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Effect of Permanent Hypoinsulinemia on Appetite, Performance, Carcass Composition, Blood Metabolites and Leptin Concentrations in Lambs

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ABSTRACT : Insulin has crucial roles in energy metabolism in all mammals but has been less studied in ruminants. An experiment was conducted to investigate the effects of hypoinsulinemia induction on appetite, performance, carcass composition and blood metabolite levels in sheep. Treatments were intravenous injection of four doses of streptozotocin; 0, 25, 50 and 75 mg/kg BW named C, L, M and H, respectively. Twenty male lambs were divided into four treatment groups. Animals in group H could not continue the experiment because of abnormalities. The duration of the experiment was eight consecutive weeks, and injection was performed at the end of week 3. Feed and water intakes were measured weekly and weight changes of animals were recorded and used for calculation of other growth parameters. Blood samples were collected weekly via venipuncture at fasting and 2.5 h post-prandial and analyzed for hormones and blood metabolites. Results showed a marked hypoinsulinemia in group M with significant decrease in fasted and post-prandial insulin concentrations and also fasted leptin concentrations vs. the control group C ($p<0.05$). Group M showed significant increases in blood glucose, triglycerides, cholesterol, total protein, blood urea nitrogen and ketone body levels vs. group C ($p<0.05$). After injection, animals in group M showed diabetic hyperphagia and enhanced water intake as compared to group C ($p<0.05$) but, despite increased feed intake, they did not gain more weight than controls ($p<0.05$), and consequently, their feed conversion ratio was greater. Protein and fat contents of meat and liver were not significantly different among groups ($p>0.05$). In conclusion, the results suggested a regulatory role of insulin in energy metabolism of ruminants by exerting two opposing effects; central catabolic and peripheral anabolic. (**Key Words :** Hypoinsulinemia, Leptin, Appetite, Performance, Carcass, Lamb)

INTRODUCTION

Appetite is one of the most important aspects of energy homeostasis that is influenced by a variety of internal and external factors. On the basis of the kind of effect, internal factors are divided into two major series of signals; orexigenic signals, such as neuropeptide Y (NPY), agouti gene-related peptide (AgRP), ghrelin and orexin, that stimulate appetite, while anorexigenic signals, such as insulin, leptin, Pro-opiomelanocortin, inhibit appetite and consequently the resultant interaction determines appetite (Schwartz et al., 2000; Gerozissis, 2004; Broberger, 2005).

Insulin and leptin are two potent anorexigenic signals that agonistically suppress appetite. In the periphery, both of them are secreted in proportion to body fat mass (Niswender et al., 2004; Shan et al., 2008) and enter into the brain as adiposity signals (Woods and Porte, 1977; Niswender et al., 2004). In the brain, especially in the arcuate nucleus of the hypothalamus, they exert their effects by interacting with two opposing sets of neurons; NPY/AgRP neurons that are suppressed (Schwartz et al., 1992; Sipols et al., 1995; Sorensen et al., 2002), and other, POMC neurons that are stimulated by them (Benoit et al., 2002; Sorensen et al., 2002; Breen et al., 2005).

Insulin is mainly known for its putative glucoregulatory roles as an anabolic hormone while in the center, it exerts a catabolic effect by reducing appetite that was shown in some species (Woods et al., 1979; Foster et al., 1991; Air et al., 2002). On the other hand, insulin acts as a potent secretagogue of leptin from adipocytes (Barr et al., 1997; Patel et al., 1998). Therefore, their circulating levels are positively correlated.

Hypoinsulinemia is a useful situation for investigating the roles of insulin and also some related hormones, especially leptin, in control of appetite and growth parameters. In this situation, peripheral anabolic and also central catabolic effects of insulin and leptin are more

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clearly observed. These effects have been frequently studied in rodents but few studies have been carried out in ruminants, despite prominent differences in neuroendocrine pathways, metabolic fuels (Bergman, 1990; Gabel and Sehested, 1997), feeding patterns and insulin sensitivity (Prior and Smith, 1983; Sasaki and Takahashi, 1983; Sano et al., 1985) between ruminants and rodents. Thus, in this study we induced hypoinsulinemia using streptozotocin in growing lambs to investigate changes in appetite, growth parameters, carcass composition and other related blood metabolites.

