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Effects of polymorphisms in the bovine growth differentiation factor 9 gene on sperm quality in Holstein bulls

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ABSTRACT. Members of the transforming growth factor-β (TGFβ) superfamily are critical regulators of germ cell development that act as extracellular ligands of the signal transduction pathways regulating proliferation, differentiation, apoptosis, and other aspects of cell behavior. Growth differentiation factor 9 (GDF9) is a member of the TGFβ superfamily that plays a critical role in ovarian follicular development and ovulation rate in females; however, its role in the testis has not been well elucidated. Therefore, in this study we investigated the effects of *GDF9* mutations on the quality of fresh and frozen semen of Holstein bulls. Two reported single nucleotide polymorphisms of *GDF9*, A485TA and A625C, were analyzed in 129 Holstein bulls. Analysis of variance revealed that the A485T polymorphism had significant effects on the acrosome integrity rate ($P < 0.05$), whereas the A625T polymorphism was significantly associated with sperm concentration ($P < 0.05$). In addition, a significant additive effect on sperm concentration was detected for the A485T polymorphism ($P < 0.05$), whereas the polymorphisms

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A485TA and A625C had significant dominant effects on acrosome integrity rate and sperm motility in frozen semen, respectively ($P < 0.05$). This study is the first to show a significant association of *GDF9* with sperm quality traits, and the results implied that GDF9 is involved in the initiation or maintenance of spermatogenesis; however, further verification is needed.

Key words: Holstein bulls; Polymorphism; Sperm quality; Growth differentiation factor 9

INTRODUCTION

Artificial insemination was the first advanced biotechnological technique applied to improve the reproduction and genetics of farm animals, and it has made an enormous impact on many species worldwide, particularly dairy cattle. However, at the same time, its use has been hindered by the fact that it is very difficult to predict the fertilizing ability of sperm when artificial insemination is used, since it leads to the selection of only those bulls with good reproductive performance (Gadea, 2005). In addition, it is difficult to perform direct selection for semen quality traits, including semen volume per ejaculate, sperm motility, sperm concentration, and so on, because of their low heritability (Rothschild and Bidanel, 1998). It will, therefore, be meaningful to use a candidate gene approach to identify genes that influence semen quality traits. Recently, genes of the hypothalamic-pituitary-testicular axis have been widely studied as candidate marker genes for sperm quality in bulls (Dai et al., 2009; Yang et al., 2011; Sang et al., 2011; Sun et al., 2012); however, only a few studies have examined this for growth factor genes in bulls.

Growth differentiation factor 9 (GDF9), which belongs to the transforming growth factor-β (TGFβ) superfamily, known as FecG on sheep chromosome 5 (Sadighi et al., 2002), plays a critical role in ovarian follicular development and ovulation rate in females (Elvin et al., 1999; McNatty et al., 2005). In males, GDF9 expression has been detected in the testis (Fitzpatrick et al., 1998; Pennetier et al., 2004; Nicholls et al., 2009); however, its role in the testis has not been well elucidated. Itman et al. (2006) reported that signals from TGFβ ligands have been implicated in germ cell specification and migration, Sertoli cell proliferation, spermatogonial growth and differentiation, and spermiogenesis. Therefore, Fitzpatrick et al. (1998) and Pennetier et al. (2004) suggested that GDF9 could potentially regulate testicular function, and this concept was supported by Itman et al. (2006, 2008). However, Dong et al. (1996) found that GDF9-knockout males are fertile and show no gross physical or behavioral defects, which indicated that the actions of GDF9 in the testes are not essential for the initiation or maintenance of spermatogenesis. However, the effects of GDF9 on the function of tight junctions, sperm quality, including number, maturation, mobility, and viability, or the circulating concentrations of the key hormones testosterone, follicle-stimulating hormone (FSH), and inhibins have not been analyzed in these knockout models, and a change in any of these parameters could affect male fertility. Nicholls et al. (2009) reported that GDF9 is germ cell-specific factor in the testis, and demonstrated that GDF9 can modulate key functions of Sertoli cells by affecting the function of tight junctions and the expression of inhibin B. To date, no study has reported the effects of GDF9 on sperm quality. Herein, this study aimed

to elucidate the effects of *GDF9* polymorphisms on the quality of fresh and frozen semen in Holstein bulls.

