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Full Length Research Paper

Association between GH encoding gene polymorphism and semen characteristics in Iranian Holstein bulls

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The objective of this present study was to investigate relationships between the growth hormone gene restriction fragment length polymorphism (RFLP) and bull sperm characteristics. A total of 89 bulls from two semen evaluation stations were genotyped for the bovine growth hormone (bGH)-AluI polymorphism by polymerase chain reaction and followed by 4% metaphor agaros gel electrophoresis. The overall allele frequencies for two L and V alleles were 0.50, respectively. The relationship between the bGH-AluI polymorphism and semen characteristics was evaluated according 1500 ejaculated records. Five sperm characteristics were analyzed. Sperm characteristics showed significant variability in relation to bGH genotypes. LL bulls had a lower ejaculated volume and higher percentage of live sperm, and VV bulls had higher fresh sperm concentration and minimum effect after cryopreservation. This polymorphism could be further used for semen evaluation process in artificial insemination program in Iranian Holstein bulls.

Key word: Semen characteristics, growth hormone (GH) polymorphism, Holstein bulls.

INTRODUCTION

The world-wide market for Holstein semen is now so large that there seems to be an assurance of future genetic improvement through the competitive activities of international breeding organizations. In Iran, over 880,000 Holstein cows have been registered. Each year, approximately 80 young Holstein bulls are entered into the progeny testing program, in which 12 to 20 bulls would be selected as proven sires (Animal Breeding Centre, Personal communication).

Artificial insemination (AI) is the oldest and currently the most common assisted reproductive technology and an important tool in animal production (Vishwanath, 2003). The economic importance of a high breeding efficiency in dairy cows emphasizes the benefit of accurate prediction of fertility of bull semen (Parmentier et al., 1999). Semen quality is important because low-fertile semen requires more number of services to get a cow pregnant. Hence, open day and subsequent calving interval increases (Mathevon et al., 1998). The semen analysis routinely includes an immediate assessment of volume, appearance (that is, color, contamination, etc.), sperm concentration and motility, as well as later determination of sperm morphology and the presence of foreign cells (Colenbrander et al., 1993).

In addition, semen with poor fertility requires more units of semen to establish a successful pregnancy and produce a live offspring. Nonetheless, both impacts are economically important factors which are considered by the producers leading to rejection of bulls with poor semen quality and investing mainly on highly fertile sires (Januskauskas et al., 1996; Correa et al., 1997; Rodriguez-Martinez, 2003). Therefore, understanding the

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Abbreviations: GH, Growth hormone; **bGH,** Bovine growth hormone; **RFLP,** restriction fragment length polymorphism; **Leu,** leucine; **Val,** valine; **AI,** artificial insemination; **PCR,** polymerase chain reaction; **bp,** base pair; **dNTP,** dinucleotide triphosphate; **$MgCl_2$,** magnesium chloride; **ng,** nano-gram; **1X,** one time; **kDa,** centigrade kilodalton; **GLM,** generalized linear model.

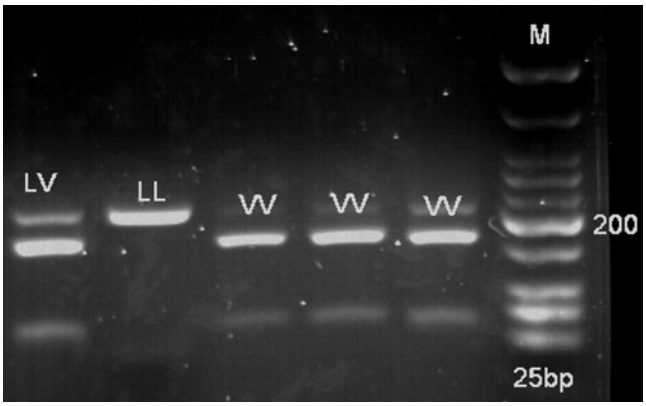

Figure 1. PCR-RFLP within bovine growth hormone gene. Three genotype of GH gene after digestion with AluI and 25 bp ladder.

sources of variation in semen quality and development of markers for early detection of highly fertile bull should interest and will be beneficial to AI companies. Although there are many tests for examination of semen quality, these tests are often subjective and have low repeatability, thus, they are time consuming. Hormone and hormone receptors are presumed to be good candidate genes for the reproductive traits because they modulate limiting steps in many reproductive pathways (Vincent et al., 1998).

