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Full Length Research Paper

Effects of restricted nutrition on biochemical parameters of liver function in pregnant Ghezel ewes

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This study was conducted in order to determine the effects of low energy diets (that is food restriction) on the liver function of Gezel ewes in late pregnancy. Twenty-four clinically intact ewes with an average weight of 50±3 kg used for the study. Twenty one pregnant ewes (clinically intact ewes with an average weight of 50±3 kg) were divided into 3 groups (Treatments I, II and III). The ewes in Treatment 1 (control) were fed rations containing 2. 4 MCal ME/kg of dry matter [(DM)-11.2% crude protein (CP)], the ewes in Treatment 2 (mild feed restriction) were fed rations containing 2 MCal ME/kg of DM-11% CP and the ewes in Treatment 3 (sever feed restriction) were fed rations containing 1.74 MCal ME/kg of DM-4. 8% CP. Serum concentration of glucose, triglyceride, cholesterol, albumin, total protein, bilerubin, total bilerubin, beta-hydroxy butyrate (BHB), non-esterified fatty acids (NEFA), AST and GGT concentration were measured on days 130 and 145 of pregnancy. No significant differences for glucose, albumin, total protein and GGT were detected among the groups on days 130 and 145 of pregnancy. The results showed that feed restriction statistically increased (p<0.05) serum triglyceride, total bilerubin, BHB, NEFA and decreased (*p*<0.05) serum cholesterol concentration in ewes fed sever restricted ration during late pregnancy. Feed restriction resulted in mild to moderate ketosis; however the Gezel_ewes revealed an excellent ability to resist against mild and sever feed restriction by compensatory mechanism.

Key words: Liver function, feed restriction, Gezel ewes, biochemical parameters, pregnancy.

INTRODUCTION

Forage is the major and cheaper ingredient of sheep diet. So, the usual method of feeding in sheep rearing systems in Iran especially when concentration ingredients are expensive is naturally grazing on pasture. Practically, it is impossible to meet metabolizable energy requirements of 3.6 Mcal/day DMI for maintenance and pregnancy in a given sheep herd. National Research Centre recommends (NRC, 1985) energy requirements of pregnant ewes should be increased by 20%. Though pasture grazing results in a feed restriction state and negative energy and nitrogen balance especially in late pregnancy period. Sheep have a seasonally oesterus cycle and winter is the lambing season in our province. Naturally, the quality of food decreases in winter. Then a sheep with a negative energy and protein balance should graze on a poor pasture (or eat low quality hay) and tolerate different levels of food restriction. An early embryo makes very little quantitative demands on the energy balance of the mother, but it may nevertheless influence the metabolic responses of the mother to nutritional signals at key moments of early pregnancy. Metabolic profiles have been used to predict prepartum and postpartum metabolic problems, and for the diagnosis of metabolic diseases and the assessment of the nutritional status of animals. So, for example, blood glucose is the major metabolite used by the sheep fetus and the energy

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Table 1. Feed ingredients of diets fed to pregnant ewes in different treatments.

	Treatment $_1$ [1] (%)	Treatment $_2$ [2] (%)	Treatment $_3$ [3] (%)
Alfalfa hay	42.8	66.6	-
Barley straw	22.2	23.4	100
Corn grain	14.3	–	–
Barley grain	14.4	–	–
Wheat bran	3.5	–	–
Soybean meal	1.8	–	–
Vitamin-mineral premix [4]	0.4	–	–
Lime stone	0.2	–	–
Die calcium phosphate	0.2	–	–
With Salt	0.2	–	–
Dry Matter intake (kg)	1.5	1.5	1.5

[1] Normal ration, [2] Mild feed restriction, [3] Sever feed restriction. [4]Vitamin-mineral premix provides (per kg of concentrate): 11,000 mg Cu, 10 mg Co, 100 mg I, 350 mg Fe, 800 mg Mn, 600 mg Zn, 8 mg Se and 2,000,000 IU vitamin A, 200,000 IU vitamin D3, 2,200 mg vitamin E and 15,000 mg antioxidant.

requirements of the ewe increase during late pregnancy due to rapid growth of the fetus (Fırat and O¨ zpınar, 2002).

