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Research Article

The association of two single nucleotide polymorphisms (SNPs) in growth hormone (*GH*) gene with litter size and superovulation response in goat-breeds

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Abstract

Two active mutations (A 781 G and A 1575 G) in growth hormone (*GH*) gene, and their associations with litter size (LS), were investigated in both a high prolificacy (Matou, n = 182) and a low prolificacy breed (Boer, n = 352) by using the PCR-RFLP method. Superovulation experiments were designed in 57 dams, in order to evaluate the effect of different genotypes of the *GH* gene on superovulation response. Two genotypes (AA and AB, CC and CD) in each mutation were detected in these two goat breeds. Neither BB nor DD homozygous genotypes were observed. The genotypic frequencies of AB and CC were significantly higher than those of AA and CD. In the third parity, Matou dams with AB or CC genotypes had significantly larger litter sizes than those with AA and CD ($p < 0.05$). On combining the two loci, both Matou and Boer dams with ABCD genotype had the largest litter sizes when compared to the other genotypes ($p < 0.05$). When undergoing like superovulation treatments, a significantly higher number of corpora lutea and ova, with a lower incidence of ovarian cysts, were harvested in the AB and CC genotypes than in AA and CD. These results show that the two loci of *GH* gene are highly associated with abundant prolificacy and superovulation response in goat breeds.

Key words: DNA polymorphism, growth hormone (*GH*) gene, litter size, superovulation response, goat.

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Introduction

It has been reported that metabolic hormones, such as growth hormone (GH), are directly involved in mediating nutritionally-induced changes in follicular development (Downing and Scaramuzzi, 1991; Prunier and Quesnel, 2000; Armstrong *et al.*, 2003). The growth hormone of mammals plays an important role in the control of reproduction, in those aspects involving cell division, ovarian folliculogenesis, oogenesis and secretory activity (Schams *et al.*, 1999; Gong, 2002; Hull and Harvey, 2002; Ola *et al.*, 2008). By acting through specific receptors within the ovary, GH is expedient in controlling proliferation and apoptosis, oocyte maturation, and the expression and synthesis of receptors to hormones and related substances (Sirotkin *et al.*, 1998; Schams *et al.*, 1999; Hull and Harvey, 2000; Sirotkin *et al.*, 2003). The addition of bovine GH during *in vitro* maturation (IVM) of bovine oocytes has been found to induce cumulus expansion and accelerate nuclear maturation, besides promoting subsequent fertilization, cleavage and early embryonic development, as shown by enhancing the number of resultant blastocysts (Izadyar *et al.*, 1996, 1998; Joudrey *et al*., 2003). Further studies have revealed that the effect of GH on ovary function is mainly through inducing the development of small antral follicles in the gonadotrophin-dependent stages and stimulating oocyte maturation (Silva *et al.*, 2009).

As abundantly illustrated in the literature, numerous attempts have been made to investigate the effects of the *GH* gene on mammal growth and milk production (Wang *et al.*, 2003; Gupta *et al.*, 2007; Hua *et al.*, 2008; Balogh *et al.*, 2009; McCormack *et al.*, 2009), with only few reported studies of this in oogenesis and spermiogenesis (Kmie *et al.*, 2007; Murphy *et al.*, 2008), and no mention of the effects on litter size and superovulation response. To date, little has been divulged on the major gene associated with litter size in goats, these few studies involving the inhibin alpha-subunit gene (*INHA*) (Hua *et al.*, 2008; Wu *et al.*, 2009), the gonadotrophin releasing hormone receptor gene (*GnRHR)* (An *et al.*, 2009), the bone morphogenetic protein receptor-IB gene (*BMPRIB*) in the prolific Indian Black Bengal goat (Polley *et al.*, 2009), and the bone morphogenetic protein 15 gene (BMP15) in Jining Grey goats (Chu *et al.*, 2007).

