IJAS Iranian Journal of Applied Animal Science

Different Sources of Protein Effect in the Flushing Rations on some Blood Parameters and the Reproductive Performance of Ghezel Sheep

Research Article

H. Daghigh Kia[1*], A. Ahmad Fazel[1] and A. Hossein Khani[1]

[1] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Received on: 28 Oct 2014
Revised on: 1 May 2016
Accepted on: 15 May 2016
Online Published on: Sep 2016

*Correspondence E-mail: daghighkia@tabrizu.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

Sixty Ghezel ewes (age 3.5 years) were used to study the effect of different nitrogenous supplements in flushing ration on reproductive performance. Experimental treatments were divided in five groups: group A: urea; group B: soybean meal (SBM); group C: corn gluten meal (CGM); group D: barley grain and group E: control or basal diet (no flushing). The four flushing rations had nearly the same amounts of nitrogen and energy (crude protein (CP)=10.5% and metabolizable (ME)=2.1 Mcal/kg). CGM and control groups had the highest and lowest number of progeny, respectively (16 *vs.* 9 lambs). Flushing rations increased average blood glucose, insulin and blood serum protein compared to the control group; however, glucose, insulin, cholesterol and BUN reached their highest levels in SBM, CGM, barley and urea treatments, respectively. Using suitable protein sources especially bypass or rumen undegradable protein (such as CGM) in the flushing ration can improve the reproductive performance and herd recovery efficiency, maximizing profitability of sheep farming enterprise.

KEY WORDS flushing ration, Ghezel sheep, protein degradability, reproductive performance.

INTRODUCTION

Manipulation of reproduction through nutrition is a practical management procedure to increase lambing rate of extensive sheep production systems particularly in the semi-arid regions such as Iran. Nutritional flushing is defined as a short-term provision of extra feed to increase nutrient intake (particularly energy and / or protein) prior, during or after mating. According to Rattray (1982), the reproductive performance can be improved by increasing body weight during the flushing period (two week prior and three week after mating). Nutrition affects reproductive performance through two ways; directly by supplying certain nutrients which are necessary for the oocyte development, ovulation, embryo survival and conception; and indirectly through its impact on some hormones and metabolites (Robinson *et al.* 2006). Improved ovulation and fertility rates by increasing dietary energy and protein before and after mating have been reported by several researchers (Al-Haboby *et al.* 1999; Daghigh Kia *et al.* 2012). Daghigh Kia *et al.* (2012) used different sources of fat and carbohydrate in the flushing rations of Markhoz goat and observed an improved reproductive performance; while Al-Haboby *et al.* (1999) reported high fertility, pregnancy, lambing and twinning rates in the Awasi ewes which fed cotton seed meal and urea-containing block as different sources of nitrogen. The mechanism by which nutrition influences the ovulation rate is very complex and unclear. It appears that protein and energy act through separate mechanisms to affect the ovulation rate. In sheep, follicle populations are very sensitive to nutritional input and folliculogenesis and ovulation rate can be readily increased by nutritional manipulation

(Scaramuzzi *et al.* 2006). Improvement of nutrient supply can affect the hypothalamus pituitary axis thereby influencing gonadotropins, change progesterone, estrogen, insulin, growth hormones concentrations and some metabolites amount (such as glucose) leading to higher ovulation rate and oocyte quality (Scaramuzzi *et al.* 2006).

There have been numerous reports on the positive effect of protein rich supplementations on ovulation rate of ewes and cows (Kaim *et al.* 1983; Rhind, 1992). Thomas *et al.* (1984) reported that an increase in nutrient intake, particularly protein, effectively increases levels of hepatic steroid metabolizing enzymes. Higher clearance rate and decreases of steroids is associated with an increase in gonadotropins which can improve ovulation rate. Ocak *et al.* (2006) demonstrated that short term (15-17 days) changes in protein supplementation could have a beneficial effect on non return rate, lambing rate and litter size in ewes grazed on range land. They revealed that embryonic losses are diminished by a high protein diet during the post mating period. Marais (2011) used different sources of nitrogen as the flushing supplements in grazing Dohne merino ewes; however, using those supplements had no effect on ovulation, conception and lambing rate. Marais (2011) used flushing supplements in the semi intensive managing system. Little is known about the effect of different sources of protein in flushing ration of ewes in the intensive system. The aim of present experiment was to investigate the effect of different protein sources in the flushing rations of Ghezel sheep on reproductive performance.

MATERIALS AND METHODS

Animals, experimental design and sampling

The experiment was carried out at the Breeding and Research farm of Ghezel sheep in Miandoab (1371 m above sea level, longitude 36˚ 9' east and latitude 36˚ 58' north). Sixty Ghezel ewes (50±3 kg), 3.5 years old, having two parity history were used. Animals were divided into five groups: 12 ewes in each group. The used protein supplements were urea (Urea), soybean meal (SBM), corn gluten meal (CGM) and barley grain (Barley). There was also a group of animal (12 ewes) under basal feeding (Control). The four flushing rations were formulated to have approximately the same amounts of nitrogen and energy (10.2-10.6%, CP) and (2.08-2.11 Mcal/kg, ME) in which protein content in Urea, SBM and CGM groups was equivalent with the protein of 400 g barley grain. Only the source of nitrogen was different among flushing diets to emphasize the effect of protein quality on reproductive performance. Experimental rations were formulated based on NRC (1985) recommendations and ewes were fed total mixed ration three times daily (Table 1).

Animals had free access to fresh water. Flushing period started 3 weeks before mating and was continued 2 weeks later. At the beginning of the experiment, all ewes had an average body condition scores (BCS) of 2.5 and reached to 3 at the time of breeding.