Feed restriction could induce pregnancy toxemia of inadequately fed single bearing ewes (Smith, 1996). The determination of changes in the metabolism of such sheep in underfeeding status during pregnancy could help producers to predict abnormal metabolic states, and the prediction of some metabolic disorders, such as pregnancy toxaemia and fatty liver. In this study, we induced mild and higher level of food restriction similar to what commonly happen in sheep rearing systems of Iran. The question is 'how mild and sever food restriction in pregnant Ghezel ewes (especially in the last 8 weeks of pregnancy) might affect on biochemical parameters of liver function in? So the purpose of the present work was to study clinical signs and some biochemical blood constituents in Gezel pregnant ewes in late pregnancy in undernutrition conditions.

MATERIALS AND METHODS

Animals and oestrus synchronization

The study was carried out in Tabriz (east Azerbaijan province of Iran). Winter is the reproduction season in this region (around January). The oesterus synchronization was in March that was out of reproduction season. Ewes had not an access to pasture and we used manual nutrition system. Thirty Iranian Ghezel ewes with an average weight of 51±6 kg were selected for this study. Prior to synchronized for oestrus and in order to boost ovulation, conception and embryo implantation rates, the ewes flushed for 5 weeks. They were fed a ration of 2.4 Mcal ME/Kg of dry matter (DM) and 11% crude protein (CP) (1.5 Kg/day per head) in 3 weeks before and 2 weeks after mating (NRC, 1985). One week after the flushing diet, an EAZI-BREED CIDR (containing 0.3 g progesterone) placed in vagina. According to recommendation of the commercial company in 14th day, CIDRs expelled and 500 IU PMSG (Intervet, Poland) injected intra-muscularly. Two rams introduced to herd for natural conception. After a 4 weeks period, 14 pregnant ewes of close body condition score were chosen and were used up to the end of study.

Experimental diets

After flushing period, all the ewes were fed a ration of 2.12 Mcal ME/kg of dry matter (DM) and 8.9% crude protein (CP) (1400 g/day per head) in the first 12 weeks of gestation. On day 100 after mating, the ewes were examined by ultrasound for pregnancy and 21 single bearing pregnant ewes were selected and were randomly assigned into Treatments 1, 2 and 3 (7 ewes each). Treatment 1 received a normal diet until their parturition but Treatments 2 and 3 fed a restricted ration (Table 1). Eight randomly selected ewes were assigned to each group and held under the same environmental conditions. All animals fed twice a day (6:00 am and 03:00 pm) according to Table 1. Drinking water and salt and Vitamin-mineral lick provided *ad libitum*. All the rations were formulated according to NRC (1985). The feeds given to each group were analyzed for CP and ME (Table 2). Chemical analyses of the ration performed as AOAC (1995) methods.

Blood sampling

Blood samples were taken from the jugular veins of the ewes on days 130 and 145 of gestation. The samples were collected by jugular venipuncture in tube without anticoagulant. Tubes for serum collection allowed clotting at room temperature for 2 h. Then centrifuged at 2000 g for 20 min. The supernatant serum was frozen at –70℃ until analyed into vacuumed-tubes c ontaining heparin.

Analytical procedures

All biochemical parameters measured on UV/VIS spectrophotometer (Alycon 300). Serum BHB and NEFA were measured using standardized kits supplied by Randox Laboratories Ltd. Serum glucose was measured using commercial kit (Pars

Table 2. Chemical analyses of diets fed to ewes in different treatment.

Variable	Treatment $_1$ [1] (%)	Treatment $_2$ [2] (%)	Treatment $_3$ [3] (%)
Dry matter (%)	88.7	88.2	91.1
Crude protein (%)	11.2	11	4.8
Crude fat (%)	2.5	2.1	1.8
Crude ash (%)	7.4	8.9	7.2
Crude fibre (%)	18.7	22.1	36
Calcium (%)	0.83	0.99	0.18
Phosphorus (%)	0.27	0.18	0.05
Metabolisable energy (MJ/kg DM)	2.4	2	1.74

[1] Normal ration, [2] Mild feed restriction and [3] Sever feed restriction.

Azmoon, Karaj, Iran). TAS was estimated using Randox Total Anti Oxidant Status test in serum (Randox Laboratories Ltd, United Kingdom, Cat. No. NX 2332, Lot. No.: 023195). ABTS® (2,2´-Azino-di-[3-ethylbenzthiazoline ulphate]) was incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS®. This has a relatively stable blue-green colour which is measured at 600 nm. Antioxidants in added sample cause suppression of this colour production to a degree which is proportional to their concentration.

