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Relationship Between Plasma Ghrelin and Leptin Concentrations on Food Intake and Feeding Frequency in Scheduled Meal-Fed Male Lambs During Long Term

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Abstract: Ghrelin, a novel acylated peptide is the endogenous ligand for Growth Hormone Secretagogue (GHS) receptor. Ghrelin has been reported to increase feed intake and BW gain. Ghrelin also has been identified in bovine oxyntic glands of the abomasum and in the stomach of nonruminant animals this peptide may also function in the regulation of feeding or energy balance in ruminants. For this reason; the lambs were randomly assigned to the following 4 groups of 4 animals each according to the administration rhythm of the ration and to the ghrelin treatment: in the Group I, animals were fed *ad libitum*, in the Group II, they were also fed *ad libitum* and were intravenously injected with the ghrelin twice a week, in the Group III they were fed once a day (09:00) and in the Group IV, the lambs were fed twice a day (09:00 and 16:00). The daily food allowance was adjusted to metabolic energy in each day and an average body weight of 43 kg was maintained. Blood samples collected 30 min before feeding (08:30) and 60 min after feeding (10:00) at were analyzed for plasma ghrelin, growth hormone and leptin levels. Researchers have collected the blood samples 15 days intervals until 45 days. There was the highest peak values of ghrelin and leptin levels at the second period in the whole groups but there was no significant changes between sampling times in the groups. Researchers have obtained feed consumption, weight gain and feed conversion of each lambs and each groups. Researchers determined that systemic (i.v.) injection of ghrelin might affect feed consumption and body weight but not affect feed conversion rate.

Key words: Lamb, feeding frequency, food intake, ghrelin, leptin

INTRODUCTION

Ghrelin, a novel acylated peptide is the endogenous ligand for Growth Hormone Secretagogue (GHS) receptor. Ghrelin is produced mainly in the oxyntic glands of the stomach but also produced in the intestines, kidneys, hypothalamus and pituitary gland (Kojima *et al*., 1999). Changes in blood ghrelin levels are associated with feeding behavior in rats (Date *et al*., 2002) and goat (Hayashirda *et al*., 2001). A large preprandial rise and a postprandial fall in plasma ghrelin levels were observed in man (Cummings *et al*., 2001) and ruminants (Sugino *et al*., 2002). Ghrelin has been reported to increase feed intake and BW gain (Nakazato *et al*., 2001; Wern *et al*., 2001). In humans (Tschop *et al*., 2000; Cummings and Foster, 2003) plasma ghrelin levels increase transiently before a scheduled meal and then decline postprandially. Furthermore, administration of exogenous ghrelin causes hyperphagia in rats (Wren *et al*., 2000, 2001) and humans (Wren *et al*., 2001). Ghrelin also has been identified in bovine oxyntic glands of the abomasum and in the stomach of nonruminant animals, this peptide may also function in the regulation of feeding or energy balance in ruminants (Hayashirda *et al*., 2001). The ruminant presents an interesting model because the gut is not emptied between periods of feeding. However, understanding the regulation of feed intake in ruminant species would minimize economic loss and maximize animal well-being in production situations where feed intake. Research has demonstrated that plasma ghrelin concentrations fluctuate relative to feed intake in sheep (Sugino *et al*., 2002) it has failed to demonstrate an effect of exogenous ghrelin injection on feed intake (Iqbal *et al*., 2006). It remains to be determined whether the function and secretion of ghrelin in ruminants is the same as that in non-ruminants.

It was found that changes in the level of ghrelin in plasma are correlated with the level of leptin (Cummings and Foster, 2003). During fasting, plasma ghrelin concentrations increase with a simultaneous decrease in leptin concentrations and during feeding, the situation is reversed. In analyzing the interactions

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between leptin and ghrelin, the finding of greatest interest is opposite effects of their actions are found not only in relation to the regulation of food intake but also to the season in which adjustments in food intake occur. Notably, leptin has also been suggested to have influence on circulating ghrelin levels. The mechanisms controlling ghrelin secretion during fasting and postprandial suppression are unknown (Nakai *et al*., 2003). Researchers have investigated the role of ghrelin in the control of feeding in ruminants using sheep as an experimental model. This research describes mechanisms regulating ghrelin secretion at feeding frequency time and also focuses on the reletionship between ghrelin and leptin.

MATERIALS AND METHODS

Experimental animals and treatments: This study was conducted and validated at the Animal Welfare and Animal Production Research and Application Center in Uludag University, Department of Veterinary Medicine (Protocol No.: 26.07.2004/020/333). Twenty Awassi male lambs were applied to homogeneity test according to similar weight and age. The animals were 2 months old and with an average body weight of 26 kg and they were put into individual paddocks. The lambs were randomly assigned to the following 4 groups of 4 animals each according to the administration rhythm of the ration and to the ghrelin treatment: in the Group I, animals were fed *ad libitum*, in the Group II, they were also fed *ad libitum* and were intravenously injected with the ghrelin peptide (1 μg kg^{-1}, Ghrelin rat, 24160 Anaspec) twice a week, in the Group III, they were fed once a day (09:00) and in the Group IV, the lambs were fed twice a day (09:00 and 16:00).

