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Effects of Peripheral Ghrelin Treatment and the Associations Between Leptin and Ghrelin in Growing Ram Lambs

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Abstract: Leptin an adipocytes-derived hormone is an important regulator of bodyweight and energy metabolism. Ghrelin another hormone, influences energy and could directly influence the deposition of fat. In this study, researchers aimed to investigate the effect of long term ghrelin treatment the relationships between leptin and ghrelin and leptin per carcass measurements in Awassi ram lambs. The lambs were randomly assigned to the following 4 groups with 4 animals per group according to both the frequency of food administration and ghrelin treatment: in group I, animals were fed *ad libitum* in the group II, animals were fed *ad libitum* and intravenously injected with the ghrelin twice a week in the group III, animals were fed once a day and in the group IV, animals were fed twice a day. Blood samples were collected 30 min before feeding and 60 min after feeding to be analyzed for plasma ghrelin and leptin hormone levels. The study was completed using the slaughter weight (43 kg) which was determined when the Awassi lambs in each group were slaughtered after 12 h of fasting. These results suggest that in ruminant species, ghrelin levels are affected by long-term programmed meal feeding; however, the relationships between leptin and carcass weight and MLD measurements showed no significant differences.

Key words: Leptin, ghrelin, lambs, MLD area, blood

INTRODUCTION

Ghrelin influences energy metabolism (Tschop *et al*., 2000) and could directly influence the deposition of fat (Patel *et al*., 2006). Altough, leptin has been reported to fluctuate relative to body composition in cattle, it remains unknown whether ghrelin levels fluctuate in response to body composition. It was found that changes in the level of ghrelin in plasma are correlated with the level of leptin (Cummings and Foster, 2003). The finding of greatest interest with regard to the interactions between leptin and ghrelin levels is that an inverse relationship occurs in response not only to the regulation of food intake but also to the season during which adjustments in food intake occur. Notably, leptin has also been suggested to have influence circulating ghrelin levels.

Leptin increases energy expenditure and provides negative feedback that inhibits its own gene expression. Leptin which secretion is highly correlated with body fat mass and adipocyte size potentially contributes to inter-animal variation regarding appetite, energy balance and body composition; leptin is also considered to be a metabolism modifier (Houseknecht *et al*., 1998). Some studies have reported a correlation between serum leptin and carcass quality in bovines (Wegner *et al*., 2001; Geary *et al*., 2003). However, studies on the relationship between leptin measured at an early growth phase and in the final carcass are scarce (Altmann and Von Borell, 2007). Due to the associations between plasma leptin concentrations and body fat, leptin could be used as an indicator for the *in vivo* evaluation of carcass composition in breeding management programs.

The mechanisms controlling ghrelin secretion during fasting and postprandial suppression are unknown (Nakai *et al*., 2003). In rodents and humans, ghrelin has been studied for both its short and long term effects but researchers have not information about long term effects of ghrelin and the general relationships between ghrelin and leptin levels in ruminant species. Therefore, researchers studied ghrelin's long term effects and the relationships between leptin and ghrelin levels in lambs to clarify whether ghrelin secretion is caused by feeding frequency over the long term. Researchers also determined plasma ghrelin and leptin concentrations and their relationships to the fattening period.

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MATERIALS AND METHODS

Experimental animals and treatments: This study was conducted and validated at the Animal Welfare and Animal Welfare and Application Center of Faculty of Veterinary Medicine in Bursa (Protocol No.: 26.07.2004/020/333). Sixteen male Awassi lambs were tested for homogeneity with respect to weight and age. The animals were 2 months old with an average body weight of 26 kg and each lamb within each group was housed individually 100×150×120 cm pen inside a closed shed. The lambs were randomly assigned to the following 4 groups with 4 animals per group according to both the frequency of food administration and ghrelin treatment: in group I, animals were fed *ad libitum* in the group II, animals were fed *ad libitum* and intravenously injected with the ghrelin (1 μg kg^{-1}, Ghrelin rat, 24160 Anaspec) twice a week in the group III, animals were fed once a day (09:00) and in the group IV, animals were fed twice a day (09:00 and 16:00).

The daily food allowance was adjusted to the metabolic energy in per day to maintain an average body weight of 43 kg. The animals were given alfaalfa hay as roughage. Water was available *ad libitum*. The concentrate feed ingredients are shown in Table 1 and 2. The dry matter content of the dietary samples was determined by drying at 105°C for 12 h and the crude protein content was determined by the Kjeldahl Method (AOAC, 1990). Ash was determined by combustion at 550°C for 6 h. The Neutral Detergent Fibre (NDF) contents were determined using the methods described by Van Soest *et al.* (1991).