Statistical analysis

All variables were subjected to analysis of variance using a mixed model that included the fixed effects of nutritional treatment (mild restriction, sever restriction and control), reproductive status (cyclic or pregnant), day of pregnancy and their interactions. For the analysis of repeated measurements (metabolites), the covariance structure was modeled to consider the correlation between sequential observations on the same animal (Littell et al., 2000). Data are presented as least square means±pooled standard errors. Statistical analyses were performed using the Statistical Analysis System program (SAS Institute, 2004). The level of significance was considered to be $P < 0.05$.

RESULTS

The serum glucose, cholesterol, triglyceride, total protein, albumin, globulin, total bilirubin, bilirubin, AST (Aspartate Aminotransferase); and GGT (Gamma Glutamyl Transferase) concentrations of the ewes in treatments (control, mild and sever energy restriction) on days 130 and 145 are presented in Table 3. No significant differences in glucose, total protein and, albumin, bilerubine and AST measurements were found between the treatments on days 130 and 145 of pregnancy (p>0.05). The concentration of BHB, NEFA, triglyceride and cholesterol of treatments differed significantly on days of 130 and 145 (p<0.05). The serum concentration of triglyceride of the ewes fed diet with sever energy restriction [diet energy 27.5% lower than NRC (1985)] increased while the serum concentration of cholesterol decreased compared to the ewes in control and the ewes fed diet with mild energy restriction [diet energy 17% lower than NRC (1985) recommendation] on days 130 and 145. The serum concentration of BHB of the ewes fed diet with mild and sever energy restriction was higher than the ewes in control group on days 130 and 145. On day 125, the serum NEFA levels of the ewes fed diet with mild and sever energy restriction has increased significantly ($P < 0.05$) compared to the control groups and there was not statistically difference between ewes fed mild or sever energy restricted diets. While on day 145, the serum NEFA level of the ewes in mild energy restricted group was higher than sever energy restricted and control group ($P < 0.05$).

Feed restriction had not statistically significant effect ($P > 0.05$) on serum concentration of AST and total bilerubin between ewes in different treatments on day 125. Serum total bilerunin levels of ewes in sever energy restriction were higher ($P < 0.05$) on day 145. Serum concentration of AST in the ewes fed diet with sever energy restriction increased significantly compared to the ewes in control and the ewes fed diet with mild energy restriction.

DISCUSSION

In this study, we decreased the total energy of animal's diet 16.5 and 27.5% from recommended level (NRC, 1985) in the last 8 weeks of pregnancy. However, only one ewe aborted and ceased her pregnancy in the last week of gestation. This can be mean as a relative resistance of Ghezel sheep to food restriction or could be a true result of flushing at the beginning of pregnancy. Robinson et al. (2002) stated that if nutrition before mating was good and there was a sufficient fat reserve, resistant of ewes to food restriction is more than those ewes with insufficient fat reserves. Undoubtedly, the most sensitive period for nutritional modification of placental growth with an associated effect on lamb birth weight is between 50 and 90 days of gestation, the period of rapid proliferating growth of the placenta (Robinson et al., 2002). As we exerted a food restriction from 14th week of gestation so, may be this is the reason why restricted diet

Table 3. Mean (±S.E.) blood metabolite concentrations at day 130 of pregnancy.

GGT (mmol/l)	AST (mmol/l)	D. bilerubin (mg/dl)	T. bilerubin (mg/dl)	Albumin (mg/dl)	T. protein (mg/dl)	NEFA (mmol/l)	BHB (mmol/l)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)	
37.4±7.1	112.6±24.2	0.15 ±0.07	0.55 ±0.06[b]	3.89±0.22	8.82±0.64	0.41 ±0.15[b]	0.30 ±0.11[b]	21.6 ±4.6[b]	88.1 ±17.2[a]	46.1±6.6	T_1
38.8±8.2	98.2±18.8	0.16±0.03	0.51±0.13[b]	3.82±0.32	8.24±0.78	0.78±0.30[a]	0.44 ±0.10[a]	22.6 ±4.6[b]	78.0 ±13.2[ab]	45.2±5.4	T_2
36.5 ±6.3	99.0 ±21.8	0.19±0.04	0.65 ±0.10[ab]	3.76 ±0.34	8.47 ±0.83	0.70±0.23[a]	0.48 ±0.21[a]	28.4 ±5.9[a]	74.1 ±11.3[b]	45.3 ±4.8	T_3