MATERIAL AND METHODS

Samples and data collection

All procedures involving animals were approved by the Animal Care and Use Committee of Huazhong Agricultural University. A total of 129 normal mature Holstein bulls were examined in this study; 54 bulls were obtained from the Beijing Dairy Center and 75 bulls were obtained from the Shanghai Bright Dairy & Food Co., Ltd., China. We collected 40 to 78 ejaculates (more than 88% of bulls produced more than 70 ejaculates) at intervals of 3 to 6 days from each bull from January to December 2007, and the repeated measurements of sperm quality traits were available. The semen was collected using an artificial vagina. Immediately after collection, the ejaculates were stored at 37°C in a water bath to evaluate the quality traits of fresh semen including semen volume per ejaculate [VOL (mL)], sperm motility [MOT (%)], and sperm concentration [SCON (x 10^8 mL)]. The fresh semen was then diluted using glycerol-egg yolk-citrate and made into frozen semen straws. After storage in liquid nitrogen for 5 to 7 days, two straws were randomly obtained from each ejaculate and thawed at 38°C for 20 s, and the quality traits of frozen semen were evaluated immediately including sperm motility [FMOT (%)], acrosome integrity rate [AIR (%)], and abnormal spermatozoa rate [ASR(%)] using light microscopy according to the guidelines of the World Health Organization. Genomic DNA was extracted from the sperm using a standard phenol-chloroform extraction protocol. The DNA samples were dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and stored at -20°C until used.

PCR conditions and genotyping

We selected two single nucleotide polymorphisms (SNPs), A485TA and A625T (Tang et al., 2013), which are located in intron 1 of the bovine *GDF9* gene, to evaluate their effects on the quality traits of sperm from 129 Holstein bulls. Primer sequences (F: 5'-AACAGAAGCC ACCTCTACAAC-3' and R: 5'-CTGGACAAGATGCTAACCTC-3') and reaction conditions for these two polymorphisms were selected according to a previous study (Tang et al., 2013). These polymorphisms were genotyped by sequencing according to Tang et al. (2013).

Statistical analysis

The allele frequencies of the polymorphisms were determined by direct counting and the Hardy-Weinberg equilibrium was analyzed by the chi-square test using the SAS 8.1 software (SAS Institute Inc., Cary, NC, USA). Pairwise linkage disequilibrium was measured using the online SHEsis software (Shi and He, 2005). Both additive and dominant effects were estimated using models in the REG procedure of SAS 8.1 (Liu, 1998). The associations of *GDF9* genotypes with sperm quality traits including VOL, MOT, SCON, AIR, FMOT, and ASR were calculated using the General Linear Model of SAS 8.1, which included the fixed

effects of age and origin of the bull. Their effects on sperm quality traits were analyzed using the GLM procedure and compared by the Duncan multiple range test (SAS 8.1). Only factors that affected the records significantly ($P < 0.05$) were fitted in the final statistical model:

$$y_{ikj} = u + G_i + A_k + P_j + e_{ikj}$$

where y_{ikj} is the phenotypic value of the traits; u is the population mean; G_i is the fixed effect of the genotypes; A_k is the fixed effect of age [k = 2-10, (1) 2 to 3 years; (2) 4 to 5 years; and (3) 6 to 10 years]; P_j is the fixed effect of the origin of bull; and e_{ikj} is the random residual error.