MATERIALS AND METHODS

Animals

Twenty male Zel lambs (three months-old) weighing 19.4±1.6 kg were assigned to the experiment and identified with ear plaques (Shirang research station of Zel breed, Gorgan, Iran). All animals were maintained in a common pen and fed the same ration. Two weeks prior to the beginning of the experiment, animals were randomly divided into four treatment groups and housed in individual, sheltered pens (2.25 m^2) under ambient temperature and photoperiod. Animals were fed *ad libitum* with a mixture of alfalfa hay (60% of DMI) and concentrate (40% of DMI) in the form of a total mixed ration containing 2.6 Mcal of ME and 14% CP per kg DM to meet their requirements (NRC, 1985). Mineral blocks and water were available at all times.

After two weeks of adaptation to experimental conditions and ration, the main stage of the experiment was initiated involving eight consecutive weeks- three weeks considered as pre-injection interval and the following five weeks as post-injection interval. At the end of the third week, STZ was injected intravenously and then animals entered the post-injection interval.

Hypoinsulinemia induction

Hypoinsulinemia was induced by single intravenous injection of STZ (Sigma Chemical Co., St. Louis, MO, USA) as previously described (Higdon et al., 2001; Ramanathan et al., 2004). Briefly, STZ was dissolved in citrate buffer (pH = 4.5) in three final concentrations and then, fresh solutions were immediately injected via the jugular vein using sterile syringes within five minutes after preparation. The treatments were single injection of four doses of STZ; 0, 25, 50 and 75 mg/kg BW and named C, L, M and H, respectively. Control group (C) received buffer solution alone.

Forty eight hours after STZ injection, animals in treatment H showed severe diabetic symptoms, such as cachexia, hypophagia and polyurea, and therefore needed insulin therapy and electrolyte therapy for survival. Indeed, they posed such abnormal conditions that they could not continue the experiment. Insulin therapy produced data not suitable for assays of hormones and other metabolites. Thus, the experiment continued with C, L and M treatment groups.

Data collection

During each week, daily feed and water intakes (WI) were measured on a specific day and extended to all days of the respective week. Hence, feed and water were weighed and offered *ad libitum* to animals twice daily at 8:00 and 14:00 and that remaining was assessed at 07:00 to 08:00 of the next day when animals avoided eating or drinking and were recumbent. These data were calculated as g feed or water offered to each animal minus g of remaining. Animals were weighed weekly at 07:00 before morning feeding and their weekly weight gain was recorded. Feed conversion ratio (FCR) was calculated individually by dividing feed intake into weight gain.

Blood samples were collected twice on a specific day of each week via jugular venipuncture using ice-cooled sterile vacuum tubes (Pars Khavar Co., Qazvin, Iran); once when animals were fasted and the second at 2.5 h after feeding (post-prandial). These days were discrete from those on which feed intake was measured because of the stress that blood collection causes on feeding behavior of animals. Samples were immediately centrifuged at 3,500 RPM for 15 min. Sera were harvested and stored in sterile tubes, then frozen at -25°C until measurements were made.

All blood metabolites were measured by spectrophotometric methods (Thomas, 1998). Briefly, glucose concentrations were measured via glucose oxidase method, triglycerides via glycerol-3-phosphate method, cholesterol via cholesterol oxidase method, blood urea nitrogen (BUN) via bertholet method, albumin via bromocresol green method, ketone bodies (KB) via nitroproside strip, (all with commercial, diagnostic kits of Pars Azmun Co., Tehran, Iran) and total protein (TP) was measured via modified biuret method (with diagnostic kit of Zistchem Co., Tehran, Iran).

Serum insulin and leptin were measured via radioimmunoassay (RIA) kits (TYN Co., No. 212, Gupre St. Bruxele, Belgium) based on a double antibody RIA method with antisera raised against ovine insulin and leptin. Intra- and inter-assay coefficients of variation were, respectively, less than 5% and 9% for insulin and less than 5% and 6% for leptin.

At the end of the experiment, animals were killed to determine empty body weight (EBW) and carcass composition. The fillet of vertebra 12 and the cranial lobe of liver were removed and analyzed to determine DM, CP and crude fat contents. Urine samples were collected directly from the bladder and used for urinalysis. All the experiments were approved by the animal care committee of Gorgan University of Agricultural Sciences and Natural

Resources.