The daily food allowance was adjusted to metabolic energy in each day and an average body weight of 43 kg was maintained. The animals were given trefoil as dry matter. Water was available *ad libitum*. Concentrate feed ingredients are shown in Table 1 and 2. Dry matter content of dietary samples was determined by drying at 105°C for 12 h and crude protein content was determined by the Kjeldahl Method (AOAC, 1995). 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).

Plasma ghrelin, leptin hormones determination: Blood samples were obtained from the puncture of the jugular vein and collected in vacutainer tubes containing EDTA, 30 min before feeding (08:30 and 15:30) and 60 min after feding (10:00 and 17:00) at a body weight of 43 kg for ghrelin measurements. Researchers have collected the blood samples 15 days intervals until 45 days. Blood was

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:12000,000 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	Alfaalfa 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

centrifuged at 2,200 g for 10 min at 4°C and plasma were collected and stored in microtubes containing an protease inhibitor, aprotinin (0.6 TIU (Trypsin Inhibitor Unit)/mL of blood) 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) with 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 as standard, human leptin (Linco, Multispecies Leptin RIA kit, Cat#XL-85K) following the manufacturer's instructions. The quantification was carried out in 100 μL of serum.

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 Deviation (SD). Within-group and between-group interactions with time were analyzed using univariate ANOVA. Significance was determined with Tukey's Honest Significant Differences (HSD) test with a cut off of $p<0.05$.

RESULTS AND DISCUSSION

Changes in plasma ghrelin and leptin levels in male lambs subjected to the four different feeding regimens are presented in Table 3 and 4. Mean plasma ghrelin levels of the series 240 samples obtained from each of the groups

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

	Feeding regimen groups			
Sampling time	*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±1.90	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

$\bar{X} \pm SD$ shows the ±values; 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 Afernoon Feeding (15:30), 60 AAF: 60 min After Afternoon Feeding (17:00)

Table 4: Plasma Leptin concentration during pre-feeding and post-feeding and periods (15 days intervals) in male lambs subjeced to the three 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

$\bar{X} \pm SD$ shows the ±values; 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 Afernoon Feeding (15:30), 60 AAF: 60 min After Afternoon Feeding (17:00)

were analysed. In the scheduled fed lambs, plasma ghrelin levels had a significant change ($p<0.01$) between fed twice a day lambs and the other groups. Ghrelin levels in the highest peak values reached II period of fed twice a day lambs.

In twice fed animals showed the highest peak values as the other groups and this values again decreased third period. Concentrations of ghrelin as a feeding regimens, mean (±SD) ghrelin showed significant changes ($p<0.001$) between II period and the other periods. There was the highest peak values of ghrelin levels at the second period in the whole groups. Otherwise, there was no significant changes between sampling times in the groups (Table 3).

Table 5: Performance of the lambs

Groups	Body weight gain (kg)	Feed consumption (kg)	Feed conversion rate
Ad libitum	15.700±0.84	101.101±6.25	6.51±0.50
Ad libitum + Ghrelin	16.420±0.68	105.579±7.64	6.44±0.43
Once a day	15.560±1.55	88.980±8.16	5.77±0.27
Twice a day	16.175±0.61	98.98±6.840	6.11±0.29

$\bar{X} \pm SEM$ shows the ±values

Plasma leptin hormone concentrations in lambs fed once a day group and fed *ad libitum* had a statistically different ($p<0.05$) while between lambs fed twice a day and the other groups had significant changes ($p<0.001$). Leptin levels in the highest peak values reached II period of each experimental gropus. Moreover, concentrations of leptin showed significant changes ($p<0.001$) between II period and the other periods like ghrelin concentrations. Also there was no significant changes between sampling times in the groups (Table 4).

Researchers have obtained feed consumption, weight gain and feed conversion of each lambs and each groups. The results showed that lambs administered i.v. ghrelin injection group (*ad libitum* + ghrelin) were the highest values on average body weight gain. This group also had peak value on feed consumption because they fed as *ad libitum* feeding. Although, *ad libitum* + ghrelin group had the high feed consumption and body weight gain had poor feed conversion rate. Besides fed once a day and fed twice a day lambs had lower feed consumption compare to other groups, they had better feed conversion rate than other group, especially fed once a day lambs had the best values on feed conversion rate. The results of this research showed no significant differences between lamb performance on feeding frequency (Table 5).

