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INVESTIGATION OF LEPTIN GENE POLYMORPHISM IN IRANIAN NATIVE CATTLE

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Summary

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Leptin, a peptide hormone secreted by adipose tissue cells, has been implicated in regulation of feed intake, energy balance, fertility, immune functions, and the neuroendocrine axis in rodents, humans and large domestic animals. The objective of present study was to determine the polymorphic patterns of leptin gene in Iranian native cattle using the PCR-RFLP technique. Bull semen specimens were collected from 132 Iranian native cattle and genomic DNA was extracted. PCR-RFLP method was used for amplification and determination of the leptin gene polymorphism. PCR was carried out between exon 2 (intron 2) and exon 3 (intron 3) and a 422 bp fragment was amplified. Two alleles *(A* and *B)*, and three genotypes – *AA*, *AB* and *BB* – were observed in the studied population. The frequencies of *A* and *B* alleles were 77.27% and 22.73%, respectively. Furthermore, the frequencies of *AA*, *AB*, and *BB* genotypes were 59.09%, 36.36% and 4.55%, respectively. The results of this study showed that the *AA* genotype and the *A* allele were highly prevalent ($P<0.01$) in Iranian native cattle and that the homozygosity for *AA* genotype of leptin gene could be used as a genetic marker in Iranian native cattle.

Key words: Iranian native cattle; leptin, PCR-RFLP, polymorphism

INTRODUCTION

Leptin is a globular protein with a tertiary structure similar to a haemopoietic cytokine synthesized by adipose tissue. It is involved in regulation of feed intake, foetal growth, energy balance, fertility, and immune functions (Glaum *et al.*, 1996). The leptin molecule (16 kDa) is made up of 167 amino acids with an N-terminal secretory signal sequence of 21 amino acids (Glaum *et al.*, 1996). It has a four-helical structure of about 5–6 turns long, which exhibits an up-up-down-down folding pattern (Zhang *et al.*, 1997). There are two long loops connecting helices B to C and the connecting loops wrap around the BD face of the helix bundle and the inter-helical angles (Imagawa *et al.*, 1998; Mantzoros & Moschos, 1998).

In cattle, the leptin gene is located on chromosome 4. It consists of three exons and two introns. Only two exons are translated into the protein. The coding region of the leptin gene (501 nucleotides in length) is contained in exons 2 and 3, which are separated by an intron of approximately 2 kb. The leptin gene promoter region spans approximately 3 kb (Zadworny & Kuhnlein, 1990).

Leptin has a role in the onset of puberty and the sexual development (Hoggard *et al.*, 2001). It stimulates the reproductive system in both sexes through an

increased release of the pituitary luteinising hormone and the hypothalamic gonadotropin releasing hormone (Barash *et al.*, 1996). Based on these findings it had been suggested that food availability is the most important factor influencing mammalian reproduction (Hileman *et al.*, 2000). Leptin circulates in blood serum in both free and bound forms (Mantzoros & Moschos, 1998). The free form is the biologically active form, while the other is bound to a carrier protein. The balance between free and bound leptin is a potential regulator of leptin bioavailability (Lahlou *et al.*, 2000). Leptin is released into the blood and transported to the brain. The brain then determines the amount of energy the body will expend. Rodriguez *et al.* (2002) discuss the relationship between serum leptin levels in the blood and body fat in both humans and rodents.

Variations at DNA level contribute to the genetic characterization of livestock populations and this may help identifying possible hybridization events as well as past evolutionary trends. Variation in the exonic region of a gene may lead to changes in amino acids which alter the expressed protein, and although intronic variation does not change the amino acid sequence of the protein it may play a significant role in gene splicing or the binding of regulatory proteins during transcription. In livestock, such variations in DNA may also be associated with, or linked to, economic traits, which are governed by many genes each having a small effect (Gelderman, 1997).

Polymorphisms in the bovine leptin gene have been described in association with fat deposition in beef cattle, feed intake, foetal growth, energy balance, fertility, and immune functions (Fitzsimmons *et al.*, 1998; Haegeman *et al.*, 2000). Selection for milk production has a negative influence on the fertility of dairy cows (Pryce *et al.*, 2000). Dairy cows have a slight to severe negative energy balance during early lactation, which influences the duration of the postpartum anoestrus. Since evidence suggest a genetic correlation between start of luteal activity and energy balance, milk yield and live weight, in which only phenotypic records were analysed, it could be hypothesised that polymorphisms at the leptin gene locus might play a role. If associations between leptin polymorphisms and milk yield, live weight, feed intake, or fertility exist, these associations will provide insight into the underlying mechanisms of leptin, and results may be used in future breeding programs (Veerkamp *et al.*, 2000).

Until recently, direct selection of bulls for specific alleles has been limited, mainly because of the lengthy and costly progeny-testing procedures required. However, currently available molecular genetic techniques allow for direct genotyping for candidate genes using polymerase chain reaction (PCR) (Sharifzadeh & Doosti, 2010).

The aim of present study was to determine the frequency of leptin gene polymorphism in Iranian native cattle using PCR restriction fragment length polymorphism (RFLP) technique.

