Molecular Characterization of Ghrelin gene in sheep of Saudi Arabia

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Abstract
The main role of Ghrelin hormone is the regulation of feed intake, body weight and gastrointestinal motility. It stimulates appetite by acting on the hypothalamic arcuate nucleus. Ghrelin is orexigenic that transmits a hunger signal from the periphery to the central nervous system and is involved in mealtime hunger and meal initiation. The aim of the present study was to characterize the GHRL gene in two Saudi sheep breeds locally named as Najdi and Naeimi. A fragment of 112 bpcomprisingparts of exon 1 and intron 1 of the Ghrelin gene was amplified and sequenced. Our mutations were detected at positions (74,92, 99 and 108) of the intron1. In addition, the four mutations were used to construct different haplotypes, three haplotypes were recovered: H1 (TAAA), H2 (CGGG), and H3 (TGAA).

The H1 (TAAA) was the most common haplotype found in Najdi and Naeimi individuals with frequency of 0.73 for both sheep breeds. The H2 haplotype (CGGG) was unique for Najdi sheep with frequency of 0.21. H3 haplotype (TGAA) was represented in Najdi and Naeimi sheep with frequencies of 0.06 and 0.27 respectively. The present study provides basic information to understand the genetic Characterization of local sheep breeds in Saudi Arabia.

Keywords: Ghrelin, Najdi and Naeimi sheep, Single nucleotide polymorphism.

Introduction

The *GHRL* gene containing 2 exons and 5 introns produces mature ghrelin mRNA which has four exons. The mRNA is processed into a 117-amino acid preproghrelin. The latter is cleaved to produce proghrelin which is cleaved to produce a 28-amino acid ghrelin (unacylated) and C-ghrelin (acylated). Obestatin is presumed to be cleaved from C-ghrelin[19].The 28-amino acid ghrelin hormone is activated in which the third amino acid, usually a serine but in some species a threonine, is modified by a fatty acid[4]. It becomes active by the enzyme ghrelin O-acyltransferase (GOAT) located on the cell membrane of ghrelin cells in the stomach and pancreas[4]. The desacyle form of ghrelin does not activate the GHSR receptor but does have other effects e.g. cardiac[2], anti-ghrelin[3], appetite stimulation[23] and inhibition of hepatic glucose output[10]. The main role of this hormone is the regulation of feed intake, body weight, and gastrointestinal motility[23]. It stimulates appetite by acting on the hypothalamic arcuatenucleus[14], a region known to control food intake[18].

Ghrelin is orexigenic that transmits a hunger signal from the periphery to the central nervous system[6] and is involved in mealtime hunger and meal initiation[18]. The more ghrelin is injected into experimental animals, the more food is consumed[25]. However, ghrelin does not increase meal size, only meal number[9]. In ruminants, several reports have been published to investigate the physiological characteristics of ghrelin. The direct evidence of ghrelin to secrete growth hormone from anterior pituitary cells in cattle was offered in the *in vitro* study[13]. Sugino et al[21] showed that a transient surge in plasma ghrelin levels occurred just prior to a scheduled meal and pseudo-feeding in sheep and that this transient surge was modified by the feeding regimen. Sheep are one of the most important domestic livestock species in Saudi Arabia with more than 11.5 million head[8] and play an important role in the livelihood of local community since they are a good source of meat, milk and coarse wool.

Several sheep populations are named in Saudi Arabia according to their area of origin or based on some morphological characteristics. These breeds are known for their hardiness and adaptability to the prevailing adverse environment of Saudi Arabia. Najdi sheep is considered the breed of choice followed in order by Naeimi and harri breeds[1]. Genetic variability assessment of Saudi Arabian sheep is important to preserve genetic resources and to develop future breeding programs to improve sheep populations. The genetic diversity and population genetic structure of sheep are poorly documented in Saudi Arabia.

Therefore, manipulation of ghrelin axis has potential for improving economically valuable traits in production animals and polymorphisms in the ghrelin (GHRL) and ghrelin receptor (GHSR) genes have been associated with growth and carcass traits. The aim of this study was to characterize the polymorphism in Ghrelin gene in parts of exon 1 and intron 1 in Najdi and Naeimi sheep breeds of Saudi Arabia and benchmark the results with some of other populations in the world.