RESULTS

Genotypic and allelic frequencies

PCR products were detected using 1.5% agarose gel electrophoresis. The amplified products were consistent with the target fragments, had good specificity, and could be used directly for genotyping. The frequencies of the g.485A, g.485T, g.625A, and g.625T alleles were 0.717, 0.283, 0.558, and 0.442, respectively, in the population analyzed (Table 1), and these two polymorphisms were in Hardy-Weinberg equilibrium (Table 1). The linkage disequilibrium status of the two SNPs was weak ($r^2 = 0.165$); thus, we did not perform further haplotype analysis.

Table 1. Allelic and genotypic frequencies of *GDF9* polymorphisms in Holstein bulls.

Locus	Genotype	Genotype frequency	Allele frequency	χ^2 value (Hardy-Weinberg equilibrium)
A485T	AA (66)	0.512	A 0.717	0.020 (P > 0.05)
	AT (53)	0.411	T 0.283	
	TT (10)	0.078		
A625T	AA (40)	0.310	A 0.558	0.005 (P > 0.05)
	AT (64)	0.496	T 0.442	
	TT (25)	0.194		

The values in parentheses are the number of cows.

Association of the genotypes with sperm quality traits

The results of the association analysis between the *GDF9* genotypes and sperm quality traits are given in Tables 2 and 3. For the polymorphic locus 485, bull heterozygous for the g.485AT genotype had a significantly higher AIR ($P < 0.05$) than those homozygous for the g.485TT genotype (Table 2). For the polymorphic locus 625, bulls with the g.625TT genotype had significantly higher SCON when compared to those homozygous for the g.625AA ($P < 0.05$) genotype (Table 3). The additive and dominant effects of the genotypes are shown in Tables 2 and 3, and the A625T polymorphism had a significant additive effect on SCON ($P < 0.05$). Furthermore, the A485TA and A625C polymorphisms had significant dominant effects on AIR and FMOT, respectively ($P < 0.05$).

Table 2. Effects of the *GDF9* A485T polymorphism on semen quality traits in Holstein bulls.

Trait	Genotype			Additive effect	Dominant effect
	g.485AA (66)	g.485AT (53)	g.485TT (10)		
VOL (mL)	6.369 ± 0.218	6.537 ± 0.229	6.628 ± 0.556	0.130 ± 0.293	-0.019 ± 0.189
SCON (x 10^8 mL)	12.349 ± 0.360	11.868 ± 0.425	11.701 ± 0.944	-0.324 ± 0.509	0.078 ± 0.327
MOT (%)	78.00 ± 1.47	80.525 ± 1.210	80.363 ± 2.646	1.179 ± 1.784	-0.671 ± 10148
FMOT (%)	40.569 ± 0.247	40.188 ± 0.412	39.798 ± 1.031	-0.386 ± 0.46	-0.0023 ± 0.288
ASR (%)	15.656 ± 0.269	15.224 ± 0.703	15.894 ± 0.560	0.119 ± 0.623	0.275 ± 0.401
AIR (%)	46.892 ± 0.637^{ab}	48.235 ± 0.771^{a}	45.098 ± 0.810^{b}	-0.567 ± 1.14	-1.285 ± 0.733*

Data are reported as means ± SE. Values with different letters within the same line indicate significant differences ($P < 0.05$). *Significance level at $P < 0.05$. VOL = semen volume per ejaculate; SCON = sperm concentration; MOT = sperm motility; FMOT = frozen sperm motility; ASR = abnormal spermatozoa rate; AIR = acrosome integrity rate.

Table 3. Effects of *GDF9* A625T polymorphism on semen quality traits in Holstein bulls.

