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Ghrelin Gene Expression in Broiler Proventriculus Tissue are Changed by Feed Restriction, Different Dietary Energy and Protein Levels

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Abstract: Problem statement: The aim of this study was to investigate the effects of feed restriction and different energy and protein contents of diet on ghrelin gene expression in broiler chicken. **Approach:** Feeding programs consisted of *ad libitum* and feed restriction, two energy levels (3100 and 2800 kcal ME kg^{-1}) and three protein levels (22.3, 19.3 and 16.3% CP). Feed restriction was applied during 22-32 days of age. Proventriculus samples were collected at 21, 32 and 49 days of age. Ghrelin mRNA expression in proventriculus tissue was quantitate using Real Time quantitative PCR. **Results:** We found that ghrelin gene expression was increased in restricted chicks compared with those fed *ad libitum* at 32 days of age ($p = 0.09$) but feed restriction had no effect on ghrelin gene expression at 49 days of age. A positive response in ghrelin gene expression was achieved by decreasing energy level in the diet at 21 days of age ($p<0.07$) and at 32 days of age ($p<0.05$). Also, we showed that dietary protein had no effect on ghrelin gene expression, whereas there was a tendency for an increase in ghrelin gene expression as protein decreased at total period. **Conclusion:** The present study, we investigated the effects of feed restriction and different energy and protein contents of the diet on ghrelin gene expression in broiler chicken. We have characterized chicken ghrelin cDNA in proventriculus tissue in broiler chicken. We also found that ghrelin gene expression is differently suppressed by diet manipulations. Additional studies are necessary to investigate the role of nutrition on ghrelin gene expression in proventriculus tissue in broiler chicken.

Key words: Feed restriction, energy, protein and ghrelin gene expression

INTRODUCTION

Chicken ghrelin was isolated from the proventriculus as endogenous ligand for the growth hormone secretagogue receptor (Kojima *et al*., 1999), which contains 26 amino acids with the third serine residue from the N-terminus of the mature peptide being acylated with n-octonoic or n-decanoic acid (Kaiya *et al*., 2002). Ghrelin-producing cells have also been detected in the arcuate nuclei of the rat hypothalamus, which is a feeding control center (Kojima *et al*., 1999; Cowley *et al*., 2003). Therefore, ghrelin is thought to have a physiological role in meal initiation by acting as a hunger signal (Cummings *et al*., 2001). Also, ghrelin affects feeding, gastrointestinal function, energy metabolism and cardiovascular function (Kaiya *et al*., 2002). However, it should be emphasized that the final growth expression is the result of interactions between nutritional, environmental and genetic factors interacting with endocrine secretions. The purpose of this study was to determine the effect of feed restriction on ghrelin mRNA expression in proventriculus tissue in broiler chicken. There is no a study on the effect of dietary energy and protein levels on ghrelin gene expression in broiler chicken. Thus, this experiment was conducted to determine whether different dietary energy and protein levels alters ghrelin mRNA expression in broiler chicks.

MATERIALS AND METHODS

Animals and housing: This experiment took place at Poultry Research Station and biotechnology laboratory in Ferdowsi University of Mashhad, Iran, 2009.

Five hundred and seventy six, old Ross male broiler chicks were randomly allocated in equal numbers in 48 floor pens. The six diets were prepared daily and diets were fed from 1-49 days of age.

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Table 1: Feed ingredients and composition of experimental diets (0-49 days of age)

	Treatment diets					
Ingredients (%)	1	2	3	4	5	6
Corn	54.50	61.87	69.38	51.98	56.38	61.73
Soybean meal 44%	28.60	24.92	21.00	34.83	25.17	19.80
Corn gluten	7.95	4.71	1.61	3.00	3.00	0.57
Vegetable oil	3.50	3.00	2.46	1.00	1.00	1.00
Wheat bran	1.50	1.50	1.50	5.67	10.89	13.28
Dicalcium phosphate	1.69	1.74	1.79	1.32	1.39	1.44
Limestone	1.17	1.21	1.24	1.16	1.17	1.20
Vit Min. Premix [1]	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.44	0.44	0.44	0.41	0.41	0.40
DL-Methionine	0.09	0.08	0.07	0.13	0.09	0.08
L-Lysine	0.06	0.03	0.01	-	-	-
Total	100.00	100.00	100.00	100.00	100.00	100.00
Compositions (calculated)						
ME (kcal kg^{-1})	3100.00	3100.00	3100.00	2800.00	2800.00	2800.00
Crud protein (%)	22.30	19.30	16.30	22.30	19.30	16.30
Calcium (%)	0.97	0.97	0.97	0.88	0.88	0.88
Available P (%)	0.44	0.44	0.44	0.39	0.39	0.39
Sodium (%)	0.19	0.19	0.19	0.18	0.18	0.18
Arginine (%)	1.27	1.12	0.97	1.41	1.18	1.01
Lysine (%)	1.07	0.92	0.78	1.14	0.92	0.78
Methionine + Cystine (%)	0.87	0.75	0.64	0.87	0.75	0.64