Statistical analyses

Data measured over weeks were analyzed as a repeated measures design using the mixed model procedure of SAS software (SAS, 1996).The tested effects were treatment (citrate buffer vs. STZ), week (time) and two-way interaction (treatment×week). The main effect of treatment was tested using animal within treatment as the error term and the residual error was used to test week and interaction effects. Multiple covariance structures were tested and the model fitting statistics were compared to determine the best fitting model. Mean separation was performed using the LSMEAN statement and comparisons between treatments were performed with the Tukey-Kramer test. Data are shown as least-squares mean±SEM. Other data were analyzed as a completely randomized design. Mean comparison was performed by Duncan's multiple range test

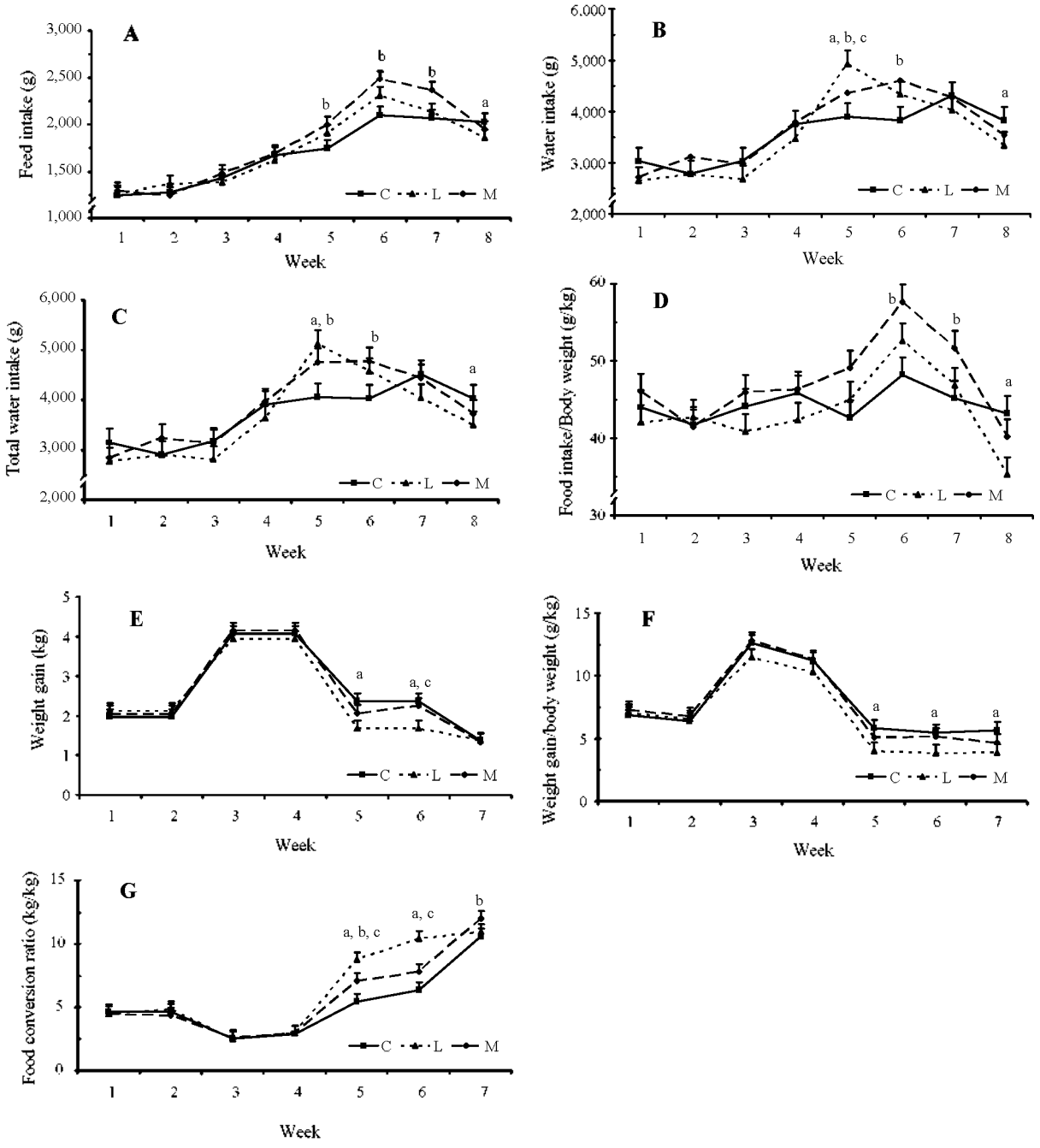

Figure 1. Means of feed intake (A), water intake (B), total water intake (C), feed intake/body weight (D), weight gain (E), weight gain/body weight (F), and feed conversion ratio (G) over the experiment. [a, b, c] Significant differences between control group and group L, control group and group M, and between groups L and M, respectively ($p<0.05$). Group H could not continue the experiment.

and a p value less than 0.05 was considered statistically significant.

RESULTS

In the testing of the main effects of some variables such as FI, WI and weight gain, the effects of week and interaction were significant; that was expected and is due to the nature of these variables and to the environmental effects. Thus, comparison between treatments in each week was considered as an appropriate contrast.

Data analyses showed that hypoinsulinemia caused a dose-dependent increase in FI where it was greater by 242, 388.8 and 290 g in group M vs the control group in weeks 5 to 7, respectively (Figure 1A). However, group L showed an incremental effect but this was not significant ($p>0.05$). WI in groups M and L was greater than in the control group in weeks 5 and 6 (Figure 1B). In addition, total WI (as the sum of WI and 10% of FI) in group M was also greater than in the control group in week 5 (Figure 1C). FI/BW ratio in