T_1: Control [NRC (1985) recommendation] ;T_2: Mild feed Restriction [Diet energy 16.5% lower than NRC (1985) recommendation]; T_3 Sever feed restriction [Diet energy 27.5 lower than NRC (1985) recommendation]. BHB: β-hydroxybutyrate; NEFA: Non Esterified Fatty Acids; AST: Aspartate Aminotransferase; GGT: Gamma Glutamyl Transferase. Different superscripts within column indicate significant differences (p<0.05).

Table 4. Mean (±S.E.) blood metabolite concentrations at day 145 of pregnancy.

GGT (mmol/l)	AST (mmol/l)	D. bilerubin (mg/dl)	T. bilerubin (mg/dl)	Albumin (mg/dl)	T. protein (mg/dl)	NEFA (mmol/l)	BHB (mmol/l)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)	
38.1±5.4	103[a]±17.4	0.17 ±0.06	0.53 ±0.07[b]	3.90±0.46	8.88±0.72[a]	0.43 ±0.21[c]	0.28 ±0.08[a]	23.1 ±5.9[b]	80.1 ±11.3[a]	46.9±4.7	T_1
36.9±7.2	96.5[a]±20.9	0.18±0.04	0.64±0.0.04[ab]	3.87±0.38[a]	8.64±0. 90[a]	0.86 ±0.17[a]	0.51 ±0.27[b]	23.9 ±5.9[b]	75.6±10.9[a]	43.9±5. 7	T_2
39.1 ±8.5	90.4[b]±19.5	0.22±0.05	0.68±0.11[a]	3.80 ±0.51	9.57 ±0.59[ab]	0.69±0.26[b]	0.53 ±0.16[b]	30.4 ±9.0[a]	68.1 ±15.5[b]	42.0 ±7.2	T_3

T_1: Control [NRC (1985) recommendation] ;T_2: Mild feed Restriction [Diet energy 16.5% lower than NRC (1985) recommendation]; T_3 Sever feed restriction [Diet energy 27.5 lower than NRC (1985) recommendation]. BHB: β-hydroxybutyrate; NEFA: Non Esterified Fatty Acids; AST: Aspartate Aminotransferase; GGT: Gamma Glutamyl Transferase. Different superscripts within column indicate significant differences (p<0.05).

could not affect conceptus growth and did not cease the gestation. The average serum concentration of glucose in treatments in comparison to the control group on day 130 and 145 (Tables 3 and 4) did not show statistically significant difference (P > 0.05). The overall mean concentration of serum glucose in sheep is 50 to 80 mg/dl (Kaneko et al., 2008). Mean value of serum glucose of the ewes in this study was 42.0 to 46.9 mg/dl and a little lower than those mentioned range (Tables 3 and 4).

It seems feed restriction was tolerated by the ewes and could not decrease serum concentration of glucose. Ruminants appear to be well adapted to a carbohydrate economy based on the endogenous synthesis of glucose from non-carbohydrate sources – that is gluconeogenesis (Kaneko et al., 2008). In ruminants, little glucose is absorbed from the gut, so the overwhelming bulk of glucose is synthesized (Lindsay, 1959; Otchere et al., 1974). Most (approximately 90%) of this synthesis occurs in the liver with the remainder occurring in the kidney (Bergman, 1982). The chief substrates are propionate and amino acids, with the former being most important in animals on a high-grain diet. Other precursors are branched chain volatile fatty acids (VFAs) and lactate absorbed from the rumen and glycerol released during lipolysis (Bergman, 1975). On the other hand, fetal glucose demands increase with increasing body size (Kaneko et al., 2008) and it can increase susceptibility to pregnancy toxemia. A fetus of sheep in late pregnancy utilizes about one-third to one-half of the daily glucose turnover of 100 g (Kaneko et al., 2008). However, the mean value of serum glucose did not decreased in our study. Therefore, we can conclude that during food-restricted period of pregnancy, gluconeogenesis powerfully supplies lamb and maternal requirements to glucose. Our finding is in agreement with Schlumbohm and Harmeyer (2004) findings and they believe that due to a metabolic adaptation, efficiency of hepatic gluconeogenesis from glucose precursors increases during pregnancy.

