mann, 1981; Rodriguez-Rigau and Steinberger, 1982) and the presence of testicular transporters for urea suggests that it may play a role in spermatogenesis (Tsukaguchi *et al.*, 1997). In analogy to this, the transport of urea is facilitated by urea transporters (You *et al.*, 1993) and also by aquaporins that are relatively small (about 26 kDa) pore-forming membrane proteins and transport water passively in response to osmotic driving forces (Litman *et al.*, 2009). Some of the aquaporins are able to transfer urea to testis (Verkman 2005; Wu and Beitz, 2007). High urea concentration in the testes may change multiple biological activities by destroying hydrophobic bonds of protein structure or causing protein carbamylation (Zou *et al.*, 1993; Kraus and Kraus, 2001). Therefore, this study was carried out to determine the relationship between nitrogen intake, plasma urea and testis urea concentration in ram lambs.

MATERIALS AND METHODS

Animals

Twenty ram lambs of an Iranian fat-tailed breed were used in this study. The mean (±SD) age and body weight were 180±20 days and 36.7±6.3 kg, respectively. The lambs were initially treated against internal and external parasites by administering Albendazole (ICI Pharmaceuticals, Iran) and dipping with Neguvon (Bayer, Leverkusen, Germany), randomly allotted to five experimental diets, and fed individually (in metabolic cage) on an ad libitum basis.

The experiment was carried out at 51˚27_Nlatitude in summer (temperature was between 15 and 30°C). To prevent deficiency of vitamins and minerals, salt and mineral blocks were offered throughout the experiment (one kg mineral blocks supplied 16 g Mg, 4000 mg Fe, 1400 mg Zn, 1200 mg Mn, 300 mg Cu, 16 mg I, 10 mg Co, 10 mg Se, 600 mg S and 1 mg NaCl), and at the start of the experiment, each lamb was injected intramuscularly with 5 ml Adeject® (ErfanDaroo, Tehran, Iran) vitamin supplement containing 50000 IU vit. A, 10000 IU vit. D_3 and 20 mg vit. E. The lambs were kept in isolation to avoid exposure to the sight and smell of the females.

Experimental Diets

Diet Ingredients

Straw

Sun-dried wheat straw was chopped into 5 to 5-cm long pieces. Before each meal, a solution containing sucrose and urea (65g sucrose and 35g urea dissolved in 750 g water per kg wheat straw) was sprayed on to the chopped straw, and uniformly mixed to be fed free choice to lambs as the forage source.

Pellets

Five iso-energetic and iso-nitrogenous pelleted diets (Table 1) were formulated each containing a single protein source, including urea (as the control, CONT), barley (Bar), corn gluten Meal (CG), cotton seed meal (CSM) and canola meal (CM). Wheat straw, processed [washed (each kg wheat straw with 10 L water), and processed with sulfuric acid (each 960 g wheat straw with 1 kg acid solution 4% w/w) and heat (70º C for 2 min)] milled and passed through a 4 mm sieve, was used as the carrier in feed pellets. Pelleted feed components were ground using a hammer mill to pass through a 4-mm sieve, mixed uniformly, and processed into 6-mm die.

The diets were fed twice daily at 08.00 and 16.00 hours at an amount to leave five percent orts, based on feed consumption during the adaptation period. Consequently, feed consumption was measured before the morning meals during the experiment. Freshwater was offered ad libitum after feeding for 1/2 hour to avoid any dilution of the rumen content before sampling.

Sampling

Rumen Fluid Sampling

On day 75, rumen fluid samples (20 mL) were collected at 2, 4, 6, and 10 hours after morning feeding by rumenocentesis technique, kept on ice and transferred to the laboratory immediately. Rumen fluid was strained through four layers of cotton gauze and centrifuged once at 1000×*g* for 10 min. The supernatant fluid was carefully decanted and named as the strained rumen fluid without protozoa.

Blood Sampling

Jugular venous blood (10 ml) was collected into heparin-containing vacuum tubes on day 76 of the experiment following a 3-4 hours after the afternoon feeding. Blood plasma was separated after centrifugation for 10 minutes at 3000 g and stored at -80°C for subsequent analyses.

Testis Sampling

At the end of the experiment (on day 76) the lambs were slaughtered at about two hours after morning feeding. The right testis was removed, weighed, homogenized with phosphate buffer solution (pH=7), and centrifuged (1000 × g, 10 min) to collect supernatant for future analysis.