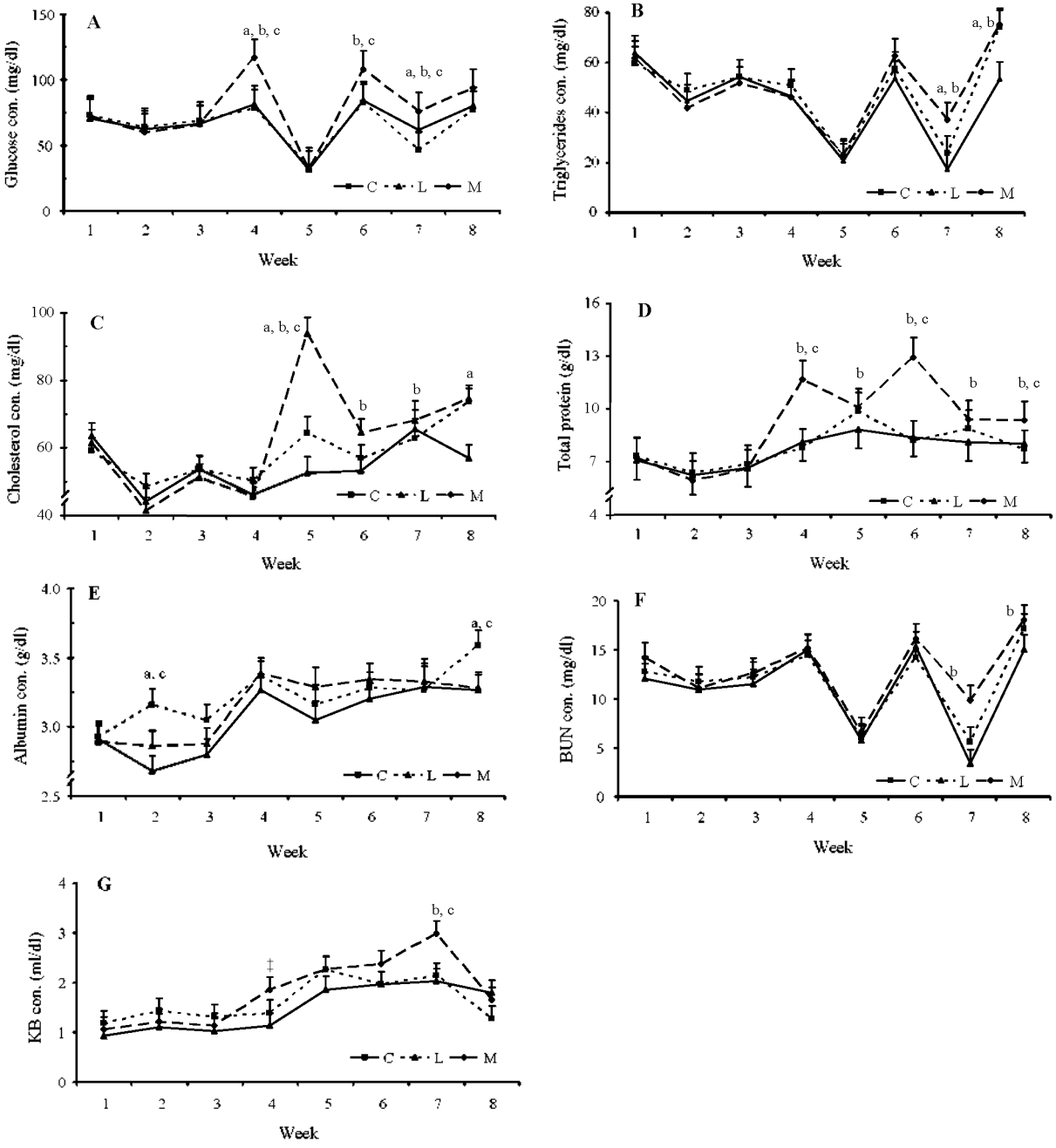

Figure 2. Means of blood metabolite concentrations over the experiment (A: Glucose; B: Triglycerides; C: Cholesterol; D: Total protein; E: Albumin; F: Blood urea nitrogen (BUN); G: Ketone bodies (KB)). [a, b, c] Significant differences between control group and group L, control group and group M, and between groups L and M, respectively ($p<0.05$). Group H could not continue the experiment.

group M significantly increased in weeks 6 and 7 compared to the control group (Figure 1D). STZ-groups gained less weight than the control group ($p<0.05$), especially in group L, where it was 2.06, 1.67 and 2.36 kg in week 5 and 2.24, 1.67 and 2.36 kg in week 6 for groups M, L and the control, respectively (Figure 1E). Weight gain /body weight ratio in group M was lower than in the control group at non-significant levels in weeks 5 to 7 (Figure 1F), while it was significantly lower for group L in weeks 5 to 8. FCR in group M was greater than in the control group in weeks 5 and 7. Interestingly, FCR in group L was markedly greater than in groups M and C in weeks 5 and 6 (Figure 1G).

As expected, blood glucose concentrations were increased by STZ injection in a dose-dependent manner ($p<0.05$), especially in weeks 4, 6 and 7 (Figure 2A). Glucose concentration reached 116.6 mg/dl in group M vs. 81.2 mg/dl in the control group in week 4. Hypertriglyceridemia was observed in groups L and M when compared with the control group in weeks 7 and 8 (Figure 2B). Blood cholesterol