Generally, researchers believe that BHB concentration is a golden marker for diagnosis of pregnancy toxemia and/or ketosis in ewes and cattle (Kaneko et al., 2008) but there are different ideas about cut-off point of BHB. Smith et al. (1996) introduce levels higher than 1 mmol/l as a cut-off point. According to this criteria, mean value of all treatments in our study were lower than this point and could not be classified as pregnancy toxemia (albeit serum BHB concentration were a few more than 1 mmol/l in one case in T_2 and one case in T_3). Kaneko et al. (2008) proposed 0.55 ± 0.04 mmol/l as a reference value of serum BHB concentration. Considering the latter criteria feed restriction resulted in a mild to moderate Ketosis in T_2 and T_3 groups. These mentioned changes were statistically significant ($p<0.05$) in comparison to the corresponding control group. It means that feed restriction clearly increased serum BHB and caused ketosis. This rise in serum BHB is a compensatory mechanism and a reflectionary response to carbohydrate deficiency and inhibition of Kreb's cycle (Reece, 2004). In fact, following β-oxidation of ketones, some acetyl-CoA is combusted in the citric acid cycle. However, during fasting, gluconeogenesis is quite active in the liver, and much of the mitochondrial oxaloacetate is used for that purpose and is unavailable for citrate formation with acetyl-CoA; consequently, large quantities of acetyl-CoA are shunted into ketogenesis (Kaneko et al., 2008) and hyperketonemia occurs.

Acetone, acetoacetate and BHB are three main product of ketogenesis, the latter is more important in ruminants (Murray et al., 2003; Dhanotiya, 2004). A negative feedback of hyperketonemia on glucose production renders the pregnant or lactating ruminant into a vicious circle (Schlumbohm and Harmeyer, 2004). An increase in serum NEFA levels of ewes of T_2 and T_3 were recorded on days 125 and 150 of gestation compared to control group. This increase in serum NEFA may be ascribed to the fact that the deposit fat is used to generate energy for the energy restricted sheep and fetus and growth of the fetus which increases exponentially during late pregnancy. A gradual increase ($P < 0.05$) in serum triglyceride and decrease in cholesterol levels was recorded on day 130 and 145 of pregnancy for the ewes in T_2 and T_3 treatments when compared to control groups. Krajnicakova et al. (1993) reported higher cholesterol, triglyceride, HDL-cholesterol and VLDL-cholesterol concentrations during late pregnancy. This increase during late pregnancy may be due to insulin which plays a direct role in adipose tissue metabolism during pregnancy and its responsiveness is significantly reduced in ewes during late pregnancy (Jainudee and Hafez, 1994). The diminished responsiveness of the target tissue to insulin during late pregnancy predisposes the ewes to increase of cholesterol, triglyceride and lipoproteins concentrations (Nazifi et al., 2002).

The reference range of serum AST activities in sheep is reported as 60 to 280 U/L (Kaneko et al., 2008). So the activity of this enzyme in all treatments of this study was in normal range. The serum GGT activities of the ewes were not statistically significant in different treatments. It seems feed restriction could not cause liver malfunction in this study. In the present study, serum total bilirubin levels were recorded to be significantly higher in sever energy restricted pregnant sheep than control pregnant sheep on day 145. Certain researchers also recorded higher plasma bilirubin levels in pregnant sheep (Shetaewi and Ross, 1991; Fırat and O¨ zpınar, 1996). The higher plasma urea levels on 145 pregnancy compared to the control group values in this study may be due to the fact that blood bilirubin levels during pregnancy increased as a consequence of additional bilirubin derived from degradation of fetal haemoglobin or due to inadequate glucuronic acid synthesis. It is suggested that this may indicate liver damage.

Conclusion

In conclusion, undernutrition affected the metabolic profile specially serum beta-hydroxy butyrate, non-esterified fatty acids and triglyceride concentration of the Gezel ewes in late pregnancy. Feed restriction resulted in mild to moderate ketosis in the ewes although they could maintain their glucose homeostasis and end their pregnancy period by using fat reserves and gluconeogenesis. According to the observed result it could be suggested that Gezel ewes might have a genetically potential to tolerate feed restriction during late pregnancy regarding their challenged carbohydrate and lipid metabolism.

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