Estrous cycle synchronization was achieved using intravaginal progesterone-releasing devices (CIDR) (EAZI BREED type G controlled internal drug releases; Pfizer New Zealand Ltd., Auckland, New Zealand) for 14 days. Ewes were naturally mated using Ghezel rams that were introduced 36 h after CIDR removal. Pregnancy rate, lambing rate and total offspring were recorded. Blood samples were collected 24 h before and 48 h after CIDR removal and 10 days after mating. Serum was separated by centrifugation at 1700 g for 15 min and stored at -20 ˚C until being used.

Commercially available kits were used to measure serum glucose (017-500-1; Pars Azmun Laboratory, Tehran, Iran), total protein and blood urea nitrogen (BUN) (Darman kave, Isfahan, Iran). Estrogen, progesterone and insulin levels were estimated using ELISA (Awareness model, WA, USA), kit no. ELA-2693 (DRG, Marburg, Germany), kit no. ELA-1561 (DRG) and kit no. ELA-24k2D10 (Monobind, USA), respectively.

Statistical analysis

The present experiment was laid out in a completely randomized design (CRD). The pregnancy rate and other reproductive traits were analyzed applying FREQ, LOGISTIC procedure; hormones and blood metabolites were analyzed using general linear model (GLM) (MODEL 1) and Mixed (MODEL 2) procedures of statistical analysis software (SAS) software (SAS, 2003). In the Mixed procedure, minimum Bayesian Information Criterion was selected in a variety of variance co-variance structures and comparison of means was performed applying the same procedures within the squares separately for each trait. Statistical models were as follow:

Model 1: $Y_{ij} = \mu + Treat_i + \beta(Weight_{ij}\text{-}Weight) + e_{ij}$

Model 2: $Y_{ijk} = \mu + Treat_i + Animal_j(Treat_i) + Time_k + (Treat \times Time)_{ik} + e_{ijk}$

Where:

Y_{ijk}: animal performance.

μ: population mean.

$Treat_i$: i treatment effect.

$\beta(Weight_{ij}\text{-}Weight)$: effect of weight as a covariate.

$Animal_j(Treat_i)$: effect of j animal in i treatment.

$Time_k$: effect of k time.

$(Treat \times Time)_{ik}$: treatment by time interaction.

e_{ijk}: residual or error.

RESULTS AND DISCUSSION

Flushing rations improved reproductive traits of ewes and had significant effects on the twining rate (χ^2=66.66, P<0.0001) (Table 2). Among the flushing groups, CGM and urea supplemented treatments had the most and least offspring number, respectively (Table 2). Since all of the experimental treatments had nearly the same amounts of nitrogen and energy (Table 1) then protein quality could be the major reason for different responses of animals. The quality of the dietary protein depends on the amino acid profile as well as the digestibility. On the other hand the protein requirement of a ruminant depends on its physiological status and production level. All of the flushing diets in the present study received approximately the same amount of crude protein, but there were substantial differences in the quality of the protein. Rumen undegrdable part of CP was 0.27 for barley grain, 0.35 for SBM and 0.55 for CGM (NRC, 1985), while urea is completely converted into ammonia in the rumen. Urea is considered just as a non-protein source of nitrogen and can only be incorporated in the microbial protein synthesis, whereas the majority of the CGM protein can escape the degradation in the rumen. The main source of absorbable amino acids in the ewes fed by high amounts of rumen degradable protein is microbial protein which relatively is poor in branched chain amino acids (BCAA) (Merchen and Titgemeyer, 1992). Moreover, high rumen degradable protein has long been suspected to have a deleterious effect on fertility in dairy cows (Ferguson *et al.* 1986). Conversely CGM is rich in BCAA and has low ruminal degradable protein. Therefore, it can increase the duodenal flow of BCAA and contributes to the intestinal absorption of these AA (Tagari *et al.* 1995). Total amounts of BCAA as a percent of CP are 15.34, 16.87 and 25.54 in barley grain, SBM and CGM, respectively (g BCAA/100 g CP) (NRC, 2001).

There is a good evidence to indicate that BCAA has positive effect on reproduction. BCAA can improve the ovulation rate directly through affecting ovarian function (Downing *et al.* 1995) and indirectly by elevating insulin levels. Since BCAA (especially leucine) can stimulate insulin secretion, it may contribute in higher ovulation rate (Kuhara *et al.* 1991). Elevated plasma concentrations of insulin has been confirmed in sheep infused with BCAA (Kuhara *et al.* 1991) or fed excess dietary protein from SBM (Molle *et al.* 1995) but not urea (Madibela *et al.* 1995). Hinch and Roelofs (1986) demonstrated an increased ovulation rate in ewes through infusion of insulin. Studies of Landau *et al.* (1996) on follicular dynamics of sheep revealed that the growth and development of follicles in the group receiving SBM with high degradation in the rumen and containing BCAAs, occur faster than the group receiving CGM with low degradability in the rumen and higher amounts of BCAAs.

Serum hormones and metabolites

The variations of some plasma metabolites and hormones which can alter reproductive efficiency are represented in Table 3. At the start of the experiment, amount of all hormones and metabolites were the same and no significant differences were found among treatments. Consumption of flushing rations resulted in different responses of ewes regarding hormones and metabolites. Insulin, glucose and cholesterol which associated with energy status showed considerable variation around ovulation (24 hrs before and 48 hrs after CIDR removal). There was a significant variation among the experimental groups for insulin levels at 24 hrs before CIDR withdrawal (P<0.05). This situation continued until the third sampling time (ovulation or 48 hrs); however, no difference was observed at the last sampling (10 days after CIDR removal). Glucose fluctuations were occurred with a delay compared to the insulin fluctuations (48 hrs *vs.* 24 hrs after CIDR removal respectively) (Table 3). Therefore, it seems that glucose changes were affected by the fluctuation of insulin (P<0.01). Diets had more obvious influence on the BUN and serum protein levels, as these metabolites were significantly affected by the rations for the period of 24 hrs before CIDR removal to the end of the flushing period (P<0.01). Experimental diets had no effect on estrogen and progesterone concentration.

