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Full Length Research Paper

A MspI PCR-RFLP within bovin growth hormone gene and its association with sperm quality traits in Iranian Holstein bulls

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The present study was aimed to examine the association of bovine growth hormone gene polymorphism with sperm quality traits including sperm volume (SV), sperm concentration (SPCO), total sperm (TS), fresh sperm motility (FSM), total fresh motile sperm (TFMS), post thaw sperm motility (PTSM), total post thaw motile sperm (TPTMS), total sperm dose (TSD) and testis biometry trait as average testis length (ATL), average testis width (ATW) and scrotum circumference (SC) in Iranian Holstein bulls. PCR-RFLP method with Msp-I restriction enzyme was used for genotyping. The frequency of the MspI^{+}(C) and MspI^{-}(D) alleles are 0.883 and 0.117, respectively. The genotype frequency for CC, CD and DD were 0.787, 0.191 and 0.022, respectively. The DD genotype was omitted of analysis. Mixed model analyses of sperm quality traits considering genotype and environment as fixed effects and animal as a random effect suggested that sire was a significant source of variation (P < 0.001) in all traits. The CC genotype resulted in a significant increase in SV (p = 0.022), FSM (p < 0.0001), TFMS (p < 0.0001), PTSM (p < 0.0001), TPTMS (p = 0.0067), TSD (p = 0.025) traits greater than CD genotype. However, CD genotype had significant effect on ATL (p = 0.0223) and ATW (p = 0.0544) traits, but not on SPCO (p = 0.3319), TS (p = 0.3818) and SC (p = 0.3841). These results indicate that new molecular markers associated with sperm quality traits can be used in marker-assisted selection in bulls.

Key words: Iranian Holstein bull, PCR-RFLP, bGH-Msp-I, polymorphism, sperm quality trait.

INTRODUCTION

Artificial insemination (AI) from superior sires is a main tool for genetic improvement of the traits with economic importance in dairy cattle herds (Parmentier et al., 1999). The conception rate with AI depends on the quantity and quality of semen affected by environment, management, physiological status (especially hormones, e.g. FSH, LH and GH) and genetics factors (Mathevon et al., 1998). Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al., 1993). However, laboratory assays which examine the quality of sperm sample are still unable to predict its fertility consistently (Braundmeier and Miller, 2001). Molecular techniques like quantitative trait loci (QTL) and candidate genes approach allow detecting variation or polymorphisms existing among individuals in the population for specific region of the DNA and increase rate of response to selection especially in trait with low heritability (e.g. reproductive trait) (Mathevon et al., 1998; Rothschild et al., 1996; Linville et al., 2001; Parmentier et al., 1999). 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).

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Growth hormone (GH) has been known as the main regulator of postnatal growth, cell differentiation and metabolism in mammals (Carnicela et al., 2003). The GH affects growth rate, milk production (Baumann, 1991; Daughaday, 1975), body composition, health and aging by modulating the expression of many genes (Sumantran et al., 1992; Ho and Hoffman, 1993; Lincoln et al., 1995). It also has an important role in mammalian reproduction (Hull and Harvey, 2000). Endocrine GH from the pituitary glands may be involved in the strategic maintenance of male reproduction; whereas testicular GH may be involved in emergency modulation of testicular function. Pituitary GH and/or testicular GH affect steroidogenesis by stimulating the activity of several steroidogenic enzymes in Leydig cells and also alter gametogensis in Sertoli cells by increasing the synthesis and/or modification of proteins such as IGF-1, IGF-binding proteins and androgen-binding proteins (Kerry and Harvey, 2000). Therefore, GH can be used as a candidate gene for marker-assisted selection programs in cattle reproductive traits, especially sperm quantity and quality.

Bovine growth hormone (bGH) is a single peptide with 190 or 191 amino acids and molecular weight equal to 22-KD (Walies and Davies, 1976; Lingappa et al., 1977; Dybus, 2002). bGH gene with 1800 bp length, 5 exons and 4 introns is a part of multiple gene family that contains prolactin and placental lactogens (Hediger et al., 1990; Gordon et al., 1983). Several polymorphic regions have been reported at different regions of bGH gene by SSCP and RFLP methods (Zakizadeh et al., 2006). The two most important polymorphisms are mutations at intron 3 (transition T to C) and exon 5 [transversion C to G (substitutes Leu by Val in protein)]; which are detected by MspI and AluI restriction enzymes, respectively (Lucy et al., 1993; Zhang et al., 1993; Yao et al., 1996).