levels in group M were significantly greater than in the control group during weeks 5 to 8 (Figure 2C); significantly increased cholesterol level was also observed for group L in weeks 5 and 8. Blood TP levels in group M showed significant increase as against the control group in weeks 4 to 8 (Figure 2D) and also vs. group L in weeks 4, 6 and 8 ($p<0.05$). Albumin concentrations in groups M and L, however, were increased after injection of STZ, but these were not significant except in week 8 (Figure 2E). BUN concentrations (Figure 2F) in group M were elevated significantly compared to the control group in the two final weeks (9.78 vs. 2 and 17.92 vs. 14.89 in weeks 7 and 8, respectively). KB concentrations in group M were greater than in the control group in weeks 4 and 7 (Figure 2G).

Fasted insulin levels in group M showed a dose-dependent decrease ($p<0.05$), especially in weeks 4, 6 and 8 (Figure 3A). In diabetic animals of group M, insulin level declined up to 52, 65 and 20 percent of that in control animals in weeks 4, 6 and 8, respectively. Post-prandial insulin levels in groups M and L declined significantly compared with the control group in week 4, but in the two final weeks the control group showed lower insulin levels than other groups (Figure 3B). Leptin concentrations in groups M and L markedly declined compared to the control group in the post-injection interval except in week 5 (Figure 3C). However, this decline was not observed for post-prandial leptin concentrations (Figure 3D).