Trait	Genotype			Additive effect	Dominant effect
	g.625AA (40)	g.625AT (64)	g.625TT (25)		
VOL (mL)	6.147 ± 0.298	6.588 ± 0.220	6.623 ± 0.253	0.238 ± 0.219	-0.102 ± 0.153
SCON (x 10^8 mL)	11.570 ± 0.505^{a}	12.080 ± 0.372^{ab}	13.006 ± 0.508^{b}	0.718 ± 0.378*	0.104 ± 0.265
MOT (%)	79.427 ± 1.369	78.401 ± 1.570	81.001 ± 1.391	0.787 ± 1.345	0.906 ± 0.942
FMOT (%)	40.052 ± 0.501	40.771 ± 0.332	39.741 ± 0.368	-0.156 ± 0.332	-0.437 ± 0.239*
ASR (%)	16.113 ± 0.864	14.892 ± 0.319	16.061 ± 0.375	-0.026 ± 0.463	0.598 ± 0.324
AIR (%)	47.679 ± 0.947	47.661 ± 0.664	45.824 ± 0.711	-0.927 ± 0.856	-0.122 ± 0.602

Data are reported as means ± SE. Values with different letters within the same line indicate significant differences ($P < 0.05$). *Significance level at $P < 0.05$. For abbreviations, see legend to Table 2.

DISCUSSION

Members of the TGFβ superfamily are critical regulators of germ cell development (Loveland and Hime, 2005) that act as extracellular ligands of the signal transduction pathways regulating the proliferation, differentiation, apoptosis, and other aspects of cell behavior (Fan et al., 2012). GDF9, which is a member of the TGFβ superfamily, plays a critical role in ovarian follicular development and ovulation rate in females (Elvin et al., 1999; McNatty et al., 2005); however, the role of GDF9 in the testis has not been well elucidated. Fitzpatrick et al. (1998) and Pennetier et al. (2004) detected expression of *GDF9* in the testis, and suggested that it could potentially regulate testicular function. This concept is also supported by the dynamic regulation of BMPRII, ALK5, and ALK6 expression throughout the seminiferous tubules, and the fact that Smad signaling pathways are vital for the control of testis development and spermatogenesis (Itman et al., 2006, 2008; Nicholls et al., 2009). However, Dong et al. (1996) found that GDF9-knockout males are fertile and show no gross physical or behavioral defects, which indicated that the actions of GDF9 are not essential for the initiation or maintenance of spermatogenesis. However, whether the action of GDF9 affects the function of tight junctions, sperm quality, including number, maturation, mobility, and viability, or the circulating concentrations of the key hormones testosterone, FSH, and inhibins has not been analyzed. Furthermore, Nicholls et al. (2009) found that GDF9 can modulate key functions of Sertoli cells by affecting the function of tight junctions and the expression of inhibin B. Therefore, in this study, we aimed to elucidate the effects of *GDF9* mutations on sperm quality traits.

Many mutations have been identified in the exons and introns of sheep or goat *GDF*9, and found to be significantly associated with litter size or ovulation rate (Hanrahan et al., 2004; Nicol et al., 2009; Barzegari et al., 2010; Polley et al., 2010; Chu et al., 2011). However, studies examining mutations in bovine *GDF*9 are relatively rare. Tang et al. (2013) first detected the two SNPs A485TA and A625C in *GDF9* and reported significant associations with superovulation traits. In the present study, these two SNPs were detected in Holstein bulls. Association analysis revealed that these two polymorphisms had significant effects on sperm quality traits. In addition, a significant additive effect on SCON was detected for the A625T polymorphisms, while the A485TA and A625T polymorphisms had significant dominant effects on AIR and FMOT, respectively. Although these two polymorphisms are located in intron 1 of *GDF9*, they may affect phenotype, gene expression, and consequent function (Van Laere et al., 2003; Krawczak et al., 2007). Thus, these two polymorphisms may have altered the function of tight junctions or the circulating concentrations of the key hormones testosterone, FSH, and inhibins by affecting signal transduction, and finally influenced sperm quality (Nicholls et al., 2009; Fan et al., 2012).

In conclusion, this study is the first to identify a significant association between *GDF9* and sperm quality traits. The results implied that GDF9 is involved in the initiation or maintenance of spermatogenesis, but further verification is needed.

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