[1]Provided per kg of diet: vitamin A, 9000 IU; vitamin D_3, 2000 IU; vitamin E, 11 IU; vitamin K_3, 2 mg; thiamine, 1.775 mg; riboflavin, 6.6 mg; vitamin B_3, 9.8 mg; vitamin B_5, 29.7 mg; vitamin B_6, 1.176 mg; vitamin B_9, 1 mg; vitamin B_{12}, 0.015 mg; vitamin H_2, 0.1 mg; choline chloride, 500 mg, Mn, 76 mg; Zn, 66 mg; Fe, 40 mg; Cu, 4 mg; I, 0.64 mg; Se, 0.2 mg

Table 2: Experimental treatments

	Without feed restriction					
Treatment	1 (control)	2	3	4	5	6
Energy level (Kcal ME kg^{-1})	3100	3100	3100	2800	2800	2800
Protein level (%CP)	22.3	19.3	16.3	22.3	19.3	16.3
	With feed restriction (22-32 days of age)					
	7	8	9	10	11	12
Energy level (Kcal ME kg^{-1})	3100	3100	3100	2800	2800	2800
Protein level (%CP)	22.3	19.3	16.3	22.3	19.3	16.3

Note: This table showed experimental treatments with and without feed restriction. Feed restriction was applied during 22-32 days of age. After the feed restriction period, the birds were fed *ad libitum* until end of the experiment (49 days of age)

Feed ingredients and composition of experimental diets were exhibited in Table 1. Broiler chickens were assigned into two feeding programs from 22 until 32 days of age. One feeding program was given free access to feed (*ad libitum*) and the other fed a limited amount of feed (restricted). The restricted birds were fed *ad libitum* every other day and that no restriction was placed on the birds during the days feed was provided. Feed restriction treatments were applied during 22-32 days of age. After the feed restriction period, the birds were fed *ad libitum* until end of the experiment (49 days of age). Also, broilers were assigned randomly to either 3100 or 2800 kcal ME kg^{-1} during different periods (0-49 days of age). Protein levels were set to 22.3, 19.3 and 16.3% during different periods (0-49 days of age). According to the treatment groups, the chickens were arranged in a 2×2×3 factorial arrangements in a completely randomized design experiment (two feeding programs, two energy levels and three protein levels) at 22-32 and 32-49 days of age but there was not feed restriction during 0-21 days of age thereby chickens were arranged 2×3 factorial arrangements in a completely randomized design experimental at 0-21 days of age (two energy levels and three protein levels). Experimental treatments had exhibited in Table 2. Each treatment group consisted of 4 replicates of 12 chickens each. The chickens were randomly allocated in cages and light was provided 24 hours daily at 0-49 days of age. At 21, 32 and 49 days of age, one chicken from each replicate of each treatment that had body weight close to the mean replicate was selected and then slaughtered to collect proventriculus tissue for evaluation ghrelin gene expression. Proventriculus samples were frozen in

liquid nitrogen and stored at -80°C. Experimental diets were formulated to provide similar nutrients content according to the broilers nutrients requirement except for protein and energy levels (Table 1). The experimental diets were based on corn-soybean meal containing vegetable oil. Chickens had free access to fresh water throughout the experiment.

RNA extraction and reverse transcription-PCR assay for ghrelin gene expression: Total RNA was isolated from the chicken proventriculus tissue using Trizol reagent procedure (Invitrogen/Life Technologies, Isogene Co, Russian) according to the manufacturer's instructions. The quantity and integrity of isolated RNA were determined for each sample by using both UV absorbances (260/280) as well as by 1% agarose gel electrophoresis. Then RNA samples were stored at -80°C until use. RNA was treated with DNase using Ambion's DNA-free kit (Fermentas/Life Science/Isogene Co, Russian) to remove any possible DNA contamination. Samples were stored at -80°C until use. Reverse Transcription (RT) Polymerase Chain Reaction (PCR) was performed using a RevertAid first strand cDNA synthesis kit (Fermentas/Life Science/Isogene Co, Russian) containing RNA (5 μg), 20 pmol gene-specific primer and DEPC-treated water. The mixture incubated at 65°C for 5 min. Then 5× reaction buffer, 20 u μL^{-1} RiboLock RNase Inhibitor, 10 mm dNTP mix and 200 u μL^{-1} RevertAid M-MuLV Reverse Transcriptase added to above mixture. After incubation (42°C, 60 min), the mixture was heated (70°C, 5 min) and then stored at -20°C.