Heart rate, rectal temperature and EBW showed no significant differences between treatment groups. Urinary glucose showed a dose-dependent increase between groups, however, decline was not significant ($p = 0.351$). Interestingly, DM of liver in group M was significantly lower than other groups but for meat, it was significantly greater. There were no significant differences between CP and crude fat contents of all groups for both liver and meat (Table 1).

It must be noted that during the final week of the experiment (week 8), a heavy snowfall occurred that led to

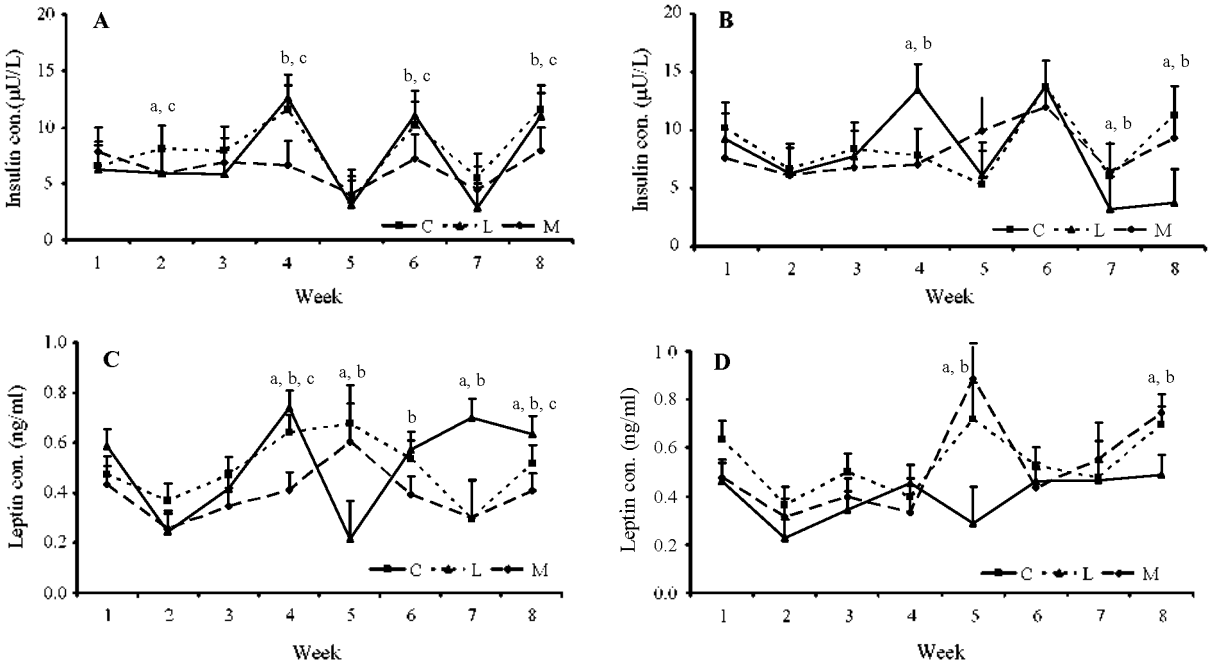

Figure 3. Means of insulin (A: Fasted, B: Post-prandial) and leptin (C: Fasted, D: Post-prandial) concentrations over the experiment. [a, b, c] Significant differences between control group and group L, control group and group M, and between groups L and M, respectively ($p<0.05$). Group H could not continue the experiment.

Table 1. Composition of liver (cranial lobe) and meat (fillet of vertebra 12) of experimental lambs in terms of DM, and CP and fat contents as a percent of DM[1]

Variable		p value	Means[2]		
			C	L	M
Liver	DM (%)	0.0061	36.23 [a]	29.68 [a]	19.20 [b]
	CP (% DM)	0.3464	58.72 [a]	60.44 [a]	60.36 [a]
	Fat (%DM)	0.3964	10.83 [a]	12.14 [a]	10.42 [a]
Meat	DM (%)	0.1346	42.90 [b]	41.64 [b]	44.61 [a]
	CP (% DM)	0.4643	55.22 [a]	50.83 [a]	54.66 [a]
	Fat (% DM)	0.9232	35.15 [a]	36.22 [a]	35.96 [a]

[1] Group H could not continue the experiment.