Table 1: Composition of concentrate food distributed to 2 months old lambs

Ingredients	Contents (%)
Corn grain	50.0
Barley	18.5
Sunflower meal	16.5
Soybean meal	13.2
Limestone	1.2
Salt (NaCl)	0.5
Vitamine-mineral premix[1]	0.1

[1]Vitamin-mineral premix (Kavimix VM) (supplied per kg): Vitamin A: 12,000,000 IU, Vitamin D3: 3,000,000 IU, Vitamin E: 30 g, Mn: 50 g, Fe: 50 g, Zn: 50 g, Cu: 10 g, I: 0.8 g, Co: 0.1 g, Se: 0.15 g, Antioxidant: 10 g

Table 2: Chemical composition of food ingredients given to 2 months old lambs

	Contents (%)	
Chemical composition	Concentrate	Alfalfa hay
Dry matter	88.30	90.30
Ash	4.67	9.93
NDF	15.09	39.40
Crude protein	15.80	15.50
Ether extract	2.56	2.26
Calcium	0.59	1.33
Phosphorus	0.41	0.25

NDF: Neutral Detergent Fibre

Determination of ghrelin and leptin hormone in plasma: Blood samples for ghrelin measurements were obtained by puncturing the jugular vein of lambs weighning of 43 kg. All samples were collected in vacutainer tubes containing EDTA at 30 min before feeding (08:30 and 15:30) and at 60 min after feding (10:00 and 17:00). Researchers have collected the blood samples at 15 days intervals until day 45. Whole blood was centrifuged at 2,200 g and 4°C for 10 min and plasma was collected and stored in microtubes containing aprotinin (0.6 Trypsin Inhibitor Unit (TIU)/mL of blood), a protease inhibitor at -20°C until analysis. Plasma ghrelin concentrations were determined by Radioimmunoassay (RIA) (DPC Gambyt CR, England 95-3/1097, Licence No. KRN0142.04.00.1N) using a Ghrelin kit (Ghrelin RIA kit, RK-0-31-31, Phoenix Pharmaceuticals).

Serum leptin concentration was measured using a double antibody RIA kit containing guinea pig multispecies leptin antibody, human [125I] leptin and human leptin as the standard (Linco, Multispecies Leptin RIA kit, Cat#XL-85K) according to the manufacturer's instructions. The quantification was carried out in 100 μL of serum.

Slaughtering procedures and measurements of carcass traits: Live weight was recorded biweekly. The final weight measurements was the slaughter weight which was 43 kg. The Awassi lambs in all the groups were slaughtered after 12 h of fasting, at which time the slaughter live weight was recorded. The Musculus Longissimus Dorsi (MLD) area and the fat thickness were determined between the 12th and 13th thoracic vertebrae. Fat content was also determined by the Soxhlet Method (Soxhlet, F.: Die gewichtsanalytische Bestimmung des Milchfettes, Polytechnisches J. (Dingler's) 1879, 232, 461).

Statistical analysis: The Statistical Package for the Social Sciences, Version 13.0 (SPSS, Chicago, IL, USA) was used for data analysis. Values are expressed as arithmetic means±Standard Error of Mean (SEM). Within-group effects and group interactions with time were analyzed using an ANOVA for repeated measures. When violations in parametric assumptions were found within the data set within-group effects and between-group interactions with time were analyzed using a univariate ANOVA. Differences in carcass traits between different groups were compared using the Kruskal-Wallis test. Significance was determined with Tukey's Honestly Significant Differences (HSD) test with a cut-off of $p<0.05$.

RESULTS AND DISCUSSION

Changes in plasma ghrelin and leptin levels in lambs subjected to the four different feeding regimens are presented in Table 3 and 4. Mean plasma ghrelin levels were analyzed for each of the groups of 240 series samples. Plasma ghrelin levels changed significantly ($p<0.01$) between lambs fed twice a day and the other groups of schedule-fed lambs. Ghrelin levels reached the highest peak values in the second period for lambs fed twice a day. In twice-fed animals, the highest peak values were the same as in the other groups and these values decreased during the third period. Within the feeding regimens, mean (±SEM) ghrelin concentrations showed significant differences ($p<0.001$) between the second and other periods. Ghrelin levels for whole groups reached the highest peak values during the second period. Otherwise, there were no significant differences between sampling times in the groups (Table 3).

