Association of Leptin Polymorphism with Production, Reproduction and Plasma Glucose Level in Iranian Holstein Cows

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ABSTRACT : The objective of this study was to evaluate the association of genetic differences in the bovine leptin gene and milk yield, reproduction, body condition score (BCS), and plasma glucose level in Iranian Holstein cows. In total, two hundred and thirty eight cows were used and genotyped for a restricted fragment length polymorphism at the leptin gene locus. Two genotypes, AA and AB, have been distinguished which have the frequencies of 0.89 and 0.11, respectively. The genotypes were distributed according to the Hardy - Weinberg equilibrium ($\chi^2 = 0.733$). During the first 12 wk of lactation, milk yield and composition, live weight, BCS and plasma glucose were measured in 50 cows. Data were analyzed based on a repeated measures ANOVA. During this period, milk yield and composition, live weight, BCS and plasma glucose level were similar among the genotypes. The first cumulative 60-d milk yield, 305-d milk yield, days to first breeding, days open and days from first breeding to conception using previous lactation records were also analyzed using Standard Least Square within mixed models. Fixed effects were year, season, parity and age at calving, and sire. For the reproductive traits the cumulative first 60-d milk yield was also added to the model. Animal was fitted as a random effect. A significant association was detected between the RFLP-AB genotype and 305-d milk yield ($p<0.05$). The first 60-d cumulative milk yield was similar for the two genotypes ($p = 0.21$) and tended to be higher in the heterozygous cows. The heterozygous genotypes at the above mentioned locus had a trend to better reproductive performance than the homozygous. The results demonstrate that the RFLP B-allele can yield a higher 305-d milk production with a trend to better reproductive performance. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 5 : 627-631)***

Key Words : Holstein Cows, Leptin Polymorphism, Milk Yield, Reproductive Performance

INTRODUCTION

Leptin is a 16-kDa protein that is synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck et al., 1998). It has been shown that leptin gene influences milk performance in cattle (Liefers et al., 2002; Madeja et al., 2004), and reproduction in beef cattle (Almeida et al., 2003) and porcine (Chen et al., 2004). Association of leptin gene polymorphisms with serum leptin concentration was found during pregnancy (Liefers et al., 2003). The plasma concentration of leptin was positively correlated with plasma concentrations of insulin and glucose, and negatively correlated with plasma concentrations of growth hormone and non-esterified fatty acids (Block et al., 2001; Leury et al., 2003; Vega et al., 2004a). Plasma leptin obtained linear relationship with body weight of purebred and backcrossed Hereford (Vega et al., 2004b). The energy deficit of periparturient cows causes a sustained reduction in plasma leptin (Block et al., 2001). It seems that leptin has a large effect in coordinating whole body energy metabolism and may be classified as a "metabolism modifier" (Houseknecht et al., 1998).

Selection for milk production has a negative influence on fertility of dairy cows (Pryce et al., 2000). Leptin is related to both energy metabolism and reproduction and it was shown that leptin polymorphisms had significant effect on calving interval and weight at first calving in beef cows (Almeida et al., 2003). It was suggested that leptin may be involved in regulating reproduction in that it may also act as the signal to the reproductive system that sufficient body fat exists to support a successful conception and pregnancy (Hossner, 1998). These observations suggest that leptin may be a candidate gene for at least some of the economically important production traits in dairy cattle. Also the relationship between body condition and reproduction performance has been proposed leptin as a candidate gene for predicting reproductive performance in livestock (Thomas et al., 2002).

In cattle, the leptin gene is located on chromosome 4 (Stone et al., 1996; Pomp et al., 1997) and consists of three exons. Several polymorphisms in this gene have been found (Madeja et al., 2004). Polymorphic microsatellites (Stone et al., 1996; Wilkins and Davey, 1997) and restriction fragment length polymorphisms (Lein et al., 1997) of the bovine leptin gene was reported.

Since there is a genetic correlation between start of luteal activity and energy balance, milk yield and live weight (Veerkamp et al., 2000), therefore it could be hypothesized that polymorphisms at the leptin gene locus might play a role in phenotypic records. If associations between leptin polymorphisms and milk yield, live weight, feed intake, or fertility exist, these associations will provide

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Table 1. Effect of the RFLP genotypes on milk production and composition during the first 12 wk of lactation

Item	Genotypes		P	
	AA	AB	Genotype	Time
Milk yield (kg/d)	32.68	34.74	0.40	0.01
Fat (%)	3.75	4.12	0.35	0.55
Protein (%)	2.89	2.75	0.08	0.003
Lactose (%)	5.28	5.20	0.65	0.06
Fat yield (kg/d)	1.21	1.55	0.06	0.10
Protein yield (kg/d)	0.95	0.93	0.81	0.008
Lactose yield (kg/d)	1.74	1.77	0.76	0.006

insight into the underlying mechanisms of leptin, and results may be used in future breeding programs (Liefers et al., 2002).