Wentzel (1986) found that blood glucose concentration increased by 60% within 48 h following flushing. According to Venter and Greyling (1994) flushing leads to a higher blood glucose concentration, which is beneficial to the reproductive performance of ewes. Another endocrinological explanation of the effect of flushing is that the high level of nutrition contributes to a greater production of insulin, encouraging the uptake of glucose and the synthesis of steroid hormones by the ovary (McDonald *et al.* 1995).

Twenty hours before CIDR removal, blood glucose concentrations showed an increase in all animals, but no differences were observed among treatments (Table 4). Forty eight hours after CIDR removal (estrus time) glucose showed small reduction, which was probably due to the increased activity of ewe and decreased feed intake around estrus. Walton and King (1986) indicated that the feed intake decreased by 15% and the magnitude of milk yield reduction is 7-35% during estrus. These results are similar to the results of Daghigh Kia *et al.* (2012). Forty eight h after CIDR removal and 10 days after mating we found that SBM and CGM groups had higher glucose levels than control group (P<0.05). Lower level of glucose in this group may be due to the lower feed intake, since this group didn't receive excess feed as flushing supplement.

Table 1 Ingredient and nutrient composition of experimental diets

Items	Treatments				
	Urea	**SBM**	**CGM**	**Barley**	**Control**
Ingredient (%)					
Alfalfa hay	27	28	28	41	36
Barley grain	15	13	13	27	13
Wheat straw	51	49	50	29	48
Molasses (sugar beet)	5	3	4	2	2
Urea	0.9	-	-	-	-
SBM	-	5.9	-	-	-
CGM	-	-	3.9	-	-
Vitamin premix	0.5	0.5	0.5	0.5	0.5
Mineral premix	0.5	0.5	0.5	0.5	0.5
Chemical composition					
Metabolizable energy (ME) (Mcal/kg)	2.08	2.12	2.11	2.19	2.07
Crude protein (CP) (%)	10.56	10.19	10.33	10.57	8.8
Crude protein (CP) (g/day)	15.36	15.01	15.12	15.37	8.8
Rumen degraded protein (RDP) (% CP)	71.51	63.22	58.29	68.17	64.03
Rumen undegradable protein (RUP) (% CP)	28.49	36.78	41.71	31.83	35.97
RUP / RDP	0.40	0.58	0.72	0.47	0.56
(ME/CP) ratio	0.20	0.21	0.20	0.21	0.24
Calcium (g/day)	5.3	5.3	5.4	5.5	5.1
Phosphorus (g/day)	2.68	2.66	2.67	2.69	2.20

Each kg of vitamin premix was as follow: vitamin A: 500000 IU; vitamin D_3: 100000 IU; vitamin E: 100 IU and Antioxidant: 400 mg.
Each kg of mineral premix was as follow: Mn: 2000 mg; Fe: 3000 mg; Zn: 3000 mg; Cu: 280 mg; I: 100 mg; Se: 1 mg; Mg: 20000 mg; Co: 100 mg; Ca: 195000 mg; P: 90000 mg and Na: 55000 mg.
SBM: soybean meal (Agro-industry Mahydasht Kermanshah, Kermanshah, Iran (42±2% CP) and CGM: corn gluten meal (Agro-industry glocossan Ghazvin. Ghazvin, Iran (64±2% CP).

Table 2 Effects of flushing treatment on offspring number and fertility rate[1]

Items	Urea	SBM	CGM	Barley	Control
Total ewes	12	12	12	12	12
Lambing ewes	10	11	12	11	9
Total offspring	10	13	16	12	9
Fertility (%)	83.3	91.66	100	91.66	75
Lambing rates (%)	100	118	133	109.09	100
Twining (%)[2]	0	16.6	33.3	8.3	0

SBM: soybean meal and CGM: corn gluten meal.
[1] Fertility rate: the number of parturitions ewes in experimental period /number of total ewes in experimental period.
[2] Twining rate: lambing's producing twins / total of lambing's.

Table 3 F-value of serum hormones and metabolites concentrations during reproductive cycle (CIDR)

Measuring time	Insulin	Estradiol	Progesterone	Glucose	Serum protein	BUN	Cholesterol
Start[1]	0.27^{ns}	0.23^{ns}	0.28^{ns}	1.06^{ns}	0.98^{ns}	1.21^{ns}	0.38^{ns}
24 (h) before[2]	2.8^{*}	0.15^{ns}	1.25^{ns}	1.95^{ns}	4.53^{**}	3.5^{**}	2.89^{*}
48 (h) after[2]	2.64^{*}	0.22^{ns}	1.73^{ns}	4.92^{**}	6.56^{**}	9.25^{**}	2.14^{ns}
10 d after mating	2.87^{*}	0.35^{ns}	0.42^{ns}	5.37^{**}	4.19^{**}	3.01^{**}	3.69^{*}

[1] CIDR insertion time.
[2] CIDR removal.
BUN: blood urea nitrogen.
* ($P<0.05$) and ** ($P<0.01$).
NS: non significant.

Blood glucose can play an important role in increasing concentrations of gonadotropins, follicle growth and its development and, ultimately, increasing ovulation rate due to its importance as fuel during proestrus, estrus and implantation periods.

In ruminants, dietary carbohydrates are fermented to short chain volatile fatty acids in the rumen and often less than 10% of the glucose requirement is absorbed from the digestive tract.

Thus, gluconeogenesis must provide up to 90% of the necessary glucose in ruminants (Young, 1977). Because of the high and moderate bypass protein content of CGM and SBM, respectively, they may be able to supply more amino acids in the intestine which can be incorporated in gluconeogenesis and increased blood glucose levels. These results are in agreement with the results of Downing *et al.* (1995), Jahani-Moghadam *et al.* (2009), Kuhara *et al.* (1991) and Molle *et al.* (1995).