Although several studies have addressed the association of bGH-MspI polymorphism with milk and meat production traits and inconsistent results have been reported (Falaki et al., 1997; Lee et al., 1996; Vukasinovic et al., 1999; Beauchemin et al., 2006; Pereira et al., 2005; Zhou et al., 2005; Thomas et al., 2007; Katoh et al., 2008). However, to date, few studies have examined its effect on reproduction traits of bulls (e.g. sperm quality trait) (Lechniak et al., 1999, 2002; Unanian et al., 2002; Balogh et al., 2008).

Therefore, the present study was aimed to estimate the allelic frequencies at the bGH-MspI loci and examine its relationship with sperm quality and testis biometry traits of Iranian Holstein bulls.

MATERIALS AND METHODS

Animals

183 bulls of North West AI center (Tabriz, Iran) and Progeny Test center of Jahed Co (Karaj, Iran), were included in the study. For each bull the repeated measurements of sperm quality traits of bulls were available since 1991 to 2008 (41890 records).

Phenotypes

Sperm volume (SV), sperm concentration (SPCO), total sperm (TS), fresh sperm motility (FSM), total fresh motile sperm (TFMS), post thaw sperm motility (PTSM), total post thaw motile sperm (TPTMS) and total sperm dose (TSD) were obtained from each ejaculate with light microscopy according to the guidelines of the World Health Organization. Testis biometry traits including average testis length (ATL), average testis width (ATW) and scrotum circumference (SC) were measured monthly for each bulls. The semen samples of bulls were collected with date and age of bull records.

Genotyping

Blood and semen samples were collected from the bulls. An anticoagulant (EDTA) was added to the blood samples and then stored at –20°C.

Genomic DNA from whole blood was purified by standard protocol using proteinase K digestion as described by Miller et al. (1988) and from semen by DNA extraction kit (DNP^{TM} kit Cinnagen Co. Tehran, Iran). The quality of the DNA was checked on 0.5% agarose gel and the quantity was measured by UV spectrophotometry at A260/A280 nm.

Genotyping for bGH polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR reaction conditions were approximately 100 ng of genomic DNA, 10 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM of MgCl2, 1 x PCR buffer [50 mM of KCl and Tris-HCl (pH 8.4)] and 0.4 U of Taq polymerase in a total volume of 25 µl. The PCR was conducted on Eppendorf Gradiant thermalcycler, HotMasterMix (EPPENDORF, Germany) using a preliminary denaturation at 94°C for 1.5 min, 62°C for 1 min and 72°C for 1 min, followed by 48 cycles of a specific temperature regime. Each temperature regime consisted of 94°C for 30 s, 62°C for 1 min, 72°C for 30 s and a final extension at 72°C for 5 min. An 891 bp fragment of bGH consisting of the of intron 2 (177 bp), exon III (117 bp), intron 3 (227 bp), exon IV (162 bp) and intron 4 (208 bp), was amplified using forward (5`ATCCACACCCCCTCCA CACAGT3`) and reverse (5`CATTTTCCACCCTCCCCTACAG3`) primers (Unanian et al., 2002).

PCR products were digested with 4 U of MspI, using the supplied buffer and maintained at 37°C for overnight. The resulting fragments were separated by vertical electrophoresis (110 W 40 min) in 8% polyacrylamide gel, stained with ethidium bromide and were visualized under UV light. The C (MspI^{+}) allele had fragment sizes of 526,193, 109 and 63 bp, whereas the D (MspI^{-}) allele had fragments of 635, 193 and 63 bp.

Statistical analysis

Allele and genotype frequencies

The bGH allele frequencies were calculated by simple allele counting (Falconer and Mackay, 1996). The possible deviations of allele and genotype frequencies from the Hardy–Weinberg equilibrium were examined with PopGene.S2 softwear by a Pearson's Chi-square test.