MATERIALS AND METHODS

Samples collection

In the present study, semen specimens were randomly obtained from 132 Iranian native cattle in animal husbandries and rural regions, between September 2010 and March 2011, being especially careful to avoid cross-contamination with bacteria

present in the prepuce. Prior to sampling, the prepuce was washed with detergent, warm water and 1% benzalkonium chloride solution and dried with sterile cotton. Semen samples were diluted according to standard procedures and sent to the Biotechnology Research Centre of Islamic Azad University, Shahrekord Branch in refrigerated boxes. All semen specimens were stored at –70°C for further use.

DNA extraction

Total DNA was extracted from semen specimens using DNP^{TM} Kit (CinnaGen, Iran), according to the manufacturer's recommendations. The total DNA was measured at 260 nm optical density (Sambrook & Russell, 2001). The extracted DNA of each sample was kept frozen at –20°C until used.

Gene amplification

Amplified region is located in the intron between two exons of leptin. The genomic bovine leptin sequences, which consist of three exons, were obtained from GenBank (accession No. U50365). The used primers were those from the study of Liefers & Veerkamp (2002) for detection of bovine leptin gene in bull's semen samples. Bovine leptin gene was amplified using the following oligonucleotide primers: Lep-F-5'-TGGAGTGGCTTGTTATTTTCTTCT-3' and Lep-R-5'-GTCCCCGCTTCTGGC TACCTAACT-3'. PCR reaction was performed in a final volume of 25 µL containing 40 ng of template DNA, 20 pmol of each primer, 10× PCR buffer (16 mM $(NH4)_2SO_4$, 67 mM TrisHCl pH 8.8, 0.1% Tween-20), 1 mM $MgCl_2$, 0.25 mM of dNTPs, and 1 U of *Taq* DNA polymerase (Fermentas, Germany). The tubes containing this solution were inserted in a Gradient Palm Cycler (Corbett Research, Australia). The amplification program consisted of an initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min, and a final extension of 72°C for 5 min. Then, amplified samples were held at 4°C.

Analysis of PCR products

The amplified fragments were detected in 1% agarose gel electrophoresis. The electrode buffer was 1×TBE containing 89 mM Tris-base 10.8 g, 89 mM boric acid 5.5 g, 2 mM EDTA 4 ml of 0.5 M EDTA (pH 8.0). Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The 100 bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragment. After electrophoresis, the gel was stained with ethidium bromide, examined under UV light and photographed in a UVIdoc gel documentation systems (UK).

Leptin gene polymorphism

Restriction fragment length polymorphisms (RFLPs) were used for analysis of leptin gene polymorphisms. Each PCR product was digested with *Sau3A*I enzyme (Fermentase GmbH, Germany) in a total volume of 20 µL (10 µL reaction solutions, 2 µL enzyme buffers, 0.2 µL enzyme, and 7.8 µL distilled water) and placed in the incubator at 37 °C for 4 h. The fragments were separated on a 2% agarose gel by electrophoresis.

Statistical analysis

Analysis of polymorphic patterns of leptin gene was performed using the SPSS version 17.0 computer software (SPSS Inc. Chicago, IL, USA). A 5% level of significance was used in the analysis and its 95%

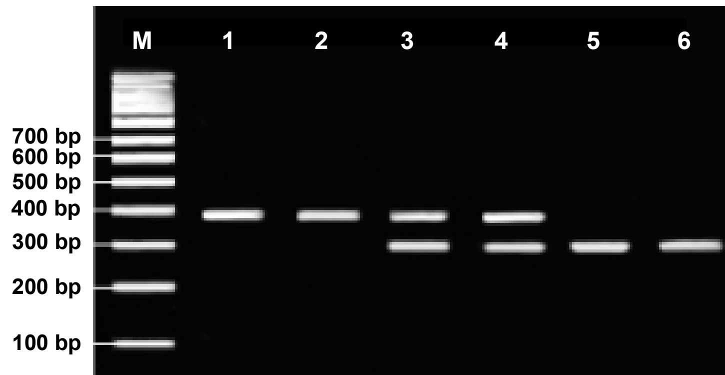

Fig. 1. Gel electrophoresis of PCR products after digested with *Sau3A*I restriction enzyme for detection of leptin gene polymorphism in Iranian native cattle. Lane M: 100 bp DNA ladder (Fermentas, Germany); lanes 1 and 2: *AA* genotype (390 and 32 bp size), lanes 3 and 4: *AB* genotype (390, 303, 88 and 32 bp size); lanes 5 and 6: *BB* genotype (303, 88 and 32 bp size).

confidence interval (CI) was calculated to measure the observed genotype and allele frequencies.

RESULTS

In this study, the PCR-RFLP technique was used to present *A* and *B* alleles of the leptin gene in Iranian native bulls. PCR products of leptin gene on agarose gel showed a fragment of about 422 bp (base pair). One RFLP in the intron between two exons of the bovine leptin gene was detected. There were two *Sau3A*I sites in amplified fragments. The *AA* genotypes showed two fragments of 390, and 32 bp and the *BB* genotype exhibited 303, 88 and 32 bp. Furthermore, 390, 303, 88 and 32 bp fragments were revealed for *AB* genotypes (only 303 and 390 bp fragments were visible on agarose gel). Fig. 1 shows the restriction patterns of the three genotypes *AA*, *AB*, and *BB* in 132 Iranian native cattle.