Flushing rations in the present study had the same CP content; however, their RUP/RDP ratio was different. There were no significant differences in glucose levels among flushing treatments throughout 3 initial weeks of flushing period until estrous stage (i.e. 48 hrs after CIDR removal) (Table 4). These results were consistent with Amanlou *et al.* (2011). They reported that any changes in the RUP/RDP ratio had no significant effect on blood glucose concentration of Afshari ewes. Applying urea as a nitrogen source when synchronized with enough fermentable carbohydrate can increase microbial protein synthesis. When the increased protein is passed to the lower part of the gut, it may enhance glucose level through gluconeogenesis. Probably higher concentration of BUN and increasing energy consumption required for ureagenesis results in reduced fertility rate and failed pregnancy incidence in the current research. Putnam *et al.* (1999) suggested that some of the changes in glucose metabolism may be related to the energy costs associated with urea synthesis. The energy cost associated with urea synthesis has been estimated at 4 ATP/mol of produced urea (Kelly *et al.* 1993) which is mainly supplied with glucose or glycogenic substrates. In our study, urea supplemented group had more fermentable carbohydrate than SBM and CGM groups because of higher barley grain and molasses which were be incorporated for the propionate production and consequently gluconeogenesis. However, probably because of the lowest RUP/RDP ratio in urea group (Table 1) and the highest levels of BUN (Table 6), some part of glucose have been used in urea metabolism. These results are consistent with the results of Madibela (1995).

Young (1977) mentioned that propionyl-CoA synthetase activity in sheep rumen mucosa increased with pregnancy. He used pregnant and lactating ewes and found that total glucose-6-phosphatase decreased during pregnancy and increased during lactation period in proportion to liver hypertrophy. However, some of the researchers have found different results. Studies of Rusche *et al.* (1993) in beef cows and Milis *et al.* (2005) in sheep showed that different sources of rumen undegradable protein decreased the blood glucose and did not affect the hormones and reproductive status. Fluctuations of serum insulin during the reproductive cycle of experimental animals are shown in Table 5. Insulin levels were the same in all treatments at the beginning of the experiment. Twenty four hours before mating, a drastic increase was observed in all of the groups except for the control group ($P<0.05$). CGM supplemented animals had the highest average insulin level in their blood during the reproductive cycle and its insulin level was almost 50% higher than the others ($P<0.05$). CGM group had higher insulin level than control in each sampling time as well ($P<0.05$). Using different sources of nitrogen in the rations elevated insulin levels obviously at the proestrus stage in all of the groups, however only the CGM showed significant increase comparing the control group ($P<0.05$) (Table 5).

McDonald *et al.* (1995) suggested an endocrinological explanation of the effect of flushing. According to them, high level of nutrition leads to higher percentages of insulin production, which encourages the uptake of glucose and the synthesis of steroid hormones by the ovary. Kaur and Arora (1995) showed that increase in dietary protein can raise insulin levels. Increased amount of dietary protein, especially bypass protein can increase protein and amino acids flow to duodenum. These amino acids can incorporate in the gluconeogenesis and enhance blood glucose and consequently insulin (Downing *et al.* 1995; Landau *et al.* 1996).

Danfaer *et al.* (1995) suggested that amino acids can potentially account for up to 20% of glucose synthesis (range 2 to 40%). Reilly and Ford (1971) also found strong correlations between daily protein intake and total glucose production rates and between the rate of glucose production from amino acids and the entry rate of amino acids in sheep.

Investigation conducted by Usami *et al.* (1982) in rat demonstrated that increased dietary protein will increase blood insulin levels by several possible mechanisms, such as increasing the protein absorption in the intestine and increased glucose uptake and absorption of arginine stimulates insulin secretion. Increasing dietary protein or amino acid increases the insulin response to glucose. This may be due to the increased susceptibility of pancreatic beta cells to arginine due to higher dietary protein. In adult ewes, insulin will be in the highest level during estrus (Landau *et al.* 1996) which can increase ovulation rates but in the young ewes it has no significant effect on ovulation rate (Beam and Holcombe, 1992), which is consistent with our findings. CGM Group showed the highest mean of insulin during the flushing period (Table 5). Higher BCAAs content of CGM can explain this change. Layman (2003) demonstrated that corn has a high amount of BCAAs, especially leucine, which plays an important role in body metabolism. Leucine is known to be an enhancer of insulin sensitivity (Garlick, 2005). Higher amounts of insulin may contribute to a higher reproductive efficiency. According to our results, the fertility percentage, lambing rate and twining in CGM treated group increased considerably owing to the highest mean of insulin level. Van Houten *et al.* (1979) have reported that insulin receptors are located in arcuate nucleus and the medial basal of hypothalamus (a region of the brain containing neurons of gonadotropin-releasing hormone). A study on sheep and rabbits revealed that there is a special need for insulin to keep normal pulses of luteinizing hormone (LH) and stimulating LH surge (Kirchick *et al.* 1982).

Table 4 Effects of flushing treatment on serum glucose concentrations (mg/dL) before and after concentrations during reproductive cycle (CIDR) removal

Treatment	Start time[1]	24 (h) before[2]	48 (h) after[2]	10 d after mating	Mean
Urea	35.76	42.34	40.65^{ab}	41.1^{ab}	41.36^{b}
SBM	36.1	47.27	45.61^{a}	47.18^{a}	46.69^{a}
CGM	31.24	43.32	43.59^{a}	44.37^{a}	43.76^{ab}
Barley	32.14	42.38	39.11^{ab}	40.13^{ab}	41.40^{b}
Control	33.41	40.27	33.24^{b}	34.53^{b}	37.54^{c}
SEM	2.13	1.93	1.96	2.25	1.30
CV	20.06	13.66	15.1	17.35	-
P-value	0.39	0.12	< 0.001	0.001	< 0.001

[1] CIDR insertion time.
[2] CIDR removal.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SBM: soybean meal and CGM: corn gluten meal.
SEM: standard error of the means.
CV: coefficient of variation.