[2] Comparison was performed by Duncan's multiple range test after ANOVA. Means within rows with no common superscript letters are significantly different.

cessation of the experiment and results for this week do not have congruity with those of previous weeks. This is completely revealed in all three figures.

DISCUSSION

The brain monitors energy states by receiving continuous information from the periphery or other regions of the brain. With regard to appetite, there are at least two major categories including orexigenic signals such as NPY, AgRP, orexin and opioid peptides that stimulate appetite while anorexigenic signals such as insulin, leptin and α-MSH exert inhibitory effects on appetite and suppress it. The regulation of appetite is derived through these opposing signals and the resultant interaction consequently determines appetite (Schwartz et al., 2000; Gerozissis, 2004; Niswender et al., 2004; Broberger, 2005; Woods, 2005).

Insulin is an important and potent anorexigenic signal in energy homeostasis. It is mainly known for its peripheral effects on the metabolism of glucose, fat and protein where it facilitates the storage of energy sources mainly in the form of fat and/or glycogen (Carlon and Campbell, 1993; Niswender et al., 2004). In the brain, insulin exerts a catabolic action by suppressing appetite. Secretion and release of two potent orexigenic peptides, NPY and AgRP, are inhibited by insulin (Sipols et al., 1995; Saho et al., 1997; Henry et al., 1999; Sorensen et al., 2002; Breen et al., 2005), while α-MSH secretion is induced (Benoit et al., 2002; Sorensen et al., 2002; Breen et al., 2005). Furthermore, insulin is a potent secretagogue of leptin that has a close partnership with insulin in control of appetite (Barr et al., 1997; Saltiel and Kahn, 2001). The opposing effects of insulin in the periphery and in the brain are evident in diabetes. In this situation, lack of peripheral, anabolic action of insulin is accompanied by the lack of its central, catabolic action that results in hyperglycemia and polyuria along with excess demand for feeding known as diabetic hyperphagia. STZ-induced diabetes in rodents caused hyperphagia, however, they could not gain weight normally because of inability to store blood metabolites in tissues (Hidaka et al., 2001; Morris and Pavia, 2004). Consequently, the metabolites are excreted via urine.

In the present study, diabetic animals - in particular group M, showed diabetic hyperphagia in weeks 5 to 7 that was consistent with results obtained in rodents (Roland and Caputo, 1985; Smith and Gannon, 1991; Hidaka et al., 2001; Barber et al., 2003; Akirav et al., 2004; Morris and Pavia, 2004). Diabetic lambs showed hyperphagia with lower severity than observed in diabetic rodents. Physical satiety, however, may be a logical reason for this, because in terms of kind of feeds consumed by ruminants, they face gut fullness that prevents more feeding. As reported for rodents and humans (Barr et al., 1997; Boden et al., 1997), in the present study the regulatory role of insulin in leptin secretion was revealed where the hypoinsulinemia in the post-injection interval paralleled hypoleptinemia, however, it was seen for fasted animals. Nonetheless, declined insulin and leptin levels in group M can be considered as an important factor in hyperphagia. Previous study in sheep showed the anorexigenic effect of leptin on appetite (Henry et al., 1999).

In spite of increased FI, diabetic animals were unable to gain weight even at the level of intact animals. Indeed, diabetic animals lost weight when compared with the control group. Similar results were observed in rodents (Hidaka et al., 2001; Morris and Pavia, 2004) and cattle (Higdon et al., 2001). It is obviously due to the inability of peripheral tissues to uptake metabolites from the bloodstream.

One week after induction of diabetes, WI and TWI increased, especially in group M. This was in agreement with increased WI observed in rats that was reported to be mainly because of elevated urine volume and, in part, of enhanced noradrenalin levels in the hypothalamus (Smith and Gannon, 1991; Barber et al., 2003; Morris and Pavia, 2004).

As observed in rodents and humans, diabetic animals in group M showed hyperglycemia, hypertriglyceridemia and (to a lower extent) hyperproteinemia that can be considered

as results of hyperphagia and also the lack of blood metabolites entering into the tissues. In fact, it revealed the lack of a peripheral, anabolic role of insulin. It was confirmed by the glycosuria and proteinuria (data not shown) observed in these animals. Similarly, induction of diabetes was accompanied by hypertriglyceridemia in rodents (Junod et al., 1969; Sambandam et al., 2000; Morris and Pavia, 2004) and sheep (Mamo et al., 1983). Proteinuria was observed in STZ-diabetic sheep reflecting increased blood TP concentrations (Ramanathan et al., 2004).