Association analysis

Statistical analysis was performed using the MIXED procedure of SAS software (SAS System for Windows, release 8.02, 1999). The following linear model was used to examine the associations between bGH-MspI, polymorphisms and SV, SPCO, TS, TMSBF,

Table 1. Gene and genotypic frequencies obtained at bGH-MspI loci in Iranian Holstein bulls.

Parameter	Genotype			Allele		Chi-square value	Pr > ChiSq
	CC	CD	DD	C	D		
Number	144	35	4				
Frequency	0.787	0.191	0.022	0.883	0.117	1.1043	> 0.05

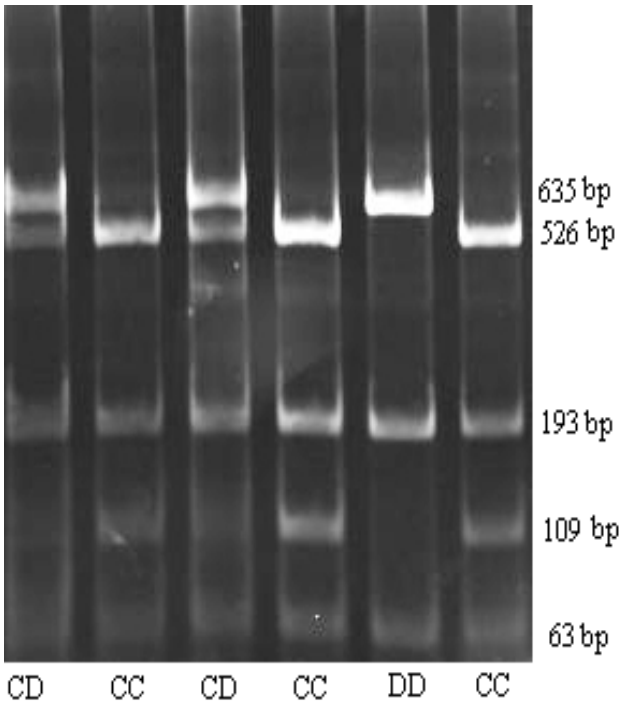

Figure 1. Representative genotyping of bGH gene at locus MspI by Polyacrylamide gel electrophoresis.

TMSAF and TPP, ATL, ATW and SC traits:

$$y_{ijklm} = \mu + a_i + YS_j + S_k + G_l + \sum_m b_m x_m + \varepsilon_{ijklm}$$

where y_{ijklm} is the observation, μ is overall mean, a_i is the random effect of the i^{th} animal, YS_j is fixed effect of the j^{th} year-season (j = 1 - 68), S_k is fixed effect of the k^{th} station (k = 1 - 2), G_l is fixed effect of the l^{th} bGH genotype (l = 1 - 3), b_m is regression coefficient of m^{th} covariate (e.g. age), x_m is fixed effect of m^{th} covariate and ε_{ijklm} is residual.

Since FSM and PTSM traits were categorical variable, hence analyzed with logistic regression using GENMOD procedure by the following model:

$$\eta_{iklm} = \log[p_i/(1-p_i)] = m + a_i + YS_j + S_k + G_l + \sum_m b_m x_m + \varepsilon_{ijklm}$$

Where η_{ijklm} is MAF and MBF traits, m is overall mean in logarithmic scale, a_i is the random effect of the i^{th} animal, YS_j is fixed effect of the j^{th} year-season (j = 1 - 68), S_k is fixed effect of the k^{th} station (k = 1 - 2), G_l is fixed effect of the l^{th} bGH genotype (l = 1 - 3), b_m is regression coefficient of m^{th} covariate (e.g. age), x_m is fixed effect of m^{th} covariate and ε_{ijklm} is residual.

Average effect of allele substitution was determined by coding genotype as 0(DD), 1(CD), 2(CC) to represent the number of C alleles present for the bGH polymorphism as described by Falconer and Mackay (1996). The regression coefficient estimates average effect of allele substitution.