The number of individuals with different genotypes and allele frequencies of leptin gene polymorphism of Iranian native cattle are shown in Table 1.

The results in native population cattle showed that the most frequent genotype for leptin gene was *AA*. The frequency of *A* and *B* alleles in Iranian native cattle were 77.27% and 22.73%, respectively. The respective numbers and frequencies of *AA*, *AB*, and *BB* genotypes in Iranian native cattle were 78 (59.09%), 48 (36.36%), and 6 (4.55%). The percentage of the *AA* genotype was statistically significantly different (P<0.01) vs those of both *AB* and *BB* genotypes.

Table 1. The genotypes and alleles frequencies of leptin gene in Iranian native cattle (n=132) after digestion with *Sau3A*I restriction enzyme

	Frequency, number (%)
Genotypes	
AA	78 (59.09) a
AB	48 (36.36) a
BB	6 (4.55) b
Alleles	
A	102 (77.27) a
B	30 (22.73) b

a,b – different superscripts indicate a statistically significant difference at P<0.01.

DISCUSSION

Leptin has been shown to have several effects on animals. Mice, with a naturally occurring mutation on the leptin gene, produce biologically inactive leptin (Kemp, 2003). When leptin was administered to these mice, reduced food intake, increased metabolism, and body weight loss resulted (Kemp, 2003). The energetic status of beef cattle has also been linked to serum leptin levels. Animals with higher levels seem to maintain an energetic homeostasis (Sansinanea *et al.*, 2001).

The purpose of this study was to determine the frequency of leptin gene polymorphism in Iranian native cattle using molecular technique. The results of current research showed that the most frequent genotype for leptin gene was *AA*. The frequency of *A* and *B* alleles in Iranian native cattle were 77.27% and 22.73%, respectively. Furthermore, *AA*, *AB*, and *BB* genotypes were observed in 78 (59.09%), 48 (36.36%), and 6 (4.55%), of Iranian native cattle, respectively.

In the last years there has been a considerable interest in leptin gene polymorphisms because of their potential use as genetic markers to improve the efficiency of selection for quantitative traits. Low bovine fertility rate is associated with suboptimal nutrition and is a major concern of livestock cattle production systems. Recently, much effort has been devoted to understand the role of the leptin protein in regulating of food intake and reproduction in ruminants (Chilliard *et al.*, 2001).

Lagonigro *et al.* (2003) suggested an association between leptin and feed intake. The analysis of genetic polymorphisms in the leptin gene and their association with fatness in four pig breeds showed that a polymorphic locus of 3,649 bp in the leptin gene was possibly related with backfat thickness of pigs (Jiang & John, 1999). The RFLP band types were significantly different in fat-type and lean-type pigs: there was a 4.3 kb band in all lean-type pigs and a 3.5 kb band in all fat-type pigs (Xi *et al.*, 2000). Liefers & Veerkamp (2002) reported that heifers with the *Sau3A*I-*AB* genotype produced 1.32 kg/day more milk and consumed 0.73 kg/day more food compared with the *Sau3A*I-*AA* genotype.

In another study in China, the association of leptin gene polymorphism with body weight and body size indexes were evaluated to find out that the allele *B* might be associated with better growth traits. The association of the leptin polymorphism with growth traits of Chinese indigenous cattle in this study suggests its feasibility as a molecular breeding marker. Cows with genotype *BB* had remarkable growth and some of them, which had better performance, could be used for the breeding of new breeds of beef cattle (Yang *et al.*, 2007). These results were different from the findings of present research in Iranian native cattle.

The study of Javanmard *et al.* (2008) on polymorphism within the intron region of the bovine leptin gene in Iranian Sarabi cattle (Iranian *Bos taurus*) showed that the *A* allele was frequent in this population (Javanmard *et al.*, 2008). The method used in their study is similar to that in the present research and confirmed the higher frequency of *A* allele in Iranian native cattle. The research of Dubey *et al.* (2008) on leptin gene polymorphism using single strand conformation polymorphism showed that Indian Sahiwal cattle exhibited a high genetic variability in the entire leptin gene (Dubey *et al.*, 2008). Furthermore, Sharifzadeh & Doosti (2010) observed three genotypes: *AA* (60.71%), *AB* (37.5%) and *BB* (1.79%), in leptin gene polymorphism of Iranian Holstein cattle

and suggested that this polymorphism could be further evaluated for marker-assisted selection.

Considering the importance of leptin for regulation of food intake, body adiposity and reproductive performance, studies on determination of leptin gene polymorphism in Iranian native cattle are necessary. The results of present research showed that the *A* allele was more frequent in the studied population. In conclusion, the homozygosity for *AA* genotype of leptin gene could be used as a genetic marker in Iranian native cattle.

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