A chicken ghrelin fragment (203 bp) was amplified with a sense primer (5-CCT TGG GAC AGA AAC TGC TC-3′) and an anti-sense primer (5′-CAC CAA TTT CAA AAG GAA CG -3′) reported by Richards *et al*. (2006). Chicken 18S as an internal control (fragment size: 148 bp): Sense primer (5′-CGC GTG CAT TTA TCA GAC CA-3′) and an anti-sense primer (5-′ACC CGT GGT CAC CAT GGT A-3′) reported (Paczoska-Eliasiewicz *et al*., 2003). All the PCR products were sequenced.

Ghrelin mRNA quantitation in proventriculus tissue by real-time RT-PCR: A master mix containing SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom), 10 pmol forward primer, 10 pmol reverse primer, cDNA, water was prepared to perform real-time PCR. The following PCR protocol was used on the ABI (Applied Biosystems) 7300 apparatus. Initial steps contain 2 min at 50°C and 10 min at 95°C, followed by two-step amplification program (15 sec at 95°C followed by 1 min at 61°C) repeated 40 times. Quantification was performed using ABI integrated software as previously described (Pfaffle, 2001). 18S ribosomal RNA was chosen as a reference gene. Each PCR run included a no template control and replicates of control and unknown samples. Runs were performed in duplicate. The chicken ghrelin (203 bp) and 18S (148 bp) cDNA were run on a 1% agarose gel and visualized by etidium bromide staining using a UV illuminatore.

Statistical analysis: All analyses were conducted using General Linear Model procedures (GLM) of SAS. Significant differences among individual group means were determined with Duncan's multiple range test (SAS Institute, 2001). Relative expression of Ghrelin mRNA determined using sample delta CT to control delta CT ratio method and then were conducted using GLM of SAS.

RESULTS

Ghrelin mRNA expression: Ghrelin mRNA expression in the broiler chicken proventriculus was detected (Fig. 1). The specificity of the amplified cDNA fragment of chicken ghrelin was verified by agarose gel 1% and sequencing PCR product ghrelin fragment (203 bp).

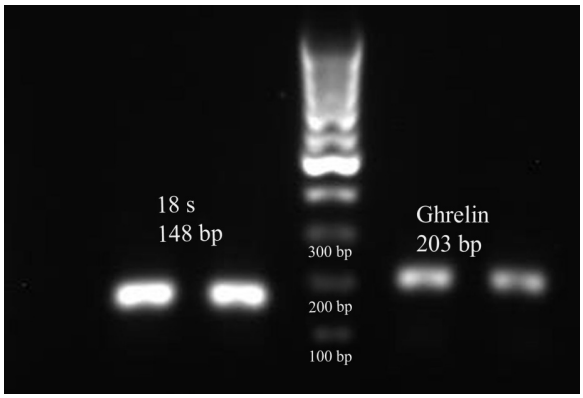

Fig. 1: Expression of ghrelin and 18S ribosomal RNA in proventriculus in broiler chicken. RT-PCR amplified fragments of ghrelin and 18S ribosomal RNA were separated by gel electrophoresis (1%) and produced a 203 and 148 bp fragment of the ghrelin and 18S RNA, respectively

Table 3: Effect of feed restriction and dietary energy and protein levels on ghrelin gene expression in broiler chicken

	Feeding program		Energy (kcal ME kg^{-1})		Protein (%)		
Variable	Feed restricted (n = 24)	*ad libitum* (n = 24)	3100 (n = 24)	2800 (n = 24)	22.3 (n = 16)	19.3 (n = 16)	16.3 (n = 16)
Delta ghrelin/delta 18S ratio, 21 day	-	-	0.94±0.04	1.06±0.04	1.01±0.05^{ab}	0.92±0.05^{b}	1.09±0.05^{a}
Delta ghrelin/delta 18S ratio, 32 day	1.34±0.06	1.19±0.06	1.17±0.06^{b}	1.36±0.06^{a}	1.24±0.07	1.28±0.07	1.27±0.07
Delta ghrelin/delta 18S ratio, 49 day	0.91±0.06	0.98±0.06	0.98±0.06	0.92±0.06	0.91±0.07	0.92±0.07	1.01±0.07