Table 5 Effects of flushing treatment on serum insulin concentrations (IU/mL) before and after concentrations during reproductive cycle (CIDR) removal

Treatment	Start time[1]	24 (h) before[2]	48 (h) after[2]	10 d after mating	Mean
Urea	25.87	39.12^{ab}	33.25^{ab}	24.50^{b}	32.29^{b}
SBM	31.25	39.37^{ab}	37.87^{ab}	29.12^{b}	35.45^{b}
CGM	23.75	51.25^{a}	52.50^{a}	41.25^{a}	48.33^{a}
Barley	28.12	37.90^{ab}	39.25^{ab}	27.00^{b}	34.72^{b}
Control	29.50	22.75^{b}	26.75^{b}	23.75^{b}	24.67^{c}
SEM	2.05	2.78	2.89	2.21	2.07
CV	33.19	32.65	34.14	33.97	-
P-value	0.083	0.011	0.047	0.059	< 0.001

[1] CIDR insertion time.
[2] CIDR removal.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SBM: soybean meal and CGM: corn gluten meal.
SEM: standard error of the means.
CV: coefficient of variation.

Experiments carried out on sheep have shown that dietary treatments which increase blood glucose levels, subsequently will increase insulin, which can increase release of gonadotropin-releasing hormone (GnRH) (Miller *et al.* 1998); leading to high levels of ovulation rate.

However some researchers suggested there is no consistent relationship existing between blood glucose and plasma insulin levels in ruminants (Horino *et al.* 1968). Other studies on sheep and cattle reported fluctuant results in this area (Block *et al.* 2001). The reason for different data obtained from different studies is not clear, but may probably be due to applying different types of feed, energy levels in feed or feeding frequency, or differences in the physiological states of animals used in studies. According to Udum *et al.* (2008) it seems unlikely that blood glucose concentrations could be totally responsible for the changes in insulin secretion after feeding. It should also be considered that in ruminants, glucose is constantly synthesized from volatile fatty acids (VFAs), the main energy source in the liver, and fluctuations in circulating glucose is small. Treatment barley had the highest cholesterol levels among the experimental groups (P<0.05) (Table 6). Receiving the highest amount of energy could be attributed to the high amount of cholesterol.

The amount of received calories in this group was about 5% higher than the average of the other groups, (2.19 *vs.* 2.10 Mcal/kg) (Table 1), which can lead to increased cholesterol synthesis. On the other hand, the presence of higher amount of barley grain in barley group can increase cholesterol level. Barley grain can produce high amounts of volatile fatty acids (VFA) because of high fermentation rate and the volatile fatty acids are mainly acetate, propionate and butyrate. Acetyl CoA as the main precursor of cholesterol synthesis can be produced from acetate, so it can be hypothesized that higher ruminal fermentation of barley grain and consequently higher levels of produced acetate can increase cholesterol production in this group.

Serum protein and BUN concentrations of experimental animal groups are shown in Table 7. Both serum protein and BUN levels are affected by the flushing rations (P<0.05). In the present study, the flushing rations had the same CP content; however they were different in RUP/RDP ratio (Table 1). CGM group had the highest average serum protein among all treatments during the experimental period (P<0.05; Table 7). Twenty four hrs before and 48 hrs and 10 days after CIDR removal, this group with 11.2, 11.46 and 9.99 g/dL had the highest serum protein concentration, respectively (P<0.05).

Table 6 Effects of flushing treatment on serum cholesterol concentrations (IU/mL) before and after concentrations during reproductive cycle (CIDR) removal

Treatment	Start time[1]	24 h before[2]	48 h after[2]	10 day after mating	Total cholesterol
Urea	50.5	56.3^{ab}	61.5	58.3^{ab}	58.7^{b}
SBM	53.2	49.9^{ab}	64.8	51.7^{ab}	55.47^{bc}
CGM	23.75	51.3^{ab}	62.4	61.7^{ab}	58.47^{b}
Barley	53.8	66.6^{a}	69.3	63.1^{a}	66.33^{a}
Control	55.5	48.3^{b}	55.8	50.0^{b}	51.37^{c}
SEM	2.93	4.35	3.37	3.06	2.49
CV	17.44	25.3	16.99	16.97	-
P-value	0.065	< 0.001	0.023	0.012	0.001

[1] CIDR insertion time.
[2] CIDR removal.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SBM: soybean meal and CGM: corn gluten meal.
SEM: standard error of the means.
CV: coefficient of variation.

Table 7 Effects of flushing treatment on serum protein and BUN concentrations (g/dL) before and after concentrations during reproductive cycle (CIDR) removal

Factor	Treatment	Start time[1]	24 (h) before[2]	48 (h) after[2]	10 d after mating	Mean
Protein	Urea	9.19	9.48^{b}	9.12^{bc}	8.77^{bc}	9.12^{bc}
	SBM	8.13	9.96^{ab}	9.67^{b}	9.41^{ab}	9.88^{b}
	CGM	8.4	11.2^{a}	11.46^{a}	9.99^{a}	10.26^{a}
	Barley	8.21	8.86^{bc}	9.37^{bc}	8.75^{bc}	8.99^{c}
	Control	8.17	7.79^{c}	8.13^{c}	7.88^{c}	7.93^{d}
	SEM	0.20	0.43	0.36	0.29	0.33
	CV	16.85	13.54	11.44	9.77	
	P-value	0.430	< 0.0001	< 0.0001	< 0.0001	< 0.0001
BUN	Urea	14.99	27.12^{a}	28.49^{ab}	24.96^{a}	26.86^{a}
	SBM	15.99	23.35^{ab}	30.73^{a}	25.37^{a}	26.49^{a}
	CGM	15.08	20.54^{b}	25.39^{bc}	20.58^{ab}	22.16^{b}
	Barley	14.88	21.80^{b}	20.94^{dc}	22.05^{ab}	21.60^{bc}
	Control	17.31	18.91^{b}	20.06^{d}	19.79^{b}	19.59^{c}
	SEM	0.47	0.62	0.79	0.64	0.87
	CV	21.08	19.74	22.17	19.96	-
	P-value	0.435	< 0.0001	< 0.0001	0.0084	< 0.0001

[1] CIDR insertion time.
[2] CIDR removal.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SBM: soybean meal and CGM: corn gluten meal.
SEM: standard error of the means.
CV: coefficient of variation.