Material and Methods

Samples collection and DNA extraction: A total of 101 sheep were selected from eight flocks representing Najdi (68) and Naeimi (33) breeds. Blood samples (10 mL) were collected from each sheep by jugular venipuncture into vacuum EDTA tubes. Genomic DNA was extracted using the QIAgenDNeasy blood and tissue DNA extraction kit

(Hilden, Germany) following the manufacturer's instructions. The quantity and quality of DNA were checked by spectrophotometer (Jenway Genova Spectrophotometer Krackler Scientific Incorporation, USA). The O.D. ratios were between 1.7 and 1.9 indicating high quality DNA as indicated by Sambrook et al.[17]

Amplification of exon 1: A fragment of 112 bp comprising parts of exon1 and intron 1 regions of Ghrelin gene was amplified using the following two PCR primers: F-(5'CCTGCTCTGGATGGACTTGGC-3') and R- (5' GGCTTGGGGCATTTAGGACG-3') according to Tahmoorespur et al.[22] Polymerase Chain Reaction (PCR) amplifications were carried out in a 25-µl reaction volume containing 100 ng of template DNA and 2 µl of each 10 µM primer. To reduce the possibility of cross contamination and variation in the amplification reactions, master mixes containing all PCR reagents including the KapaTaq polymerase enzyme (KAPA Biosystems, Boston, MA, USA) except DNA template and primers were used. The amplification program was performed using the Gene Amp PCR system 9700 thermocycle (Applied Biosystems, Warrington, UK).

The amplification protocol was initial denaturation step for 2 min at 94°C, followed by 35 cycles at 94°C for 30s, 60°C annealing step for 30s and extension at 72°C for 1 min. The final step of the amplification protocol was the extension step at 72°C for 5 min. Electrophoresis of the PCR products was done using 1% agarose gel and bands were detected by UV lamp after syber safe staining through gel documentation system (Amersham Biosciences, Uppsala, Sweden).

DNA Sequencing and Sequence Analysis: PCR products of Ghrelin parts of exon 1 and intron 1 gene were cleaned and sequenced by the Advanced Genetic Technologies Center (http://www.uky. edu/Centers/AGTC/). The DNA sequences were edited and aligned using BioEdit software (http://www.mbio. ncsu.edu/Bioedit/bioedit.html).[12] The BLAST algorithm was used to search the NCBI GenBank database (http:www.ncbi.nlm.nih.gov) for homologous sequences. The BioEdit software was also used to detect SNPs and indel mutations.

Results and Discussion

The amplification of GHRL region spanning parts of exon1 and intron1 generated a PCR product of 112 bp in length from 68 Najdi and 33 Naeimi sheep breeds. To confirm if there are genuine single nucleotide length polymorphisms between the exogenous and indigenous sheep breeds, a genetic map was developed based on different GHRL sequences of Ovisaries retrieved from literature or GenBank database e.g. AY455983.1, GU014694.1, AY455979S1, GU071075.1, AY455944S1, AY454076S1, AY454075.1 and AY455990S1 and BioEdit software was used to align these sequences. The aligned DNA sequences of GHRL region showed no SNPs in the exon region. However, four SNPs (T74C, A92G, A99G and A108G) were detected in the intron1 region. All the detected mutations were only recorded in Najdi sheep. Almost the frequencies of the A allele in the three positions, A92G, A99G and A108G were similar in Najdi sheep breed with higher values than the G allele (Table 1). At position T74C, allele T was higher than allele C in Najdi sheep breed whereas the C allele was not present in Naeimi sheep breed. The genotypes of the four SNPs detected in GHRL region in Saudi sheep breeds are shown in table 2.

When the four SNPs were used to construct different haplotypes, three haplotypes were recovered: H1 (TAAA), H2 (CGGG) and H3 (TGAA). The H1 (TAAA) was the most common haplotype found in Najdi and Naeimi individuals with a frequency of 0.73 for both sheep breeds. The H2 haplotype (CGGG) was unique for Najdi sheep with frequency of 0.21. H3 haplotype (TGAA) was represented in Najdi and Naeimi sheep with frequencies of 0.06 and 0.27 respectively.