The possibility of realizing selection criteria on a molecular genetic level shortens the generation interval. Particularly, for fertility traits, this is of great interest. Genotyping can be performed by using blood or tissue samples of new-born calves; and as such, it is not necessary to wait for the first parities of a sire or the results obtained by the offspring of bulls. The bovine growth hormone (bGH) is a 22 KDa single-chain polypeptide hormone which is produced in anterior pituitary gland. The encoding gene is approximately 1800 base pair (bp) and consists of five exons separated by four intervening sequences (Harvey et al. 2000). Recently, several studies have investigated the association between bGH locus and reproduction traits. A substitution of cytosine (C) for guanine (G) at position 2141 causes an amino acid change from leucine (leu) to valine (val) at residue 127. This transversion enables the genotyping of this particular locus using the endonuclease AluI, since in the mutant bull, this enzyme does not recognizes its target sequence (Harvey et al. 2000).

Several point mutations in the bovine growth hormone (GH) gene have been described, and as such, the $Leu_{127}Val$ polymorphism described by Lucy et al. (1993) has been extensively investigated with regard to production and reproduction traits. Also, the relationship between individual semen quality trait and fertility has been reported by Lechniak et al. (1999). The aim of the present study is to evaluate the possible association between the Leu/Val polymorphism of the GH encoding gene and semen characteristics. If such an association could be established, polymorphism in GH could be used as a marker for early detection of fertile sire.

MATERIALS AND METHODS

Animals

A total of 89 Holstein (3 to 4 years old) bulls bred at two semen evaluation stations were used for this project. During the entire study, bulls were fed a ration of ground, cottonseed meal with water and Bermuda grass hay available *ad libitum.*

Semen collection

Each bull was carefully prepared sexually and the semen was collected with artificial vagina. Ejaculated semen was collected, on average, twice a week with 3 to 4 days interval. The semen was weighted to calculate the volume of ejaculation and each ejaculate was maintained in a water bath at 35°C, while the sperm concentration was estimated. The percentage of motile sperm was estimated subjectively after dilatation on a slide at 37°C. Semen from mature bulls was processed in milk base extender and then frozen in 0.5 ml straws, and stored in liquid nitrogen. Each straw of semen was thawed at 37°C for 30 s, emptied into a 1.5 ml polyethylene micro-centrifuge tube and incubated at 38.5°C for 3 h. The data collected for each ejaculation included five semen characteristics: ejaculation volume (ml), concentration (10^6 cells/ml), fresh sperm motility (%), percentage of live sperm after cryopreservation (106 cells/ml) and sperm motility after cryopreservation (%).

Genotyping of bulls

DNA was extracted from semen using silica gel kit (Fermentas Company) according to the modified Boom et al. (1999) procedure. Candidate fragment was 211 bp in length, consisting of 49 bp of the fourth intron and 162 bp from the fifth exon according to the published sequence by Gordon et al. (1983). The primers sequences were: Forward, 5'-GCTGCTCCTGAGGGCCCTTC-3'; Reverse, 5'-CATGACCCTCAGGTACGTCTCCG-3'.

PCR was done in 30 µl reaction solution with the following conditions: 2.25 mM $MgCl_2$, 200 µM of each dNTP's, 1 µM of each primer, 50 to 100 ng of genomic DNA and 1 unit Taq DNA polymerase. The first cycle of the PCR was 3 min at 94°C, 1 min at 65°C and 2 min at 72°C, followed by a 34 cycle of 45 s at 94°C, 1 min at 63°C, 1 min at 72°C and ending with a 10 min extension phase at 72°C. The PCR product for each sample was digested with 10 unit of AluI at 37°C for at least 2 h. Digestion products were separated in a 4% metaphor agarose gel for 1 h at 73 V. Then, the gel was stained with ethidium bromide (Figure 1).