Increased serum protein can directly or indirectly increase secretion of GnRH and LH during the metabolism through neurotransmitters. It can also affect ovarian activity directly (Sabra and Hasan, 2008). Serum protein improves fertility rate and offspring number (Daghigh Kia *et al.* 2012). Jahani-Moghadam *et al.* (2009) reported that increased RUP/RDP ratio of diet causes a linear increase in serum protein which can improve fertility.

Urea and SBM groups had the highest mean of BUN among the experimental treatments (P<0.05) (Table 7). Higher content of BUN in the urea consuming animals, which used urea as main protein source in their ration was not much surprising, however, higher content of BUN in the SBM group, was unexpected. Synchronization of dietary protein with carbohydrate in the rumen is one of the strategies which can control BUN level as well as maximizing microbial protein synthesis.

Both of the urea and SBM supplemented rations received barley grain and molasses as fermentable carbohydrate sources (15 and 5 *vs.* 13 and 3 percent respectively, Table 1). It seems that fermentable carbohydrate in the SBM group was not enough for optimum bacterial growth and resulted in elevated BUN content in this group.

No significant effects were observed in the levels and fluctuations of progesterone and estrogen hormones of experimental treatments (Figure 1 a, b). The levels of progesterone decreased following the CIDR removal (14th day of the flushing), however, the levels of estrogen increased.

CIDR was the main source of progesterone in the experimental animals and withdrawing it on day 14th resulted in an obvious decrease in the progesterone levels of all animal groups (Figure 1a). On the other hand following CIDR removal, the level of estrogen was drastically increased due to the growth of follicles (Figure 1b).

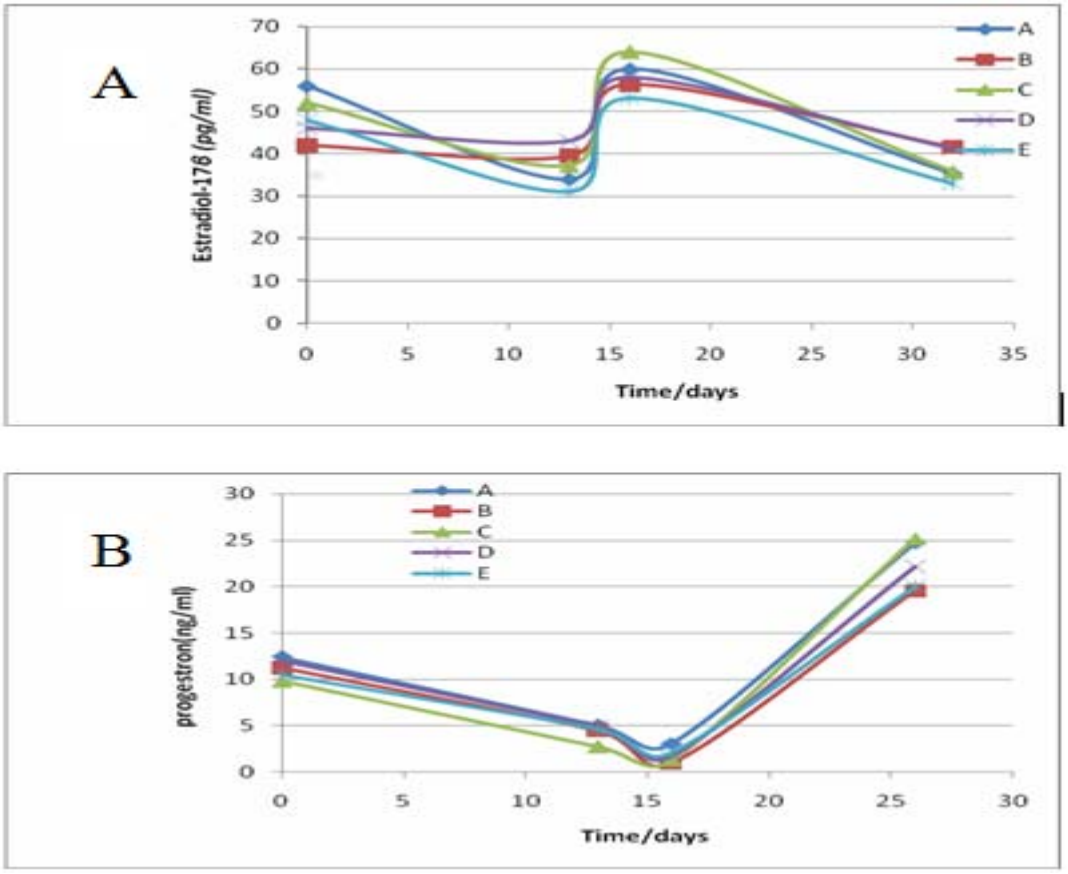

Figure 1 a: serum estrogen
b: progestron concentrations from CIDR removal to 10 days after mating (14 days after CIDR removal)
A: urea; B: SBM; C: CGM; D: barley grain and E: control group

Such fluctuations of progesterone and estrogen are a normal phenomenon in the estrous cycle. Commonly higher amount of estrogen can be an indicator of high numbers of growing follicles, leading to increased reproductive performance. Scaramuzzi *et al.* (2006) reported that the nutritional flushing alters the blood concentrations of some reproductive hormones using the short-term model: a transient increase in follicle-stimulating hormone (FSH) and a decrease in estradiol concentrations in the blood. Nutrition influences folliculogenesis in the ovary.

