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Effect of Starvation and Feeding on the Plasma Cortisol, Ghrelin and Lipid Metabolite Concentrations in Lambs

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Abstract: Ghrelin affects not only growth hormone secretion but also nutrient utilization and metabolic hormone secretion in humans and experimental animals. The mechanisms controlling ghrelin secretion during fasting and postprandial suppression are unknown in ruminant species. In this study, researchers aimed to determine the effects of starvation and feeding on the relationship of plasma ghrelin, cortisol concentrations and plasma lipid metabolites in 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 cortisol hormone levels. Ghrelin levels in the highest peak values reached 2, period of fed twice a day lambs and cortisol levels also showed high peak values in animals fed twice a day. As plasma lipid metabolites, fasting NEFA and phospholipids levels showed high values in the same group. At the present study, suggest that the ghrelin may stimulate cortisol hormone as *in vivo* ans increase NEFA and phospholipids levels in ruminant species.

Key words: Ghrelin, cortisol, plasma lipid metabolites, lambs, Turkey

INTRODUCTION

Ghrelin, a 28 amino acid hormone was recently identified in the stomach as the endogenous ligand for the Growth Hormone (GH) secretagogue receptor (Kojima *et al*., 1999). This hormone exerts multiple endocrine and nonendocrine effects, such as stimulation of Prolactin (PRL) and Adrenocorticotropic Hormone (ACTH) secretion, inhibition of the gonadal axis at both the central and peripheral level, stimulation of appetite and of a positive energy balance and influence on sleep and behavior, on gastric motility and acid secretion and on pancreatic exocrine and endocrine function as well as on glucose levels (Van der Lely *et al*., 2004; Ghigo *et al*., 2005; Broglio *et al*., 2006). Ghrelin activity at the pituitary level is not fully specific for GH because it also includes stimulatory effects on both the lactotroph and corticotroph system. Cortisolis a steroid hormone produced by the adrenal gland that increases as part of the body's response to stress. Most studies suggest that cortisol plays a very small role or is not related at all to body fat distribution and excess weight. On the other hand, the stimulatory effect of ghrelin and synthetic GHS on the hypothalamus-pituitary-adrenal axis in humans is remarkable and similar to that of the administration of naloxone, vasopressin and even Corticotropin-Releasing Hormone (CRH) (Van der Lely *et al*., 2004; Ghigo *et al*., 2005; Arvat *et al*., 2001). It seems unlikely that ghrelin plays a role in the regulation of corticotroph function in physiological conditions. In fact, 2-fold increments of plasma ghrelin which reflect physiological fluctuations in healthy subjects do not elicit ACTH levels in humans whereas they stimulate GH secretion. At least 3-fold increase in circulating ghrelin is required to stimulate corticotroph function (Lucidi *et al*., 2005).

Huston *et al*. (1997) also found that the most variable data were observed in the daily-fed group of cows due to aggressive competition during short consumption periods. Cows fed less frequently showed a reduced variation in production. On the other hand, certain evidence indicates that frequent feeding is more favourable than intermittent feeding for weight gain in sheep and cattle (Mochrie and Lucas, 1956), except in drought conditions (Franklin *et al*., 1955; Briggs, 1956;

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Rakes *et al*., 1961; Robards, 1970). These studies also indicated that frequent feeding might be more important to young animals than to mature animals.

Little is known of the role of ghrelin on the feeding frequency and cortisol hormone in species other than humans and rodents. For feeding frequency, the ruminant presents an interesting model because the gut is not emptied between periods of feding. However, there are few studies about the effect of ghrelin on ghrelin's intravenous injection effects in ruminants under feding frequency. In the present study, researchers performed measurements of ghrelin every 30 min before feeding (ghrelin values of fasting) and 1 h after feding in 16 male lambs and researchers studied effects of feeding frequency and ghrelin injection on cortisol and plasma lipids concentrations in lambs.

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). A total of 16 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).

Determination of ghrelin and cortisol 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 at 60 min after feeding (10:00). 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).

Plasma cortisol concentrations were analyzed during just pre-feeding and periods (15 day intervals) in lambs (ng mL^{-1}) and the samples were determined by Enzyme-Linked Immuno Sorbent Assay (ELISA) (ELX808IU Ultra Microplate Reader, BIO-TEK Instruments, INC) with a cortisol kit (Active Non-Extraction Cortisol ELISA DSL-10-2000).