Statistical analysis

Allele frequencies were calculated by counting the genotypes of the 100 animals, in which 11 were excluded in the statistical approach due to missing values or unreliable data. The data were analyzed by generalized linear model (GLM) on SAS program (SAS system for windows 6.12) according to the following statistical fixed model:

$$Y_{ijkmn} + \mu + M_j + YS_j + L_n + G_m + \sum b_i X_j + \varepsilon_{ijkmn}$$

Table 1. The genotypes and allele frequencies of ALuI polymorphism in the bovine growth hormone gene in Iranian Holstein bulls.

Sample	Genotype frequency			Allele frequency	
	LL (n = 30)	LV (n = 28)	VV (n = 31)	L	V
Holstein (n = 89)	0.34	0.32	0.35	0.50	0.50

Table 2. Mean ±, standard deviation and semen parameters in Iranian Holstein bull's ejaculation records.

Semen parameters	Number	Mean	Standard deviation
Semen volume (ml)	89	57.5	1.68
Sperm concentration (10^6 cells/ml)	89	1210.49	323.16
Fresh sperm motility (%)	89	76.65	6.23
Sperm motility after cryopreservation (10^6 cells/ml)	89	71.65	9.14
Live sperm (%)	89	30.53	11.12

Table 3. Least square means and standard errors for semen characteristics in Iranian Holstein bulls with different growth hormone genotypes.

Semen characteristics	LL (n = 30)	LV (n = 28)	VV (n = 31)
Semen volume after ejaculation (ml)	4.42 ± 1.41^{b}	5.16 ± 1.3^{a}	6.24 ± 1.4^{a}
Sperm concentration (10^6 cells/ml)	1251.41 ± 60.159^{c}	1344.40 ± 115.97^{b}	1772 ± 1638^{a}
Fresh sperm motility (%)	69.58 ± 1.83^{b}	71.59 ± 1.68^{ab}	74.13 ± 1.76^{a}
Sperm motility after cryopreservation (10^6 cells/ml)	36.23 ± 1.63^{b}	37.96 ± 60.1^{b}	50.89 ± 1.60^{a}
Live sperm (%)	37.26 ± 1.93^{a}	35.85 ± 1.93^{b}	23.57 ± 1.80^{c}

Values are mean ± SE (standard error).
Values with different letters on the same row are significantly different ($p < 0.05$).

where, Y_{ijkmn} is the observed value of the trait, μ is the overall mean, M_i is the effect of i^{th} ejaculation month, YS_j is the effect of j^{th} ejaculation year (j = 2003, 2004), L_n is the fixed effect of station (n = 1, 2), G_m is the effect of m^{th} ALuI genotype (m = 1, 2, 3), $\Sigma b_i X_i$ is the linear effect of bull age and e_{ijkmn} is the random residual effect.

RESULTS

The examination of PCR products confirmed 211 bp DNA fragments which have been amplified in this study. Digestion of the product with ALuI produced two fragments with sizes of 159 and 52 bp. As shown in Figure 1, the LL genotype consisted of a band with 221 bp, while VV genotype consisted of a band with 159 and 52 bp and LV exhibited 211, 159 and 52 bp. Table 1 shows estimated genotype and allele frequencies in growth hormone gene in Holstein bulls. The overall genotype frequencies for LL, LV and VV were 0.34, 0.32 and 0.35, respectively. As such, the allele frequencies for L and V were similar (0.5).

The effect of GH polymorphism in this locus on sperm quality was examined by measuring five semen characteristics. However, statistical analysis showed significant variability in some sperm characteristics in relation to bGH genotypes. Nonetheless, LL bulls tend to have a lower ejaculated volume and higher percentage of live sperm, while VV bulls showed higher fresh sperm concentration and minimum defect after cryopreservation. Table 2 and 3 shows semen parameters and assocaition study between GH genotypes and semen characteristics respectively.