The aim of this study was to indicate polymorphisms at the bovine leptin gene locus in Iranian Holstein dairy cows and its contribution to milk production, plasma glucose concentration, live weight, and body condition score (BCS) in postpartum cows and also its contribution to first cumulative 60-d milk yield, 305-d milk yield, days to first breeding (DFB), days open (DO), and days from first breeding to conception (DFBC) using previous lactation records.

MATERIALS AND METHODS

Animals and traits

Blood samples were collected by EDTA-anticoagulated vacutainer tubes from two hundred and thirty eight Iranian Holstein cows via venipuncture from coccygeal vessels. The samples were stored at 4°C for further DNA extraction.

During the first 12 wk of lactation, a random sample of fifty cows were selected and fed the same diet. Cows were milked three times a day. Milk samples were taken at a fixed day of the week for measurement of milk yield and composition. Milk composition was measured using midinfrared spectroscopy (Foss Electric, Copenhagen, Denmark). Body weight and body condition score (BCS; 1 = emaciated, 5 = obese) measures were taken weekly. Blood samples were also collected weekly via venipuncture from coccygeal vessels using heparin as anticoagulant. All the weekly blood samples were obtained prior to the morning feeding. Plasma was separated immediately by centrifugation (20 min at 1,000×g) and stored at -20°C until analysis for glucose. Plasma glucose was measured by colorimetric method using a commercial kit (Zist Chimi, Tehran, Iran).

The first cumulative 60-d milk yield, 305-d milk yield, DFB, DO and DFBC using previous lactation records were also analyzed. For DFB and DO, the follow-up period started at 35 d after calving and ended at 200 and 365 d after calving, respectively. The DFB was defined as the interval from 35 d after calving to first breeding or end of follow-up, whichever occurred first. The DO was defined as the interval from 35 d after calving to conception or end of follow-up, whichever occurred first. The DFBC was defined as the interval between DFB and DO.

PCR-RFLP

PCR-RFLP method was used to detect the polymorphism at a 423 bp fragment from intron 2 of leptin gene. 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 GeneBank (Accession Number U50365). Primers were designed (forward primer: 5′-TGGAGTGGCTTGTTATTT TCTTCT-3′; reverse primer: 5′-GTCCCCGCTTCTGGCT ACCTAACT-3′) close to the polymorphic site, which resulted in a PCR product of 423 bp (Liefers et al., 2002).

PCR was performed in 25-μl reactions with a PCR mix containing 2.25 m*M* $MgCl_2$, 200 μ*M* dNTP, 7.5 p*M* of each primer, 50-100 ng of genomic DNA and 1 U of Taq polymerase. Thermal cycling conditions were included an initial denaturation at 94°C for 3 min followed by 34 cycles of 94, 62 and 72°C (45 sec, 50 sec and 1 min, respectively) and ending with a final extension for 10 min at 72°C. PCR product for each sample was digested with 5 units of *Sau3*AI (Roche Applied Science, Mannheim, Germany) overnight at 37°C. Digested products were ran on a 2.5% agarose gel and stained with ethidium bromide which resulted in genotypes AA (389 bp) and AB (389, 303 and 86 bp).

Statistical analysis

Genetic diversity : PopGen32 software (v. 1.31) was used to estimate allele and genotype frequencies and Nei's heterozygosity. Allele and genotype frequencies were determined by direct counting. A χ^2 test for goodness-of-fit was performed to verify if genotype frequencies agreed with Hardy-Weinberg expectations. Average heterozygosity (h, (Nei, 1978)) was employed to estimate genetic diversity within the population.

Associate analysis : Analysis of variance for the data over the first 12 wk of lactation was conducted using the Mixed procedure of SAS (version 9.0; SAS Institute, NC, USA) for a completely randomized design with repeated measures. The model contained the effects of genotype, week, and the interaction of genotype by week. The overall effect of genotype was tested using cow within genotype as the error term. Except than the milk composition and plasma glucose level, other first week data were used as the covariate in analyzing the traits.