Based on H1, H2 and H3 haplotypes, three different genotypes, two homozygous (G1 and G2) and one heterozygous (G3) were observed in the present study (Figure1). G1 was the most frequent genotype represented by 74 animals including 50 Najdi and 24 Naeimi with frequency of 0.73 for both sheep breed followed by G2 that was represented by 14 animals from only Najdi breed with frequency of 0.21. The heterozygote genotype (G3) contained 4 Najdi and 9 Naeimi sheep with frequencies of 0.06 and 0.27 respectively. The *GHRL* gene parts of exon 1 and intron 1sequences in Saudi sheep were searched against other *GHRL* sequences deposited in NCBI-GenBank database (Figure 2). The representative *GHRL* gene parts of exon 1 and intron 1 sequences from sheep that deposited in GenBank under accession no. AY455983 were identical to the most frequent haplotype 1 recovered from the indigenous Najdi and Naeimi sheep breeds.

Elkorshy et al[7] used same primer of the ghrelin gene amplified from ovine genomic DNA and then sequenced to identify the polymorphism in exon1 to the ovine ghrelin gene. The samples from three Egyptian sheep breeds (Barki, Rhmani and Saidi) and two Saudi sheep breeds (Najdi and Harri) were analyzed. The sequence analysis indicated three nucleotide substitutions (T to C; A to G and A to C) at position 38, position 49, position 108, position 60 and position 71 in Rahmani (Figure 2). The six different detected variables were present in the Saidi sheep breed at different frequencies with a majority of five transversion and one transition (G to T, A to T, A to T, G to C, G to T) at position 14, position 56, position 58, position 67, position 60 and 71 respectively. However, they detected two mutations in Najdi sheep and these mutations were different from the present study in Najdi sheep breed, they found allele A at position 93 and allele T at position 96, on the other hand, they found only one mutation in position 39 (allele A) in Harri sheep.

Tahmoorespur et al[22] used same primer and showed the ghrelin gene was not polymorphic in Baluchi sheep. Similar works have been reported for variety of animal species, the representative *GHRL* gene sequences from Bos Taurus, Bubalusbubalis, Odocoileushemionus, Bison bison, Antilocapra Americana and Cervuselaplus deposited in Genebank under accession no. AY455979s1, GU071075.1, AY455994s1, AY454076s1, AY4540751.1 and AY455990s1 respectively. One SNP (G) was detected at position 99 from the indigenous Najdi and Naeimi sheep in the present study whereas the SNP(C) in position 74 in the present study was detected in five out of six of this accession no. Allele (G) in position 92 was also detected in four out of six of these accessions no. and was also detected in Genebank under the accession no. GU014694.1

Interestingly, as reported in most of studies related to this subject, the DNA polymorphisms in the *GHRL* exon 1 and intron 1 gene were associated with economical traits. Gil et al[11] studied the association between GHRL and milk traits. They identified 2 SNPs located in the intron 1 in the positions 138 and 272, constituting the haplotypes GG and AC in Murrah buffaloes. *GHRL* gene has also been analyzed on other cattle breeds and the similar frequency of the G allele was found in non coding region: 0.15, 0.12, 0.03, 0.19 and 0.24 for Angus, Charolais, Holstein, Belgian Blue and Simmental cattle respectively[5,20].

Colinet et al[5] identified eight SNPs in the bovine ghrelin gene. All of them are located in the non-coding parts of the gene. Also in goats, SNP in the GHRL gene was found and it is also located in the non-coding sequence[16]. The role of GHRL in feed efficiency is important because it has been shown to play roles in determining if fats or carbohydrates are the metabolic substrate used for maintenance of energy balance as seen in GHRL knockout mice[24]. Jarkovska et al[15] showed the role of GHRL in GH release, it has also played important roles in the stimulation of appetite and feeding activity through interactions with peptides such as neuropeptide Y.

Table 1
Allele frequencies of the 4 SNPs of *GHRL* region detected in Saudi sheep breeds

Breed/Allele	T74C		A92G		A99G		A108G	
	T	C	A	G	A	G	A	G
Najdi	0.79	0.21	0.77	0.23	0.79	0.21	0.79	0.21
Naeimi	1	00	0.27	0.73	00	1	00	1