DISCUSSION

Fertility is one of most economical traits in Holstein bulls. Reproductive performance is controlled by the genetic make-up of the dam, sire and offspring, but it is largely affected, in general, by environment. Thus, the reproductive efficiency of the breeding herd depends on the fertility of the bulls (Parmentier et al., 1999). Bull's fertility is also essential since bull's DNA is the primary mecha-nism through which genetic improvements can efficiently be accomplished. Implementation of artificial insemination (AI) in dairy cattle production allowed the improving selection of bulls for production traits. At the same time, it stresses the meaning of the individual bulls' reproductive performance and requires consequent

evaluation of fertilization potential of a semen sample for AI in Holstein breeding stations.

However, the pre-selection of the samples, the high number of sperm per dose and the high quality of the semen used in the AI programs reduces the variability, thereby giving a low probability of detecting fertility differences associated with seminal parameters (Gadea et al., 2004). Spermatogenesis is a complex process that involves stem-cell renewal, genome reorganization and genome repackaging, and as such, it culminates in the production of motile gametes. The process of spermatogenesis is regulated by reproductive hormones in gonadotropin axis and is controlled by a large number of genes. Therefore, hormone and their receptors are presumed to be good candidate genes for reproductive traits (Colenbrander et al., 1993).

The usefulness of semen parameters in measuring the fertility of a semen sample accurately is controversial (Januskauskas et al., 1996; Correa et al., 1997) and correlations between sperm motility and fertility have revealed large ranges of variation (Kjaestad et al., 1993; Stalhammar et al., 1994; Tardif et al., 1999; Januskauskas et al., 2003). Correlations between sperm morphology and fertility have also been found to vary widely, and most often, they have been statistically non-significant when the semen of AI quality grade has been assessed (Rodriguez-Martinez, 2003).

Most frequently, the semen quality of dairy bulls and boars in AI centers is evaluated using sperm concentration and motility in fresh semen and motility in post-thaw samples for bulls. While some authors established a correlation between motility and field fertility, others did not (Januskauskas et al., 1996; Correa et al., 1997; Holt et al., 1997; Christensen et al., 1999; Tardif et al., 1999). Good progressive motility of spermatozoa is an indicator of both unimpaired metabolism and intactness of membranes (Johnson et al., 2000), and as such, estimation of motility has fundamental importance in the daily quality control of semen.

Morphological abnormalities of sperm can have a detrimental impact on fertilization and embryonic development (Walters et al. 2005; Saacke 2008). Thus, bulls and boars used for commercial AI are selected to a certain degree on the basis of a low incidence of morphologically abnormal spermatozoa, so that statistical calculations concerning their correlation with fertility are not very informative (Rodriguez-Martinez et al., 1997; Johnson et al., 2000), although some evidence for a relationship between sperm morphology and fertility in bulls has been presented (Söderquist et al., 1991; Al-Makhzoomi et al., 2008).

The bovine testis has been shown to be a site of GH action, in that it influences steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotropin secretion and responsiveness (Kerry and Harvey, 2000). The results obtained from this study showed that mutation in GH is associated with sperm characteristics which are crucial for its ability to fertilize the ovulated oocyte after insemination and development after cleavage. These results are in agreement with the study of Lechniak et al. (1999) who indicated that AluI polymorphism of bGH gene had no effect on the sperm quality traits, non-return rate, number of oocytes (collected from donor ovaries) suitable for *in vitro* maturation, the number of matured oocytes, mean oocyte diameter and number of embryos produced. Sabour et al. (1997) reported the relationship of L/V locus of bovine GH gene with estimated ETA of milk and protein yields in Holstein bulls. In addition, Shariflou et al. (1998) reported that leu allele of GH favored higher production of milk, fat and protein and was dominating in the production of Val.

In summary, differences in semen parameters could have potential use in bull per-selection for AI program. This SNP beside other polymorphisms within candidate genes could be used for further evaluation of semen collection process.

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