Plasma leptin concentrations differed significantly ($p<0.05$) between lambs fed once a day and those fed *ad libitum*. The plasma leptin level for lambs fed twice daily was also different from that of the other groups ($p<0.001$). Leptin levels reached the highest peak values during the second period of each experimental group. Moreover, similar to the ghrelin concentrations, the leptin concentrations also showed significant differences ($p<0.001$) between the second and other periods. There were no significant differences between sampling times within groups (Table 4).

The carcass measurements are given in Table 5. No significant differences ($p<0.05$) were observed in fat thickness, M. longissumus dorsi area or rate.

Leptin is produced primarily in adipocytes and regulates food intake and energy expenditure. Pre-meal ghrelin hypersecretion which occurs at the onset of dark-phase ingestive behavior and precedes the time of food availability in a scheduled feeding paradigm is coincident with low circulating levels of leptin. However, a gradual rise in leptin hypersecretion precedes the postprandial decline in ghrelin secretion (Crowley *et al*., 2005; Tokuda and Yano, 2001; Xu *et al*., 1999). It has been hypothesized that the satiety-inducing effects of leptin include the suppression of ghrelin secretion (Yildiz *et al*.,

Table 3: Plasma ghrelin concentration during pre-feeding and post-feeding periods (measured at 15 days intervals) in male lambs subjected to the four different feeding regimens (ng mL^{-1})

	Feeding regimen groups			
Sampling times	*Ad libitum*	*Ad libitum*+ ghrelin	Once a day	Twice a day
Period (15 days)				
30 BMF	4.34±4.21	6.46±4.01	5.05±1.20	7.04±3.00
60 AMF	5.01±3.88	5.40±2.54	5.37±0.75	7.18±1.43
30 BAF	7.52±3.37	7.19±2.20	6.84±1.95	8.48±2.24
60 AAF	7.61±2.28	8.53±2.48	7.47±0.50	9.36±1.94
Period (30 days)				
30 BMF	7.70±0.75	7.98±2.16	7.09±0.93	10.04±3.06
60 AMF	8.62±1.32	8.94±2.07	8.50±0.88	9.88±1.99
30 BAF	7.38±1.32	8.23±1.25	8.21±0.84	8.61±1.35
60 AAF	9.31±5.90	7.97±1.96	8.22±0.76	9.49±2.16
Period (45 days)				
30 BMF	5.95±0.83	7.54±1.06	6.82±1.26	7.90±2.28
60 AMF	5.72±1.95	6.73±0.81	5.58±2.12	6.85±1.75
30 BAF	5.03±2.06	5.95±2.99	5.29±0.54	7.01±2.94
60 AAF	6.17±1.55	6.01±1.23	5.39±0.77	6.09±1.81

Feeding time: 09:00 and 16:00; 30 BMF: 30 min Before Morning Feeding (08:30); 60 AMF: 60 min After Morning Feeding (10:00); 30 BAF: 30 min Before Afternoon Feeding (15:30); 60 AAF: 60 min After Afternoon Feeding (17:00). ±values are represented by $\bar{X} \pm SEM$

Table 4: Plasma leptin concentration during pre-feeding and post-feeding periods (measured at 15 days intervals) in male lambs subjected to the four different feeding regimens (ng mL^{-1})

	Feeding regimen groups			
Sampling times	*Ad libitum*	*Ad libitum*+ ghrelin	Once a day	Twice a day
Period (15 days)				
30 BMF	2.47±0.46	2.81±0.37	2.39±0.57	2.97±0.40
60 AMF	2.37±0.70	3.03±1.08	2.28±0.46	2.94±0.58
30 BAF	2.52±0.46	2.96±0.18	2.73±0.23	3.04±0.70
60 AAF	3.27±0.49	2.94±0.50	2.76±0.29	3.07±0.57
Period (30 days)				
30 BMF	2.71±0.39	2.48±0.47	3.19±0.36	3.37±0.49
60 AMF	2.44±0.27	2.34±0.72	3.23±0.57	3.80±0.36
30 BAF	3.07±0.30	2.37±0.34	3.05±0.97	3.63±0.87
60 AAF	3.52±0.75	3.34±0.42	4.45±0.73	4.12±0.58
Period (45 days)				
30 BMF	2.60±0.53	2.87±0.88	2.51±1.02	3.43±0.45
60 AMF	2.50±0.76	2.65±0.66	2.69±0.52	3.13±0.24
30 BAF	2.03±0.87	2.78±0.55	3.39±1.25	2.88±1.03
60 AAF	1.98±0.76	2.71±0.37	2.07±0.11	3.10±0.31