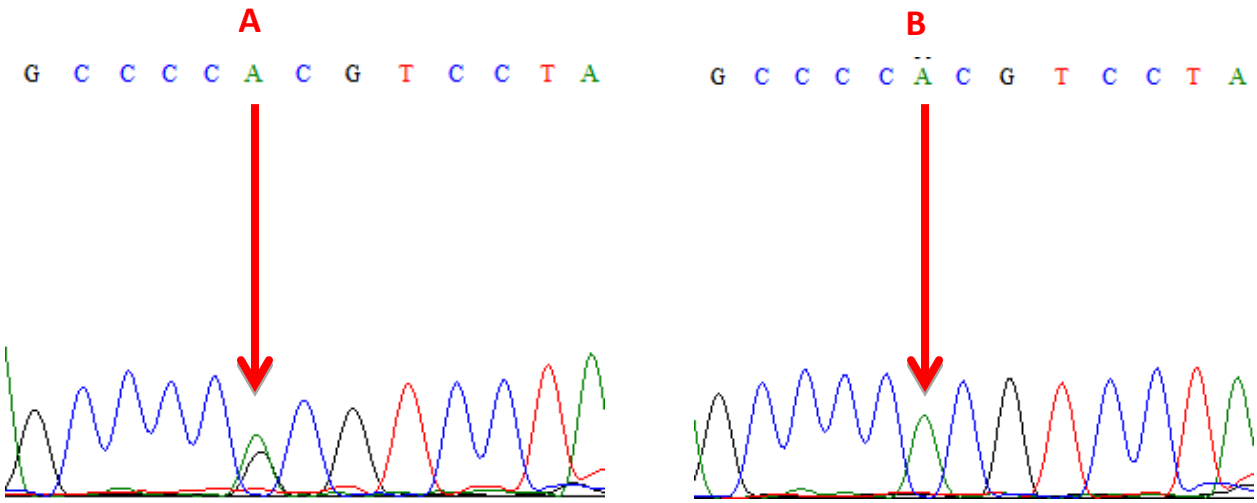

Figure 1: Chromatographs showing genetic variations in the DNA sequence of *GHRL* gene. (A)Overlapping of A/G peaks representing heterozygous individual and (B) Homozygous individual at the same position.