We did not find any significant differences for estrogen levels among the experimental treatments; however, the group receiving CGM in their flushing diet had the highest level of estrogen which is matched with the best reproductive performance in this group (Figure 1b, Table 2). The progesterone production from corpus luteum gradually increases after ovulation to support embryo survival. CGM supplemented group showed the highest (not significant) level of progesterone among experimental groups as well. It has been reported that insulin causes an increased level of progesterone and FSH in cultured luteal anterior pituitary cells (Adashi *et al.* 1981).

Higher levels of progesterone causes an improvement in fertility, maintaining fetus and increasing the lambing rate.

CONCLUSION

The results of the present study indicate that the reproductive efficiency, especially twinning rate were improved using flushing ration in Ghezel sheep flocks. The presence of corn gluten meal in the flushing diet prior to mating, increased insulin and probably certain amino acids improving ovarian function, follicular growth and ovulation rate which would result in an improved fertility rate, increased number of offspring and twinning birth. Group receiving CGM with 16 and control group with 9 lambs showed the highest and lowest numbers of offspring, respectively. CGM supplemented group had the highest level of insulin and offspring number as well. It can be concluded that insulin rise in the flushing period especially around ovulation might be an efficient strategy to improve reproductive performance.

ACKNOWLEDGEMENT

The authors thank Agro-Industry Glucosan Company (Qazvin, Iran) for providing corn gluten meal supplement.

REFERENCES

Adashi E.Y., Hsueh A.J. and Yen S.S. (1981). Insulin enhancement of luteinizing hormone and follicle stimulating hormone release by cultured pituitary cells. *Endocrinology*. **108,** 1441-1449.

Al-Haboby A.H., Salman A.D. and Abdul Kareem T.A. (1999). Influence of protein supplementation on reproductive traits of Awassi sheep grazing cereal stubble. *Small Rumin. Res.* **34,** 33-40.

Amanlou H., Karimi A., Mahjoubi E. and Milis C. (2011). Effects of supplementation with digestible undegradable protein in late pregnancy on ewe colostrums production and lamb output to weaning. *J. Anim. Physiol. Anim. Nutr*. **95,** 616-622.

Beam S.W. and Holcombe D.W. (1992). Effects of insulin administration during follicular growth serum glucose and hormone profiles in ewe-lambs. *Canadian J. Anim. Sci.* **72,** 421-426.

Daghigh Kia H., Mohamadi Chapdareh W., HosseinKhani A., Moghaddam G., Rashid A., Sadri H. and Alijani S. (2012). Effects of flushing and hormonal treatment on reproductive performance of Iranian Markhoz goats. *J. Anim. Physiol. Anim. Nutr.* **96,** 1157-1164.

Danfaer A., Tetens V. and Agergaard N. (1995). Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **111,** 201-110.

Downing J.A., Joss J. and Scaramuzzi R.J. (1995). A mixture of the branched chain amino acids leucine, isoleucine and valine increases ovulation rate in ewes when infused during the luteal phase of the oestrous cycle: an effect that may be mediated by insulin. *J. Endocrinol*. **145,** 315-323.

Ferguson J.D., Blanchard T.L., Shotzberger S. and Chalupa W. (1986). Effect of rumen degradable protein on infertility. *J. Dairy Sci.* **69(1),** 121-130.

Garlick P.J. (2005). The role of leucine in the regulation of protein metabolism. *J. Nutr.* **135,** 1553-1556.

Hinch G.N. and Roelofs J.H.W. (1986). Lupin feeding and insulin infusion during late luteal phase can increase ovulation rate in sheep. *Proc. Australian Soc. Reprod. Biol*. **18,** 43-50.

Horino M., Machlin L.J., Hertelendy F. and Kipnis D.M. (1968). Effect of short-chain fatty acids on plasma insulin in ruminant and nonruminant species. *Endocrinolology.* **83,** 118-128.

Jahani-Moghadam M., Amanlou H. and Nikkhah A. (2009). Metabolic and productive response to ruminal protein degradability in early lactation cows fed untreated or xylose-treated soybean meal-based diets. *J. Anim. Physiol. Anim. Nutr.* **93,** 777-786.

Kaim M., Folman Y., Neumark H. and Kaufmann W. (1983). The effect of protein intake and lactation number on postpartum body weight loss and reproductive performance of dairy cows. *Anim. Prod*. **37,** 229-235.

Kaur H. and Arora S.P. (1995). Dietary effects on ruminant livestock reproduction with particular reference to protein. *Nutr. Res. Rev*. **8,** 121-136.

Kelly J.M., Park H., Summers M. and Milligan L.P. (1993). Interactions between protein and energy metabolism. Pp. 341-362 in Quantitative Aspects of Ruminant Digestion and Metabolism. J.M. Forbes and J. France, Eds. CAB Int., Oxford, UK.

Kirchick H.J., Keyes P.L. and Frye B.E. (1982). Restoration of the LH surge and ovulation by insulin in alloxan-diabetic immature rats treated with pregnant mare's serum gonadotrophin. *Acta Endocrinol.* **100,** 266-273.

Kuhara T., Ikeda S., Aohneda A. and Sasaki Y. (1991). Effects of intravenous infusion of 17 amino acids on the secretion of GH, glucagon and insulin in sheep. *Am. J. Physiol.* **260,** 21-26.

Landau S., Houghton J.A., Mawhinney J.R. and Inskeep E.K. (1996). Protein sources affect follicular dynamics in ewes near the onset of the breeding season. *Reprod. Fertil. Dev.* **8,** 1021-1028.

Layman D.K. (2003). The role of leucine in weight loss diets and glucose homeostasis. *J. Nutr.* **133,** 261-267.