Several factors including body size or body composition, feeding frequency and diet composition or amount offered may contribute to differences in the responsiveness of ghrelin between these experiments. Because more pronounced differences in plasma ghrelin concentrations resulted with multiple feed offerings, feeding frequency should be considered. Sugino *et al.* (2004) reported less fluctuation in plasma ghrelin concentrations for sheep allowed *ad libitum* consumption compared with those offered feed 2-4 times daily. In the study for *ad libitum* feeding, fed once a day and fed twice a day in lambs we found that the total ghrelin preprandial and postprandial concentrations did not effect food intake throughout the day. Ghrelin concentrations didn't increase 30 min before morning feeding and didn't decrease 60 min after feeding in the experimental groups. Whereas in previous studies, a large preprandial rise and

a postprandial fall in plasma ghrelin levels were observed in human (Cummings *et al*., 2001) and ruminants (Sugino *et al*., 2002). In addition, ghrelin expression and plasma ghrelin levels increased by fasting (Kim *et al*., 2003). In contrast researchers found that there was no different before and after feeding in lambs. Researchers also studied intravenous ghrelin administration on the experimental groups which had no different the other groups in plasma ghrelin levels and feed intake but ghrelin concentrations in fed twice a day lambs were higher than other groups. Despite the consistent finding of elevated ghrelin concentrations before meal initiation, there is little evidence that elevated fasting ghrelin concentrations as representative of elevated overall 24 h ghrelin concentrations or hyperghrelinemia are related to food intake and subsequent weight gain. The results of the study also haven't had relationship between feeding frequency and ghrelin concentrations. Kim *et al*. (2003) observed that plasma ghrelin levels are increased by fasting but the study results showed that although, fed once a day lambs which have exposure fasting longer than other groups, this group were lower across to fed twice a day lambs. Iqbal *et al*. (2005), in a ruminant species and using a variety of experimental paradigms, they showed that ghrelin had consistent effects to stimulate GH secretion but they didn't observe any effect on food intake. This contrasts with results obtained in other species and suggests that ruminants may not be responsive to the orexigenic properties of ghrelin. Leptin is produced primarily in adipocytes and regulates food intake and energy expenditure. Premeal ghrelin hypersecretion at the onset of darkphase ingestive behavior and preceding the time of food availability in a scheduled feeding paradigm is coincident with low circulating levels of leptin. On the other hand, a gradual rise in leptin hypersecretion precedes the postprandial decline in ghrelin secretion (Crowley *et al*., 2005; Tschop *et al*., 2000; Xu *et al*., 1999). It has been hypothesized that the satiety-inducing effects leptin include the suppression ofghrelin secretion. Indeed, the effects of leptin on energy homeostasis are opposite (although not complementary) to those of ghrelin; leptin induces weight lossby suppression of food in take where asghrelin functions asan appetite-stimulatory signal (Nakazato *et al*., 2001; Ueno *et al*., 2005). In the present study, leptin was associated with ghrelin levels but not oppositely their plasma levels were rise and fall together in prefeeding and post feeding. Plasma ghrelin and leptin levelsinthe highestpeak values reached 2 period (30 days) and both of these hormones in fed twice a day lambs had higher than other groups. Researchers expected that lambs administered with ghrelin by peripherally had low leptin levels in that group but Researchers had not any expected result. However, several studies in humans have produced conflicting results previous studies. For example while Tschop *et al*. (2000) demonstrated that in obese patients fasting plasma ghrelin levels are negatively correlated with fasting plasma leptin levels, in another study fasting plasma leptin and ghrelin concentrations were not correlatedin obese children and adolescents. Whether an elevated level of circulating leptin causes a reduction in ghrelin levels is still not clear. However, it seems that leptin does not have a direct influence on ghrelin levels.

In the present study, *ad libitum* + ghrelin group have showed the highest feed consumption and body weight gain but this group have not showed the highest plasma ghrelin levels. Although, fluctuation in plasma ghrelin concentrations relative to feed consumption has been reported for cattle fed daily, no data have been reported thatobserve plasma ghrelin concentrations for cattle completely deprived of feed. In other experiments where peripheral ghrelin administration has resulted in increase in an feed intake in rodents, injected ghrelin concentrations were considerably greater (Tschop *et al*., 2000; Wern *et al*., 2001). Salfen reported that multiple ghrelin injections over a 6 days period resulted in increased BW in weanling pigs but no significant increase in feed intake. Likewise, Iqbal *et al*. (2006) demonstrated that neither ICV injection of ovine ghrelin nor i.v. injection of human ghrelin in sheep influenced feed intake. Iqbal *et al*. (2006) reported that multiple ICV injections of human ghrelin (2.3 g $kg^{-1}$of BW) did not influence feed intake in sheep. Additional research is needed to further establish the effects of ghrelin on feed intake in ruminants.

CONCLUSION

In this study, researchers reported that plasma ghrelin levels didin't increase before feeding and decrease after feeding in the experimental groups. Moreover, when Researchers administered ghrelin peptide by intravenous, there was no change both ghrelin and leptin levels. Researchers didn't find scheduled-frequency meal feeding effect on lambs. Researchers have not observed any effect on food intake and the results were not the same across species in the lambs. However, the data also showed that ghrelin levels in the highest peak values reached II period of fed twice a day lambs. Researchers determined that systemic (i.v.) injection of ghrelin might affect feed consumption and body weight but not affect feed conversion rate.

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