In the present study, FCR in diabetic animals was greater than in the control group, meaning that diabetic animals are less efficient in converting consumed feed into weight gain. It was not unexpected because diabetic animals were polyuric (visually but not measured quantitatively) with obvious glycosuria and proteinuria. As a result, retained-energy value (kcal of FI minus kcal of urinary glucose loss) in these animals decreased as observed in rodents (Willing et al., 1990).

Higher blood KB concentrations observed in diabetic animals (particularly group M) were not necessarily similar to those observed in diabetic rodents or humans as ketoacidosis, because diabetic animals consumed more feed producing more VFAs which a moiety of them is absorbed in the form of ketone bodies, in particular beta-hydroxybutyrate (Bergman, 1990; Gabel and Sehested, 1997). Nonetheless, it is difficult to distinguish these conflicting events from each others. The previous study in rodents showed occurrence of ketoacidosis using high doses of STZ (>100 mg/kg BW). Similar results were observed in diabetic sheep (Ramanathan et al., 2004) and cattle (Prior and Smith, 1983).

BUN concentrations in group M were greater than in the control group in the final two weeks. Prior and smith (1983) showed that diabetic cattle have higher BUN and creatinine levels. In the present study, elevated blood TP and BUN concentrations strongly imply that nitrogen metabolism was impaired with diabetes. This is mainly due to the lack of uptake of proteins by peripheral tissues and their denaturation.

Carcass and liver compositions did not differ among groups while feed intake and also insulin and leptin levels showed marked differences among them. In farm animals, fat and protein contents and their partitioning in body are influenced by nutritional states and hormonal profiles including insulin, leptin and growth hormone (Torbay et al., 1985; Olivares and Hallford, 1990; Roberts et al., 1994; Chung et al., 2008). Likely, enhanced feed intake counteracted wih lowered insulin and leptin levels in fat deposition. Lack of peripheral anabolic effect of insulin in diabetic animals of present study has no significant effect on EBW after dressing. It seems that attenuated anabolic effect of insulin in these animals was compensated by hypherphagia.

Ruminants are less sensitive to insulin than monogastric animals (Sasaki and Takahashi, 1983; Sano and Terashima, 1991). On the other hand, secretion of insulin in ruminants is stimulated by VFAs, especially n-butyrate and propionate, and IV injection of these VFA acts as a strong stimulus of insulin secretion in sheep, goats and cattle (Matsunaga et al., 1999). In ruminants, a large proportion of energy requirements are supplied as VFAs, which are produced by the fermentation of dietary carbohydrates (Gabel and Sehested, 1997). Therefore, small quantities of glucose are absorbed from the small intestine and, as a consequence, glucose utilization in the whole-body is relatively dependant upon glucose production via gluconeogenesis in liver and other tissues (Sano et al., 1985).

Regardless of these profound differences between energy metabolism in ruminants and non-ruminants, in feeding patterns (e.g. time spent to eat and the numbers of meals), and also the diet composition (mainly due to energy density and fiber content of feed), it seems that there is close similarity in energy homeostasis in diabetic conditions of both species.

In the present study, injection of STZ caused hypoinsulinemia with a wide extent of abnormalities; in group H, the severity of diabetes was so intense that they were challenged for survival, in a manner that has been previously observed for cattle (Higdon et al., 2001), confirming the crucial role of insulin in energy balance and metabolism in ruminants. Diabetic hyperphagia and the inability for weight gain were observed in sheep, establishing the opposing anabolic and catabolic actions of insulin in these animals and suggesting that diabetic sheep are a useful animal model for investigating roles of insulin in energy homeostasis.

IMPLICATIONS

Before this study, there existed no work in which diabetes was induced with varying severity and a wide range of STZ doses. More studies are needed to determine the role of insulin in energy homeostasis and growth parameters of ruminants. It seems that the role of insulin in energy homeostasis of ruminants is more prominent than presumed for these animals and their lower blood glucose levels cannot attenuate its crucial roles.

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