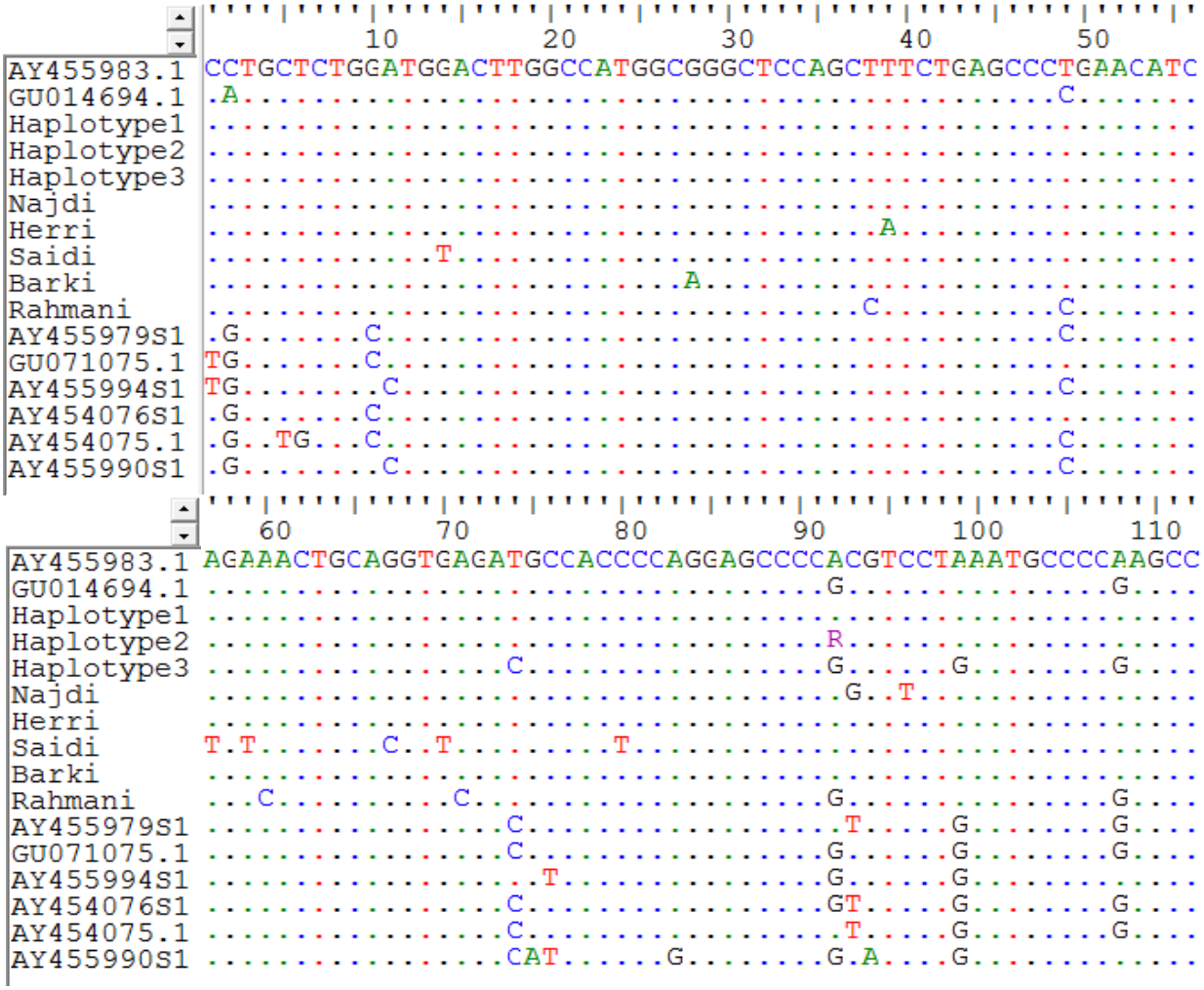

Figure 2: Alignment of *GHRL* sequences generated from sheep (this study) and other animals (retrieved either from GenBank database or literature). Identical sequences are represented by dots and polymorphism is represented by the corresponding one-letter symbol of nucleotides.

Table 2
Genotypes of the 4 SNPs detected in GHRL region detected in Saudi sheep breeds

Breed	T74C			A92G			A99G			A108G		
	Genotypes			Genotypes			Genotypes			Genotypes		
	TT	TC	CC	AA	AG	GG	AA	AG	GG	AA	AG	GG
Najdi	54	00	14	50	04	14	54	00	14	54	00	14
Naeimi	33	00	00	00	09	24	00	00	33	00	00	33
Total	87	00	14	50	13	38	54	00	47	54	00	47

Conclusion

The present study provided basic information to understand the genetic diversity of local sheep breeds in Saudi Arabia. Sequence analysis revealed four SNPs in intron 1 of the Najdi and Naeimi *GHRL* gene. Larger samples from each sheep breed are needed to be examined in order to investigate the impact of DNA polymorphisms on Ghrlein activity and function.

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References

1. Abouheif M., Abdo G., Basmaeil S. and Alsobayel A., Identification of the preference patterns of different breeds of sheep for consumption in Saudi Arabia, *Asian Australian J Anim. Sci*., **2(2)**, 129-132 **(1989)**

2. Bedendi I., Alloatti G., Marcantoni A., Malan D., Catapano F., Ghé C., Deghenghi R., Ghigo E. and Muccioli G., Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln 14-ghrelin, *Eur. J. Pharmacol.*, **476(1)**, 87-95 **(2003)**

3. Broglio F., Gottero C., Prodam F., Gauna C., Muccioli G., Papotti M., Abribat T., van der Lely A. J. and Ghigo E., Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans, *J Clin Endocrinol Metab.*, **89(6)**, 3062-3065 **(2004)**

4. Castañeda T., Tong J., Datta R., Culler M. and Tschöp M., Ghrelin in the regulation of body weight and metabolism, *Front Endocrinol.*, **31(1)**, 44-60 **(2010)**

5. Colinet F., Portetelle D. and Renaville R., Molecular characterization of the bovine GHRL gene, *Arch. Tierzucht.*, **52(1)**, 79-84 **(2009)**

6. Dickson S.L. and Luckman S.M., Induction of c-fos Messenger Ribonucleic Acid in Neuropeptide Y and Growth Hormone (GH)-Releasing Factor Neurons in the Rat Arcuate Nucleus Following Systemic Injection of the GH Secretagogue, GH-Releasing Peptide-6 1, *Endocrinology*, **138(2)**, 771-777 **(1997)**

7. Elkorshy N., Mahrous K.F. and Salem L.M., Genetic polymorphism detection in four genes in Egyptian and Saudi sheep breeds, *World Appl. Sci. J.*, **27(1)**, 33-43 **(2013)**

8. FAOstat **(2013)**

9. Faulconbridge L. F., Cummings D. E., Kaplan J. M. and Grill H. J., Hyperphagic effects of brainstem ghrelin administration, *Diabetes*, **52(9)**, 2260-2265 **(2003)**