Table 1: Ingredients and Composition of the Pelleted Diets (Dry Matter Basis)

Item	CON	Bar	CG	CSM	CM
Ingredients (%)					
Wheat Straw	27	30	40	28	34
Protein Source	--	58	28	34	33
Urea	4.8	2	--	--	--
Dicalcium Phosphate	2	1	2	1.2	1.2
Starch	57	2	11	20	13
Common Salt	2.2	4	4	4	4
Sodium Bicarbonate	1	1	1	1	1
Sucrose	5	1	13	11	13
Calcium Sulfate	1	1	1	0.8	0.8
Nutrient Composition					
Dry matter †	93.7	93.3	94.6	93.8	94
ME (Mcal/kg) ‡	2.2	2.2	2.2	2.2	2.2
CP (%)†	13.6	13.6	13.6	13.6	13.6
NDF (%)†	15.8	30.3	30.3	29.2	31.9
ADF (%)†	10.6	14	14.1	15.2	18
NFC (%)§	63.2	46.4	45.3	44.9	43.2
EE(%)†	0.3	0.7	0.96	1.73	0.93
Ash†(%)	7.05	9	9.8	10.5	10.3

† Determined in the laboratory
‡ Calculated based on NRC (2007)
§Calculated based on laboratory data (100-(CP+EE+NDF+Ash))
CON: Control, Bar: Barley, CG: Corn gluten, CSM: Cotton seed meal, CM: Canola meal.
ME: Metabolizable energy, CP: crude protein, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, NFC: None fiber carbohydrate, EE: Ether extract

Analytical Procedures

Pellet samples were analyzed in duplicate for dry matter (DM, AOAC 2000 ID934.01), total N [(AOAC 2000 ID954.01) using 6.25 as conversion factor for crude protein, CP], neutral detergent fiber (Mertens,

2002), acid detergent fiber (Van Soest *et al.*, 1991), and ash (AOAC 2000 ID 942.05). Plasmaurea N was measured by spectrophotometer (Uniqo, UV-2100, Germany) using a commercial kit (DarmanKav, Isfahan, Iran). Ruminal fluid ammonia and ammonia concentration in the testicular extract was determined as described by Pathak *et al.*, (1996).

Statistical Analysis

This experiment was designed as a completely randomized design with five treatments and four replicates. All measurements were performed in duplicate. The data were analyzed by using the GLM procedure (SAS, 2009). When a significant ($P<0.05$) main effect was detected, the main effect means were separated by the Duncan's multiple range test. Correlation between urea and ammonia were calculated using the ProcCORR (SAS, 2009).

RESULTS AND DISCUSSION

The effect of protein source on N intake, and urea concentration in the testis and blood plasma is presented in Table 2. The intake of N was significantly increased when CSM was included in the diet as the protein source, but other protein sources did not affect the N intake. Plasma urea concentration was generally lower in the CON lambs compared with the lambs which were fed with the diets containing Bar, CG, CSM, and CM, but only the difference between CON and CSM lambs were significant ($P < 0.05$). Testicular urea was not affected by the protein source in the diet. The intake of nitrogen is affected by the amount of feed intake which in turn is influenced by feed palatability. Lambs tend not to prefer the diet containing urea, canola meal and corn gluten due to their low palatability (Mereu, 2009; Rapisarda *et al.*, 2012). Several studies showed that increasing urea level in the diet caused a reduction in dry matter intake (Van Horn *et al.*, 1967; Broderick *et al.*, 1993; Brito *et al.*, 2007). It has been shown that ruminants are able to detect asynchrony in the rate of supply of nutrients in the rumen and have developed mechanism (s) to overcome or minimize its effects by altering their pattern of feed intake to avoid an excessive load of ammonia, storing carbohydrates by rumen microbes during periods of N deficit, and by higher recycling of urea to the rumen (Dawson, 1999). In fact, lambs receiving urea as nitrogen supplier formed a preference for flavor associated with the lowest dose and avoided flavor associated with the highest dose. This is a demonstration that the formation of preferences is linked to post-ingestive effects that nutrients acts in the body metabolism. The fact that animals avoided the highest dose of urea can be explained by the potential toxic effect generated by urea. This negative effect was connected by the animals to the flavor associated with the high dose of urea; thus, animals reduced feed intake on urea treated feeds when they have the possibility to choose. However, all treatments resulted in higher plasma urea compared with the CON diet (Table 2), although, it was significantly higher in lambs on CSM diets ($P<0.05$). It has been suggested that plasma urea concentration may be a good predictor of both nitrogen utilization (Kohn *et al.*, 2005) and nitrogen intake (Cheng *et al.*, 2013) in sheep. Cheng *et al.*, (2013) found a close relationship between protein intake and plasma urea levels in growing-finishing lambs. On the other hand, since the amino acid profile of microbial proteins is more consistent with the amino acid requirements of the animal (Kohn and Boston, 2000), it may decrease protein catabolism in the body. Hence, the amino acid profile of the true dietary proteins which escape microbial degradation may not resemble that of the microbial proteins. This would have increased urea production in the liver, resulting in increased plasma urea level. Therefore, increased plasma urea level in CSM lambs may be a consequence of increased N intake in CSM group. On the other hand, urea concentration in the testis was not affected by the protein source ($P>0.05$) suggesting that plasma urea concentrations do not affect the testicular urea concentration.