RESULTS

Allele frequency

Data of 183 bulls were included in the final evaluation. The genotype and allele frequencies at bGH loci calculated by PopGene.S2 softwear, are shown in Table 1. Three genotypes for bGH gene CC (526,193, 109 and 63 bp), CD (635,526,193,109 and 63 bp) and DD (635, 193 and 63 bp) was observed (Figure 1). The C allele was more frequent than D allele (0.883 vs. 0.117) and therefore most of the bulls (78.7%) were homozygous for the C allele and only 19.1% were heterozygous. The DD genotype was found in only four animals and their results weren't reported. Pearson's Chi-square test (P > 0.05) indicated that the genetic pool were in Hardy–Weinberg equilibrium.

Candidate gene effects

Least square means of sperm quality and testis biometry traits for bGH genotypes are presented in Table 2. Analysis of variance indicated significant association of bGH genotypes with SV (P < 0.022), FSM (P < 0.0001), TFMS (P < 0.0001), PTSM (P < 0.0001), TPTMS (P < 0.0067), TSD (P < 0.025) and ATL (P < 0.0223), but there was no significant association with SPCO, TS, SC and ATW (P > 0.05). Moreover year-season and age had significant effects on some sperm quality traits (P < 0.0001). In this population, bulls with CC genotype had sperm volume, sperm concentration, total sperm, fresh sperm motility, total fresh motile sperm, post tallow motility, total post tallow motile sperm and total payot product greater than bulls with CD genotype. However CD genotype had average testis length, average testis width and scrotum circumference greater than bulls with CC genotype.

The allele substitution effects on sperm quality and testis biometry traits were estimated and shown in

Table 2. Least square means (±SD) of sperm quality and testis biometry traits for bGH genotypes in Iranian Holstein bulls.

Trait	bGH genotype		P-value
	CC (n = 146)	CD (n=37)	
Sperm volume (ml)	5.064 ± 0.202	4.35 ± 0.315	0.0220
Sperm concentration(×10^8/ml)	1055.08 ± 62.93	987.58 ± 82.98	0.3319
total sperm (×10^8/ejculation)	5305.26 ± 302.23	5018.04 ± 397.56	0.3818
fresh sperm motility (%)	4.22 ± 0.0054	4.11 ± 0.01	< 0.0001
total fresh motile sperm (×10^8/ejculation)	3514.64 ± 188.53	2294.66 ± 290.71	< 0.0001
post thaw sperm motility (%)	3.54 ± 0.0402	3.50 ± 0.0409	< 0.0001
total post thaw motile sperm (×10^8/ejculation)	1885.62 ± 130.87	1123.98 ± 285.1	0.0067
total payot produce	160.41 ± 4.1810	138.83 ± 12.0586	0.025
average testis length (cm)	17.11 ± 1.7	17.99 ± 1.7	0.0223
average testis width (cm)	13.39 ± 4.22	14.54 ± 4.22	0.0544
scrotum circumference (cm)	38.78 ± 7.1	39.44 ± 7.1	0.3841

Table 3. Allele substitution effect of bGH gene on sperm quality and testis biometry trait.

Trait	bGH genotype		p-value
	α	SD	
Sperm volume (ml)	0.5638	0.1960	0.0049
Sperm concentration(×10^8/ml)	- 8.2411	51.743	0.8737
total sperm (×10^8/ejculation)	618.67	259.99	0.0190
fresh sperm motility (%)	- 0.1400	0.0434	0.0013
total fresh motile sperm (×10^8/ejculation)	507.14	187.89	0.0081
post thaw sperm motility (%)	- 0.0896	0.0406	0.0272
total post thaw motile sperm (×10^8/ejculation)	132.95	74.788	0.0782
total payot produce	0.6746	3.7029	0.8558
average testis length (cm)	- 0.884	0.377	0.0214
average testis width (cm)	- 1.168	0.592	0.0520
scrotum circumference (cm)	- 0.665	0.759	0.3828

Table 3. The substitution effects of C to D in sperm quality traits were mainly significant, but no significant allele substitution effect on SPCO, TPP and SC was observed. The substitution of C for D allele at GH locus resulted in an increase of 0.564 ml in sperm volume, 619 $\times$ 10^8 per ejaculation in total sperm and 507 $\times$ 10^8 per ejaculation in total fresh motile sperm ($p < 0.01$). However, a significant reduction ($p < 0.05$) was observed in fresh sperm (-0.14%) and post thaw sperm motility (0.09%). No significant effect of allele substitution was observed in Sperm concentration, total post thaw motile sperm and total payot produce. For testis traits, testis length decreased significantly on average by 0.884 cm ($p < 0.05$) through substitution of C allele by D allele. In spite of a decrease in testis width (- 1.168 cm) and scrotum circumference (- 0.665 cm), no significant effect of allele substitution was observed on these traits.