Feeding time: 09:00 and 16:00; 30 BMF: 30 min Before Morning Feeding (08:30); 60 AMF: 60 min After Morning Feeding (10:00); 30 BAF: 30 min Before; Afternoon Feeding (15:30) but 60 AAF: 60 min After Afternoon Feeding (17:00). ±values are represented by $\bar{X} \pm SEM$

Table 5: The carcass and MLD measurements in scheduled meal-fed Awassi ram lambs

	Feeding regimen groups				
Parameters	*Ad libitum*	*Ad libitum*+ghrelin	Once a day	Twice a day	p-value
Warm carcass weight (kg)	17.59±0.37	17.85±0.49	18.22±0.40	18.25±0.32	NS
Cold carcass weight (kg)	17.05±0.43	17.22±0.45	17.81±0.34	17.78±0.36	NS
Fat thickness (mm)	8.09±0.98	8.06±0.48	7.37±0.94	7.42±0.61	NS
M. Longissumus dorsi area (mm^2)	10.70±0.35	10.46±0.74	12.55±1.39	12.46±1.30	NS
M. Longissumus dorsi area (mm^2) rate (%)	0.63±0.13	0.61±0.05	0.70±0.08	0.70±0.08	NS

2004). Indeed, the effects of leptin on energy homeostasis are opposite (although, not complementary) to those of ghrelin: leptin induces weight loss by suppression of food intake, whereas ghrelin functions as an appetite-stimulatory signal. Moreover, leptin has been shown to be an upstream regulator of ghrelin in rodents (Nakazato *et al*., 2001; Ueno *et al*., 2005). In the study, the highest peak values for plasma ghrelin and leptin levels occurred during the second period (at 30 days) and both of these hormones reached higher concentrations in the lambs fed twice a day than in lambs in the other groups. Contrary to other study results, researchers observed a positive correlation between plasma ghrelin and leptin responses to meal intake. Plasma ghrelin and leptin levels rose and fell together during the pre-feeding and post-feeding periods, respectively. Moreover, when we administered intravenous ghrelin peptide there were no changes in leptin levels. Researchers expected that lambs treated peripherally with ghrelin would have low leptin levels but researchers did not observe the expected result. Several studies in humans have produced conflicting results. For example, Tschop *et al.* (2001) demonstrated that in obese patients, fasting plasma ghrelin levels are negatively correlated with fasting plasma leptin levels; however, in a different study in obese children and adolescents, fasting plasma leptin and ghrelin concentrations were not correlated (Ikezaki *et al*., 2002). Whether an elevated level of circulating leptin causes a reduction in the ghrelin level is still not clear. However, it seems that leptin does not have a direct influence on ghrelin levels. Intramuscular fat was reported to be correlated (ranging from 0.39-0.51) with leptin concentrations measured at slaughter (Geary *et al*., 2003) and at several weeks before slaughter (Kawakita *et al*., 2001; Wegner *et al*., 2001). Intramuscular fat content (0.2-32.1%) and total body fat varied widely in steers and a correlation of 0.59 was observed between leptin and intramuscular fat (Yamada *et al*., 2003). In contrast, no differences were observed in plasma leptin in steers of varying marbling scores (Yonekura *et al*., 2002). Furthermore, no correlations between plasma leptin and intramuscular fat were observed in lambs (Altmann *et al*., 2006), cattle (Tokuda and Yano, 2001; Higashiyama *et al*., 2003) or pigs (Berg *et al*., 2003). In the present study, no significant differences were observed in the relationships between leptin and carcass weight or MLD measurements.

Researchers found that the total preprandial and postprandial ghrelin concentrations did not affect food intake throughout the day for lambs that were fed ad libitum or once or twice a day. Ghrelin concentrations in the experimental groups did not increase 30 min before morning feeding and they did not decrease 60 min after feeding. However, in earlier studies, a large preprandial rise and a postprandial fall in plasma ghrelin levels were observed in humans (Cummings *et al*., 2001) and in ruminants (Sugino *et al*., 2002). These contrasts between the results and those obtained in other species suggest that ruminants may not be responsive to the orexigenic properties of ghrelin. However, the data also showed that the highest peak values of ghrelin levels occurred during the second period in lambs fed twice a day. Twice-fed animals showed the highest peak values compared to the other groups and these values decreased during the third period.

CONCLUSION

These results suggest that in ruminant species, ghrelin levels are affected by long-term programmed meal feeding. However, to explain the regulation of ghrelin (such as the surge before feeding and the fall after feeding), further investigations are required in ruminant species. In addition, the association between carcass characteristics and plasma leptin concentrations varies across studies. Further research should validate the obtained results before applying this information in breeding management programs.

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