The relationship between urea and ammonia in the testis, plasma and ruminal fluid is shown in Table 3. Plasma urea concentration was positively correlated with ruminal ammonia concentration at 4 ($r=0.51$; $P=0.021$) and 6 ($r=0.46$; $P=0.040$) hours after feeding but not with 2 ($r=0.14$; $P=0.554$) and 10 ($r=0.43$; $P=0.056$) hours after feeding.It has been reported that ammonia production increases for several hours after feeding that depends on the dietary protein properties (Chalupa *et al.*, 1964; Oltjen *et al.*, 1968; Bach *et al.*, 2005). However, ammonia flux through portal circulationis significantly related with ammonia

production levels in the rumen. Ammonia is detoxified to urea in the liver, with more than 60% of blood urea can be derived from ammonia absorbed from the rumen (Kennedy and Milligan, 1978). The lack of effects of nitrogen source on urea concentration in testis, despite increases in plasma urea level, may be associated with the levels not being high enough since plasma urea levels remained below the broad range (e.g. 43 to 75 mg/dl) considered to be normal in rams (Smith *et al.*, 1978; Roller *et al.*, 1982; Cortada *et al.*, 2000).

Testicular Sertoli cells can potentially produce urea (Fenton *et al.*, 2002). They contain high arginase activity that hydrolyzes arginine into urea and ornithine (Tsukaguchi *et al.*, 1997). This necessitates the efflux of urea from seminiferous tubules to maintain normal spermatogenesis, which is achieved through urea transporter B (UT-B). Sertoli cells are also part of the blood-testis barrier that prevent harmful substances from reaching the developing germ cells, most notably post-meiotic spermatids thus maintaining a unique environment for normal spermatogenesis. According to Guo *et al.*, (2007), deletion of UT-B decreases urea flux across the blood-testis barrier and blocks the efflux of urea from Sertoli cells. It seems that with increasing concentrations of urea in the testis the activity of the UT-B also increases (Guo *et al.*, 2007). Therefore, it seems that despite the increases in plasma urea concentration, the existence of these mechanisms prevent increasing urea concentration in the testis.

Table 2: The Effect of Dietary Protein Source on N Intake (mg/w$^{0.75}$/d), and Plasma and Testicular Urea Concentration (mg/dl) in Male Rams

Parameter	Dietary Protein Source					SEM*	P-Value
	CON	Bar	CG	CSM	CM		
N intake	1484^{b}	1332^{b}	1468.8^{b}	2054.5^{a}	1553.5^{b}	104.2	0.002
Plasma urea	20.7^{b}	27.8^{ab}	29.3^{ab}	32.5^{a}	25.03^{ab}	1.305	0.004
Testicular urea	0.982	0.950	0.979	1.083	1.050	0.078	0.81

* Standard error of the mean
a, b…. In each row, means with the same superscript are not significantly different (P>0.05).
CON: Control, Bar: Barley, CG: Corn gluten, CSM: Cotton seed meal, CM: Canola meal.

Table 3: Correlation Coefficients between Nitrogen Intake, Testicular Urea, Testicular Ammonia, Plasma Urea and Post-Feeding Ruminal Ammonia Concentrations in Male Rams

Parameter	Testicular Urea	Testicular Ammonia	Plasma Urea	Ruminal Ammonia Concentration (Hours Relative after Feeding)			
				2 h	4 h	6 h	10 h
Nitrogen Intake	0.9900^{a} (<0.0001^{b})	0.1809 (0.4586)	-0.2190 (0.3677)	-0.2922 (0.2247)	-0.1038 (0.6724)	0.0552 (0.8221)	-0.2130 (0.3811)
Testicular Urea		0.1809 (0.4586)	-0.2190 (0.3679)	-0.2922 (0.2247)	-0.1038 (0.6724)	0.0552 (0.8221)	-0.2131 (0.3810)
Testicular ammonia			0.3043 (0.2052)	0.0245 (0.9204)	0.0535 (0.8246)	0.1909 (0.4335)	0.0389 (0.8743)
Plasma urea				0.1405 (0.5544)	0.5122 (0.0209)	0.4636 (0.0395)	0.4338 (0.0560)

[a]*Coefficient of correlation.*
[b]*Level of probability.*

Conclusion

From the results of this study, it is evident that plasma urea concentration does not seem to affect the urea and ammonia concentration in the testicular tissues but nitrogen intake may have a significant effect on urea concentration in the testis.

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