10. Gauna C., Delhanty P. J., Hofland L. J., Janssen J. A., Broglio F., Ross R. J., Ghigo E. and van der Lely A.J., Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes, *J Clin Endocrinol Metab.*, **90(2)**, 1055-1060 **(2005)**

11. Gil F., Souza F., Fonseca L. and Tonhati H., Polymophisms in ghrlgene in murrah buffaloes (Bubalus bubalis), *Rev Med Vet.*, **21(1)**, 408 **(2010)**

12. Hall T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp. Ser.*, **41**, 95-8 **(1999)**

13. Hashizume T., Horiuchi M., Tate N., Nonaka S., Kojima M., Hosoda H. and Kangawa K., Effects of ghrelin on growth hormone secretion from cultured adenohypophysial cells in cattle, *Endocrine J.*, **50(3)**, 289-295 **(2003)**

14. Hewson A. and Dickson S., Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats, *J Neuroendocrinol.*, **12(11)**, 1047-1049 **(2000)**

15. Jarkovska Z., Krsek M., Rosicka M. and Marek J., Endocrine and metabolic activities of a recently isolated peptide hormone ghrelin, an endogenous ligand of the growth hormone secretagogue receptor, *Endocr. Regul*., **38(2)**, 80-86 **(2004)**

16. Jin Q., Fang X., Zhang C., Yang L., Sun J., Chen D., Shi X., Du Y., Lan X. and Chen H., A novel SNP of the GHRL gene in goat and its association with growth traits, *Small Ruminant. Res.*, **90(1)**, 150-152 **(2010)**

17. Sambrook J., Fritsche E. and Maniatis T., Molecular Cloning: a Laboratory Manual 2NDed Cold Spring Harbor, NewYork, Cold Harbor Laboratory Press, USA **(1989)**

18. Schwartz M. W., Woods S. C., Porte D., Seeley R. J. and Baskin D. G., Central nervous system control of food intake, *Nature*, **404(6778)**, 661-671 **(2000)**

19. Seim I., Amorim L., Walpole C., Carter S., Chopin L. K. and Herington A. C., Ghrelin gene-related peptides: Multifunctional endocrine/autocrine modulators in health and disease, *Clin Exp. Pharmacol. Physiol.*, **37(1)**, 125-131 **(2010)**

20. Sherman E., Nkrumah J., Murdoch B., Li C., Wang Z., Fu A. and Moore S., Polymorphisms and haplotypes in the bovine neuropeptide Y, growth hormone receptor, ghrelin, insulin-like growth factor 2 and uncoupling proteins 2 and 3 genes and their associations with measures of growth, performance, feed efficiency, and carcass merit in beef cattle, *J. Anim.*, **86(1)**, 1 **(2008)**

21. Sugino T., Hasegawa Y., Kikkawa Y., Yamaura J., Yamagishi M., Kurose Y., Kojima M., Kangawa K. and Terashima Y., A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep, *Biochem. Biophys. Res. Commun*., **295(2)**, 255-260 **(2002)**

22. Tahmoorespur M., Taheri A., Valeh M. V., Saghi D. A. and Ansary M., Assessment Relationship Between Leptin and Ghrelin Genes Polymorphisms and Estimated Breeding Values (EBVs) of Growth Traits in Baluchi Sheep, *J. Anim. Vet. Adva.*, **9(19)**, 2460-2465 **(2010)**

23. Toshinai K., Yamaguchi H., Sun Y., Smith R. G., Yamanaka A., Sakurai T., Date Y., Mondal M. S., Shimbara T. and Kawagoe T., Des-acyl ghrelin induces food intake by a mechanism

independent of the growth hormone secretagogue receptor, *Endocrinology*, **147(5)**, 2306-2314 **(2006)**

24. Wortley K. E., Anderson K. D., Garcia K., Murray J. D., Malinova L., Liu R., Moncrieffe M., Thabet K., Cox H. J. and Yancopoulos G. D., Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference, Proc. Natl. Acad. Sci., USA, **101(21)**, 8227-8232 **(2004)**

25. Wren A., Small C., Ward H., Murphy K., Dakin C., Taheri S., Kennedy A., Roberts G., Morgan D. and Ghatei M., The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion, *Endocrinology*, **141(11)**, 4325-4328 **(2000)**.

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