Note: All values are mean ± SEM, Means within rows with different letter superscripts are significantly different ($p < 0.05$); there is no feed restriction at 0-21 days of age thereby there is no feed restriction data at 0-21 days of age. It explained in materials and methods section

Effect of feed restriction, dietrary energy and protein levels on ghrelin gene expression in proventriculus tissue in broiler chicken: The effects of feed restriction, dietrary energy and protein levels on ghrelin gene expression of broiler chicken are presented in Table 3.

We looked at the effect of feed restriction and refeeding on ghrelin mRNA in the proventriculus tissue. Ghrelin gene expression increased in restricted birds compared with those fed *ad libitum* at 32 days of age ($p<0.1$).

There is no a study on the effect of dietary energy and protein levels on ghrelin gene expression in broiler chicken. Decreasing dietary energy increased ghrelin gene expression at 21 ($p<0.07$) and 32 ($p<0.05$) days of age. Dietary protein had no effect on ghrelin gene expression, whereas there was a tendency for an increase in ghrelin gene expression as protein decreased at total period.

DISCUSSION

This is the first report to demonstrate ghrelin gene expression influence by different dietary energy and protein levels in broiler chicken.

The purpose of this experiment was twofold (1) to further study the use of a two-step RT-PCR in a real time mode to determine ghrelin gene expression in proventriculus tissue and (2) to extend our studies on the relationship of feed restriction and dietary protein and energy status with ghrelin gene expression in broiler.

Results of the present study are in agreement with Kaiya *et al*. (2002) who reported ghrelin gene expression in proventriculus in 8-day-old chickens using RT-PCR. Wada *et al*. (2003) observed ghrelin mRNA expression only in the proventriculus of newly hatched Leghorn chicks, whereas in adult chickens, mRNA expression was also detected in proventriculus and duodenum. This is similar to the tissue expression patterns reported previously for 8-week-old White Leghorn chickens (Tanaka *et al*., 2003) and 10 day-old broiler chicks (Geelissen *et al*., 2003).

Also, Richards *et al*. (2006) determined changes in ghrelin mRNA levels in proventriculus, pancreas and brain under different energy balance states created by fasting and refeeding of 3 week-old broiler chickens. They showed fasting (negative energy balance) elevated ghrelin gene expression in proventriculus tissue. In present experiment, expression of ghrelin mRNA in the proventriculus tissue returned to the control levels after refeeding at 49 days of age. This was an expected observation, which agreement with Kaiya *et al*. (2007) and Richards *et al*. (2006) who have reported the expression of ghrelin mRNA in the proventriculus returned to the control levels after reseeding. This study demonstrated that the ghrelin mRNA expression in proventriculus were altered in response to feeding states in broiler chicken in a similar manner as in rat and human, suggesting a role of ghrelin as a hunger signal.

Effect of diet manipulation on ghrelin gene expression was studied only in rat and human. Asakawa *et al*. (2003) indicated gastric ghrelin mRNA expression during fasting was increased by a high fat diet. Therefore, these findings show that ghrelin gene expression is differently suppressed by diet manipulations. Stomach ghrelin expression parallels ghrelin secretion. Monteleone *et al*. (2003) indicated that plasma ghrelin changes were significantly associated with hunger changes. Beck *et al*. (2002) had shown that ghrelin plasma concentration was low when a high-fat diet is ingested for a long period of time and that it increased when the amount of carbohydrates in the diet increased. The levels of ghrelin were therefore linked to the fat content of the diet and the low ghrelin levels observed in rats ingesting a high fat diet may contribute to limit the energy intake provided by this calorie-rich food. Ghrelin secretion was therefore very sensitive to the diet composition.

CONCLUSION

In summary, we investigated the effects of feed restriction and different energy and protein contents of the diet on ghrelin gene expression in broiler chicken. We have characterized chicken ghrelin cDNA in proventriculus tissue in broiler chicken. We also found that ghrelin gene expression is differently suppressed by diet manipulations. Additional studies are necessary

to investigate the role of nutrition on ghrelin gene expression in proventriculus tissue in broiler chicken.

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