Determination of plasma lipids concentrations: The blood samples were taken from the jugular vein into vacutainers (Becton Dickinson, NJ, USA) 30 min before feeding at 15 days intervals until day 45. The plasma was separated by centrifugation at 2,200 g for 10 min and stored at 20°C until used. Total lipids, triglycerides, phospholipids, total cholesterol, High Density Lipoprotein cholesterol (HDL) and Non-esterified Fatty Asids (NEFAs) were analized as plasma lipids and their plasma levels were determined using commercial kits and an automatic spectrophotometer (Schimadzu UV-1601).

Table 1: Composition of concentrate food distributed to 2 month 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: 0.8g; 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

RESULTS AND DISCUSSION

Changes in plasma ghrelin and leptin levels in lambs subjected to the four different feeding regimens are shown 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.

Plasma cortisol concentrations differed significantly ($p<0.05$) between lambs fed twice a day and lambs fed *ad libitum* ($p<0.05$). Cortisol levels reached the highest peak values during the third period of each experimental group. Although in twice fed animals for all periods, cortisol concentrations decreased after feeding, there were no significant differences between sampling times within groups (Table 4).

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

	Feeding regimen groups			
Sampling time (period)	*Ad libitum*	*Ad libitum* +Ghrelin	Once a day	Twice a day
15 days				
30BMF	4.34±4.21	6.46±4.01	5.05±1.20	7.04±3.00
60AMF	5.01±3.88	5.40±2.54	5.37±0.75	7.18±1.43
30 days				
30BMF	7.70±0.75	7.98±2.16	7.09±0.93	10.04±3.06
60AMF	8.62±1.32	8.94±2.07	8.50±0.88	9.88±1.99
45 days				
30BMF	5.95±0.83	7.54±1.06	6.82±1.26	7.90±2.28
60AMF	5.72±1.95	6.73±0.81	5.58±2.12	6.85±1.75

Feeding time: 09:00 and 16:00 30BMF: 30 min before morning feeding (08:30); 60AMF: 60 min after morning feeding (10:00); $\bar{X}$±SEM represent the ±values

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

	Feeding regimen groups			
Sampling time (period)	*Ad libitum*	*Ad libitum* +Ghrelin	Once a day	Twice a day
15 days				
30BMF	2.05±0.31	6.52±1.21	4.88±0.76	8.21±2.34
60BAF	5.96±1.20	6.27±1.01	3.96±0.82	2.92±0.59
30 days				
30BMF	2.59±0.29	3.33±0.55	2.50±0.69	11.35±4.75
60BAF	5.77±0.82	6.77±1.04	7.33±2.04	6.06±0.79
45 days				
30BMF	8.78±2.97	8.17±1.47	11.36±2.76	9.18±2.44
60BAF	6.43±1.89	4.82±1.54	7.20±3.29	6.11±1.37

Feeding time: 09:00 and 16:00 30BMF: 30 min before morning feeding (08:30); 60AMF: 60 min after morning feeding (10:00); $\bar{X}$±SEM represent the ±values

The effects of feding frequency and ghrelin peptide on plasma lipid metabolites are shown in Table 5. There were no significant differences in fasting total lipid, total cholesterol and HDL-cholesterol between groups and periods. However, there were significant differences *ad libitum* and animals were fed twice a day ($p<0.01$) at 30 day for NEFA levels. The similar result for phospholipids at 30 day, *ad libitum* and animals were fed twice a day showed significantly changes ($p<0.01$) also there were significant differences *ad libitum* and ghrelin injected group ($p<0.05$) in the same period. The plasma triglyceride levels showed also significant differences at 30 day between *ad libitum* and animals were fed once a day ($p<0.001$) between *ad libitum* and animals were fed twice a day ($p<0.001$), lambs fed twice daily and *ad libitum*+ghrelin group were also different significantly ($p<0.01$).