Madibela O.R., McEvoy T.G., Robinson J.J., Findlay P.A., Aitken R.P. and Robertson I.S. (1995). Excess rumen degradable protein influences the rate of development and glucose metabolism of fertilized sheep ova. *Anim. Sci*. **60,** 536-537.

Marais W.J. (2011). The influence of flush feeding with different nitrogen sources on ovulation and conception rates in Dohne-Merino ewes. MS Thesis. University of Pretoria. South Africa.

Mcdonald P., Edwards R.A., Greenhalgh J.F.D. and Morgan C.A. (1995). Animal Nutrition. Longman, Harlow, UK.

Merchen N.R. and Titgemeyer E.C. (1992). Manipulation of Amino Acid supply to the growing ruminant. *J. Anim. Sci.* **70,** 3238-3247.

Milis C., Liamadis D., Roubies N., Christodoulou V. and Glouseljiannis A. (2005). Comparison of corn gluten products and a soybean-bran mixture as sources of protein for lactating Chios ewes. *Small Rumin. Res*. **58,** 237-244.

Miller D.W., Blache D., Boukhliq R., Curlewis J.D. and Martin G.B. (1998). Central metabolic messengers and the effects of nutrition on gonadotrophin secretion in sheep. *Reproduction.* **112,** 347-356.

Molle G., Branca S., Ligios S., Sitzia M., Casu S., Landau S. and Zoref Z. (1995). Effect of grazing background and flushing supplementation on reproductive performance in Sarda ewes. *Small Rumin. Res*. **17,** 245-254.

NRC. (1985). Nutrient Requirements of Sheep. 6th Ed. National Academy Press, Washington, DC, USA.

NRC. (2001). Nutrient Requirements of Dairy Cattle. 7th Ed. National Academy Press, Washington, DC, USA.

Ocak N., Cam M.A. and Kuran M. (2006). The influence of pre- and post-mating protein supplementation on reproduction performance in ewes maintained on rangeland. *Small Rumin. Rese*. **64,** 16-21.

Putnam D.E., Varga G.A. and Green M.H. (1999). Glucose kinetic responses to protein supplementation and exogenous somatotropin in late gestation dairy cows. *J. Dairy Sci.* **82,** 1274-1281.

Rattray P.V. (1982). Feeding management for flushing: don't miss

profitable opportunities. *New Zealand J. Agric*. **144,** 30-32.

Rhind S.M. (1992). Nutrition: its effects on reproductive performance and its hormonal control in female sheep and goats. Pp. 10-13 in Progress in Sheep and Goat Research. A.W. Speedy, Ed. CAB International, Wallingford, UK.

Reilly P.E.B. and Ford E.J.H. (1971). The effects of different dietary contents of protein on amino acid and glucose production and on the contribution of amino acids to gluconeogenesis in sheep. *Br. J. Nutr.* **26,** 249-263.

Robinson J.J., Ashworth C.J., Rooke J.A., Mitchell L.M. and McEvoy T.G. (2006). Nutrition and fertility in ruminant livestock. *Anim. Feed Sci. Technol.* **126,** 259-276.

Rusche W.C., Cochran R.C., Corah L.R., Stevenson J.S., Harmon D.L., Brandt R.T. and Minton J.E. (1993). Influence of source and amount of dietary protein on performance, blood metabolites, and reproductive function of primiparous beef cows. *J. Anim. Sci.* **71,** 557-563.

Sabra H.A. and Hasan S.G. (2008). Effect of new regime of nutritional flushing on reproductive performances of Egyptian Barki ewes. *Global Vet*. **2,** 28-31.

SAS Institute. (2003). SAS®/STAT Software, Release 9.1. SAS Institute, Inc., Cary, NC. USA.

Scaramuzzi R.J., Campbell B.K., Downing J.A., Kendall N.R., Khalid M., Muñoz-Gutiérrez M. and Somchit A. (2006). A review on the effects of supplementary nutrition in the ewe on the concentration of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. *Reprod. Nutr. Dev.* **46,** 339-354.

Tagari H., Arieli A., Mabjeesh S., Bruckental I., Zamwell S. and Aharoni Y. (1995). Assessment of duodenal amino acid profile in dairy cows by the *in situ* method. *Livest. Prod. Sci*. **42,** 13-22.

Thomas G.B., Oldham C.M. and Martin G.B. (1984). Frequency of luteinizing hormone release in Merino ewes with one or two ovulations. Pp. 41-57 in Reproduction in Sheep. D.R. Lindsy and D.T. Pearce, Eds. Australian wool corporation, Canberra.

Udum D.C., Cetin M., Balc F., Gunes N. and Hecer C. (2008). Effects of plasma insulin, glucose and NEFA concentrations of feeding frequency during long term in lambs. *J. Biol. Environ. Sci*. **2,** 45-51.

Usami M., Seino Y., Seino S., Takemura J., Nakahara H., Ikeda M. and Imura H. (1982). Effect of high protein diet on insulin and glucagon secretion in normal rats. *J. Nutr.* **112,** 681-685.

Van Houten M., Posner B.I., Kopriwa B.M. and Brawer J.R. (1979). Insulin binding sites in the rat brain: *in vivo* localization to the circumventricular organs by quantitative radioautography. *Endocrinology.* **105,** 666-673.

Venter J.L. and Greyling J.P.C. (1994). Effect of different periods of flushing and synchronized mating on body weight, blood glucose and reproductive performance in spring-mated ewes. *Small Rumin. Res.* **13,** 257-261.

Walton J.S. and King G.J. (1986). Indicators of estrus in Holstein cows housed in tie stalls. *J. Dairy Sci.* **69,** 2966-2973.

Wentzel D. (1986). Improved small stock production- collaboration between scientist and producer. *South African J. Anim. Sci*. **16,** 123-126.

Young J.W. (1977). Gluconeogenesis in cattle: significance and methodology. *J. Dairy Sci*. **60,** 1-15.