Data from previous lactations were analyzed by analyzing Standard Least Square within mixed models using JMP software (version 4.0.4; SAS Institute Inc, NC. USA). In total, 48 parental half-sib families were used in this study and the average cow number per family was 2. Apart from the genotypic effect, fixed effects were year,

Table 2. Effect of the RFLP genotypes on plasma glucose level, live weight and BCS during the first 12 wk of lactation

Item	Genotypes		P	
	AA	AB	Genotype	Time
Plasma glucose level, (mg/dl)	52.31	52.73	0.93	0.40
Live weight (kg)	660	643	0.38	<0.001
BCS*	3.20	3.03	0.60	0.90

* Body condition score (1 = emaciated, 5 = obese).

season, parity, and age at calving, and sire. For reproductive traits the cumulative first 60-d milk yield was also added to the model. Animal was fitted as a random effect. Non-significant interactions were removed from the final model. Statistical significance was declared at $p<0.05$.

RESULTS

Genetic variability

Two genotypes, AA and AB, were distinguished which had the frequencies of 0.89 and 0.11, respectively. The genotypes were distributed according to the Hardy - Weinberg equilibrium (χ^2 = 0.733). Nei's expected heterozygosity was 0.0995. Allele frequencies of allele A and allele B were 0.947 and 0.053, respectively. Allele A was the allele not digested by the restriction enzyme and allele B was the restriction enzyme-digested PCR product.

Association analysis results

During the first 12 wk of lactation, milk production and composition were similar between the homozygous and heterozygous cows ($p>0.05$, Table 1). Fat yield tended to be 0.34 kg/d higher in the heterozygous cows ($p = 0.06$, Table 1). The effect of time (wk of lactation) is also shown in Table 1.

The polymorphism had no impact on BCS and body weight changes during the first 12 wk of lactation (Table 2). Although BCS was not impacted by wk of lactation, the live weight showed a second order response in time ($p<0.05$).

Plasma glucose levels were also similar between the homozygous and heterozygous cows during the first 12 wk of lactation (Table 2). The effect of time and the interaction of time and genotype were not significant ($p>0.05$).

The 305-d milk yield was affected by leptin polymorphism and the heterozygous produced more milk than the homozygous cows ($p<0.05$, Table 3). The 305-d milk yield was also impacted by sire, and also year, season, and parity at calving ($p<0.01$). The first 60-d cumulative milk yield was similar between two genotypes ($p = 0.21$; Table 3) and tended to be higher in heterozygous cows. The first 60-d cumulative milk yield was also impacted by sire, and also year and parity at calving ($p<0.01$).

Table 3. Effect of the RFLP genotypes on milk production using previous lactation records

Item	Genotypes		P
	AA	AB	
First cumulative 60-d milk yield (kg)	2,288	2,377	0.21
305-d milk yield (kg)	9,479	9,550	0.03

The heterozygous cows tended to have a better reproductive performance than the homozygous cows (Table 4). The DFB was impacted by sire, season and year of calving and age of cow at calving ($p<0.05$). The DO was influenced by sire, DFB and age at calving ($p<0.05$). The DFBC was affected by sire and DFB ($p<0.05$).

DISCUSSION

This study shows a genetic association between one of the mutations at the leptin gene locus (genotype AB) and 305-d milk yield. In confirmation with our result, Liefers et al. (2002) showed that heifers with the RFLP1-AB genotype produce 1.32 kg/d more milk and consume 0.73 kg/d more food compared with the RFLP1-AA genotype. A hypothesis might be that the RFLP1-AB cows have lower leptin levels, which results in more feed intake and less (basal) energy expenditure, or that the efficiency of feed expenditure (net efficiency) of the RFLP1-AB cows is higher (Liefers et al., 2002). In contrast with our results, Madeja et al. (2004) did not find associations between the *Kpn2*I and *Sau3A*I polymorphisms and production traits. On the other hand, Liefers et al. (2002) did not find any effect of the *Hph*I polymorphism, although they pointed to *Sau3A*I as a possible marker for milk and protein yield. Similarly, Buchanan et al. (2003) demonstrated a strong influence of the *Kpn2*I polymorphism (the TT genotype) on milk and protein yield, which was not confirmed in the Madeja et al (2004) study. Milk production traits are quantitative traits controlled by numerous genes and environmental factors. Lindersson et al. (1998) reported a QTL for milk production traits on chromosome 4 in a region where the leptin and the serum amylase-1 genes are located but no firm evidence was found for an association between the obese locus and the milk production traits. Recently, Madeja et al. (2004) showed that the *Hph*I polymorphism (the TT genotype) had

Table 4. Effect of the RFLP genotypes on reproduction traits using previous lactation records