DISCUSSION

The results of the present study showed that the $MspI^+$ allele (C) was more frequent than the $MspI^-$ (D) (0.883 vs. 0.117), so that most of the bulls (78.7%) were homozygous for the C allele, 19.1% were heterozygous and 2.2% were homozygous for the D allele. These findings were similar to those previously reported for Holstein dairy cattle (Zhang et al., 1993; Yao et al., 1996; Sabour et al., 1997; Vukasinovic et al., 1999; Zhou et al., 2005; Zakizadeh et al., 2006; Pawar et al. 2007). Comparison of the allelic frequency in different breeds showed that $MspI^-$ (D) allele frequency is relatively low for breeds prevalent in most of European breeds, that is, zero for Herford cattle, 0.15 for Jersey and 0.14 for Angus cattle (Lagziel et al., 2000) and 0.13 for Polish Black and White cattle (Dybus and Grzesiak, 2004). For the Eastern

Europe or the Middle East cattle, these frequencies were reported to be moderate to high, that is, 0.26 and 0.39 for Ukraine Brown Carpathian and Limousine cattle (Lagziel et al., 2000); 0.45 for Iranian Sarabi cattle (Zakizadeh et al., 2006); 1.00 for Indian subcontinent and zebu breeds (Lagziel et al., 2000); 0.81 to 0.87 for Indian Zebu (Pawar et al., 2007) and 0.82 to 0.85 for Brazilian Nellore cattle (Unanian et al., 2002). These results suggested that breed of cattle is an important source of variation in allelic frequency of GH-MspI locus. Also, due to neutral and artificial selection, D and C alleles might be a characteristic of *Bos indicus* breeds (resistance to rough environmental condition) and *Bos taurus* breeds (high production), respectively.

The bovine testis has been shown to be a site of GH action; it influences the steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotropin secretion and responsiveness (Kerry and Harvey, 2000). In the present study sperm volume, sperm concentration, total sperm, fresh sperm motility, total fresh motile sperm, post tallow motility, total post tallow motile sperm and total payot product were higher in bulls with CC genotype, compare to CD by 16.4% ($P < 0.022$), 6.8% ($P < 0.3319$), 5.7% ($P < 0.3818$), 0.11% ($P < 0.0001$), 53% ($P < 0.0001$), 0.04% ($P < 0.0001$), 40.3% ($P < 0.0067$) and 15.5% ($P < 0.0067$) receptively. Moreover regarding to testis biometry traits, bulls with CC genotype had lower average testis length, average testis width and scrotum circumference, compare to CD by 5% ($P < 0.0223$), 8% ($P < 0.054$) and 1.7% ($P < 0.3841$)

Although there are several studies regarding the association of bGH-MspI polymorphism with different traits, to date few have examined the reproduction traits. Only one study assessed the effect of bGH-MspI polymorphism on sperm quality trait in bulls. The results didn't show any significant association of bGH-MspI polymorphism with fresh sperm motility, sperm concentration and minor and major defects (Unanian et al., 2002) which was in contrast with our findings. It was may be related to breed differences. [Brazilian Nellore (*Bos indicus*) vs Holstein (*Bos taurus*)].

Moreover in consistent with our findings, the study of Unanian et al. (2002) indicated that bGH-MspI polymorphism had significant effect on scrotal circumference and testicular growth after puberty. The results of Rocha et al. (1992) demonstrated a significant association of the bGH-Msp-I polymorphism with body weight gain and scrotum circumference.

The study of Lechniak et al. (1999, 2002) 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.

In conclusion, the results of the present study showed that including the bGH-MspI polymorphism in breeding program will improve the sperm quality traits in AI bulls. But it is currently unknown how this mutation alters the structure and conformation of growth hormone. However, further studies are required to test the biochemical effects of bGH's various isoforms, resulting from this polymorphism on reproduction traits.

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