The present study reports on the feding frequency and interrelationship of cortisol, ghrelin and plasma lipid metobolites, such as total lipids, total cholesterol in the ram lambs. Ghrelin values were high in the lambs fed twice a day but researchers expected high values from intravenous injected ghrelin peptid of lambs or fed once a day group. Morever, there was no any different at sampling times, fasting ghrelin values and postprandial ghrelin values were almost same in all groups. However,

Table 5: Plasma lipid metobolites during pre-feeding periods (measured at 15 day intervals) in male lambs subjected to the four different feeding regimens

	Feeding regimen groups			
Plasma lipids (mg dL^{-1})	*Ad libitum*	*Ad libitum* +Ghrelin	Once a day	Twice a day
15 days				
Total lipid	110.62±8.310	91.87±7.090	110.62±9.860	82.50±4.330
Triglycerides	58.33±12.31	58.33±11.22	50.00±4.530	52.77±9.480
Total Cholesterol	55.00±11.01	33.33±11.22	28.33±5.690	58.33±5.690
HDL-Cholesterol	13.56±1.880	11.19±2.000	10.67±1.360	10.80±1.260
NEFA	2.69±0.780	3.11±0.700	7.67±2.090	2.69±1.320
Phospholipids	78.94±8.850	77.63±7.550	56.57±12.03	71.05±6.960
30 days				
Total lipid	137.28±29.50	122.03±9.280	127.11±22.54	129.66±13.37
Triglycerides	75.00±3.580	52.77±14.07	10.20±2.630	11.22±2.560
Total Cholesterol	51.56±15.17	54.68±11.23	57.81±10.63	46.87±7.430
HDL-Cholesterol	11.00±2.940	12.40±2.440	14.23±1.690	16.58±1.060
NEFA	1.65±0.000	2.07±0.530	2.69±0.390	3.52±0.520
Phospholipids	126.38±13.86	80.55±7.340	70.83±12.70	59.72±8.590
45 days				
Total lipid	195.53±15.38	152.67±19.74	152.67±16.58	120.53±20.22
Triglycerides	11.22±4.820	13.26±5.360	19.38±5.100	10.20±2.630
Total Cholesterol	51.56±2.990	54.68±8.600	54.68±8.970	48.43±5.330
HDL-Cholesterol	18.88±2.080	20.39±5.020	16.77±0.650	14.73±3.460
NEFA	4.35±1.320	4.56±1.280	5.39±1.280	3.93±2.010
Phospholipids	108.33±17.64	115.27±14.93	69.44±11.22	90.27±13.86

in earlier studies, a large preprandial rise and a postprandial fall in plasma ghrelin levels were observed in humans and in ruminants (Sugino *et al*., 2002). Also, peripheral infusion of ghrelin in 18 days old pigs did not change feed intake however, body weight gain during the 5 days ghrelin infusion period was greater in treated piglets (Salfen *et al*., 2004) but in the study, intravenous ghrelin injected lambs did not show body weight gain. Researchers have not observed any effect on food intake and the results were not the same across species in the lambs and ruminants may not be responsive to the orexigenic properties of ghrelin.

Ghrelin also stimulates the ACTH and cortisol secretion in humans (Tassone *et al*., 2003; Broglio *et al*., 2004; Takaya *et al*., 2000; Arvat *et al*., 2001) but researchers did not observe a similar effect in sheep with peripheral ghrelin administration. However in rodents, iv injection of ghrelin did not stimulate PRL and ACTH secretion (Tolle *et al*., 2001). Igbal did not observe an effect in sheep with central administration and they concluded that any effects of ghrelin on the secretion of PRL or ACTH in sheep are likely to be at the level of the pituitary gland. Unfortunately, there is very little study about cortisol related to ghrelin in sheep. Ghrelin injection also stimulated corticotroph secretion in Holstein dairy cattle regardless of their physiological state. When we injected ghrelin pheripherally, lambs did not show any effect at cortisol concentrations. However as expected in the study, similar to the ghrelin concentrations, the cortisol concentrations also showed high values in lams fed twice a day, ghrelin and cortisol levels shoed high values in this group. Therefore, ghrelin may stimulate cortisol hormone as *in vivo* in ruminant species.

CONCLUSION

Researchers did not find any significant differences in plasma ghrelin between total lipids, total cholesterol and HDL-cholesterol. However, NEFA and phospholipid levels were high values in lambs fed twice a day like ghrelin and cortisol concentrations and showed significant differences. Therefore, NEFA and phospholipids levels may be related to ghrelin and cortisol concentrations in lambs. There is due to a few study about relationship of lipid metabolites and ghrelin in sheep, researchers could not compare to other studies. However, several studies report that after deprivation of food, plasma Free Fatty Acids (FFA) levels increase approximately 2-fold, in both rodents and humans (Menahan and Sobocinski, 1983; Bergman *et al*., 2006). Researchers found that same results like this studies, the fasting NEFA and phospholipd levels shoed high values, especially in lambs fed twice a day. Because of this, it should be noted that the close relation of ghrelin to phospholipid and NEFA levels.

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