Item	Genotypes		P
	AA	AB	
Days from parturition to first breeding (d)	71.04±8.8	67.97±12.2	0.77
Days open (d)	126.75±18.7	119.86±28.1	0.79
Days from first breeding to conception (d)	50.23±20.7	46.18±28.7	0.49

an effect on the breeding values of yield traits and animals with the TT genotype had approximately two times higher estimated breeding value (EBV) for milk and protein yields. Fat yield for this genotype also tended to be higher. The *Hph*I restriction site resulting in a change from alanine to valine is located at the conserved region of the β helix of the leptin protein. Because alanine and valine have similar nonpolar aliphatic R-groups, the substitution should not affect the protein structure or binding to its receptor. Therefore, this polymorphism should not directly influence production traits, but may be linked with other unknown milk production QTL in the vicinity (Madeja et al., 2004). It is also possible that a gene closely linked to the leptin gene, rather than the leptin gene itself, is the gene actually responsible for some of the detected effects (Liefers et al., 2002).

Milk composition was not impacted by the genotypes in this experiment. In contrast with our result, Liefers et al. (2002) reported that the AA genotypes had the lowest percentage of lactose compared with the other genotypes. In addition to genotype, many other factors influence milk yield and composition. These include environmental factors, the cow's age, lactation parity and stage, and the animal's health with special reference to the mammary gland (Mackle et al., 1999). In agreement with these results, our study also showed that the 305-d milk yield was affected by genotype and other environmental and genetic components such as sire, season and year of calving, and parity at calving ($p<0.01$).

Our results showed that the frequency of the allele A was 0.947. Frequency of this allele was 0.71 and 0.905 in other studies (Pomp et al., 1997; Liefers et al.; 2002, respectively). A rare polymorphism at an additional *Sau*3AI restriction site (the 690-bp fragment was digested into two fragments of approximately 470 and 220 bp) was observed in cattle from Simmental, Gelbvieh, and Angus breeds (Pomp et al., 1997). Our result did not show this polymorphism in Iranian Holstein dairy cows which is in agreement with other studies (Pomp et al., 1997; Liefers et al., 2002). Discrepant results about the leptin polymorphism in Holstein cows may be due to a number of factors, including the number of animals studied, population differences or breed composition, and the small number of animals with a specific genotype (Madeja et al., 2004).

Reproductive performance including days to first insemination, days open, and days from first insemination to conception were numerically better in the heterozygous than the homozygous cows. The DFB tended to be lower in the heterozygous than homozygous cows. The association of commencement of luteal activity with RFLP1 revealed a p-value of 0.777 compare with 0.043 for feed intake (Liefers et al., 2002). Therefore, the effect of polymorphism on feed intake might be the reason for the better reproductive performance in heterozygous cows. Liefers et al. (2003) showed significant relationship between postpartum leptin concentration and first observed estrus that animals with higher leptin concentrations have a better estrous expression. It was shown that leptin concentrations were different before and after ovulation and the interval from parturition to first ovulation correlated significantly with the interval from parturition to the leptin nadir in high producing dairy cows (Kadokawa et al., 2000). Their results also show that plasma concentrations of leptin decrease in early postpartum dairy cows and suggest that a delay in the recovery of leptin secretion increases the delay to the first ovulation. Specific targets of leptin in the hypothalamus are neurons expressing neuropeptide Y, proopiomelanocortin and gonadotropin-releasing hormone, but the presence of leptin receptors in peripheral reproductive structures suggests that leptin might also act at these sites (Magni et al., 2000). As the fluctuations in plasma leptin concentrations appear to be related to LH concentrations (Nagatani et al., 2000), a link between leptin and LH secretion may still explain the delay of ovulation by negative energy balance during the post-partum period (Kadokawa et al., 2000).

During the 12 wk of lactation, BCS and live weight were not affected by the genotypes. In agreement with our result Liefers et al. (2002) showed similar BW changes in the different genotypes.

Plasma glucose concentrations were similar between the genotypes and during the weeks of lactation. It was shown that leptin did not affect glucose transport in both intramuscular and subcutaneous adipose tissues (Kim and Baik, 2004). In agreement with our results, it was shown that plasma glucose level was similar in wk 1 and 3 vs. wk 8 and also wk 1 and wk 3 postpartum in dairy cows (Block et al., 2001).

In conclusion, leptin polymorphism was associated with 305-d milk production. The RFLP-AB genotype tended to have better reproductive performance than the other genotype. It seems breeding programs favoring the B-allele can yield a higher 305-d milk production without negatively affecting fertility. Although further studies should be performed to confirm these results, the association of genetic markers with better production performance is a very important finding and could be used in marker-assisted selection to improve milk production.

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