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Growth and reproduction, two crucial economic traits in production, are co-ordinated during normal puberty and the adult stages. There is evidence that normal growth-hormone secretion is required for the correct timing of the onset of puberty (Franks *et al.*, 1998). As already confirmed in our previous research, there is a significantly positive association of two polymorphisms (A 781 G and A 1575 G) of *GH* gene with growth in Boer bucks (Hua *et al.*, 2008). Consistent with the essential function of GH in puberty and ovary activity, there is the need for further validation regarding its effects on dam reproduction.

Hence, in this study, the relatively hyper-prolific Matou breed and the low-prolific Boer were used to investigate the frequency distribution of the two *GH* gene polymorphisms (A 781 G and A 1575 G), and evaluate their effects on litter size. We contemplated factors affecting litter size, including year, season and parity, besides the age of the dam, with due account also being given to their mutual interactions. Furthermore, specific experiments were designed to evaluate the superovulation response in dams with different *GH* genotypes.

Materials and Methods

Data collection from experimental goat breeds

All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture, through the Animal Care and Use Committee at the respective institution where the experiment was undertaken.

A total of 534 adult females from two goat breeds differing in prolificacy, viz., 352 Boer dams with records of 1188 parities and 182 Matou dams with records of 583 parities, were analyzed in the present study. Those from the Boer breed, the low-prolific line (LS = 1.42) obtained from the Yidu Boer-Goat Breeding Station, had four depth levels of generation in the pedigree consisting of 129 sires and 552 dams, whereas the Matou breed, the native hyper-prolific line (LS = 2.14), collected from farms in Shiyan county, presented three generation depth levels in the pedigree.

Blood collection and genomic DNA preparations were carried out according to Hua *et al.* (2008). The data regarding litter size (LS) were collected in consecutive parities, considering repeated measurements in the same individual. Due to the significant effect of parity on litter size in goats, and its stability following the third parity (Moaeen-ud-Din *et al*., 2008; Wu *et al.*, 2009; Zhang *et al.*, 2009), the data were calculated separately for primiparity, third parity and all the parities together.

Superovulation dams and sampling

A total of 57 adult native, fertile females in healthy conditions were used in superovulation experiments. The dams, selected under similar and uniform conditions, taking into account age (3 to 5 years) and body weight (35 to 40 kg), were raised for about 2 weeks prior to the outset of superovulation. All the animals were barn housed and under controlled nutrition. The diet was a mixture of cured hay and grains, with a daily vitamin supplement. Fresh water and a mineral supplement were available *ad libitum*.

Superovulation procedure and response

The synchronization of estrus was induced by the administration of a single minuscule injection of Cloprostenol Sodium (PG-Cl), in a dose of 0.2 mg, on the day prior to initiating the experiment. Beginning on the 2nd day, Pituitary Follitropin for Capra (cFSH) was given twice daily at intervals of 12 h in eight decreasing doses (40, 40, 30, 30, 20, 20, 20, and 20 IU). The last cFSH dose was given concurrently with a single 25 ug dose of Luteinizing Hormone Releasing Hormone A3 (LHRH-A3). All the experimental dams were slaughtered on the 7th day, whereupon the ovaries and oviducts were collected and kept in incubation casks (37 °C) containing PBS, and brought to the laboratory within 1 h. The whole experiment, which took place in November, was undertaken with hormones provided by the Ningbo Sansheng Pharmaceutical Co., Ltd., Ningbo, China, all from single batches.

The ova were recovered by aspiration from the oviducts, and searched immediately after filtering the aspirated follicular fluid medium. The numbers of follicles of different sizes on ovarian surfaces were evaluated, based on a classification of follicle diameters as small (< 2.0 mm), medium (2.0 ~ 4.0 mm), large (4.0 ~ 8.0 mm) and ovarian cyst (> 8.0 mm) (Valasi *et al.*, 2007). The total number of ova, corpora lutea and ovarian cysts, per dam and in each class, were recorded. All the observations were done by one and the same person using the same methodology throughout.

Primer synthesis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

Our previous report (Hua *et al.*, 2008) formed the base for designing primer and setting PCR conditions. Two amplicons of 422 bp and 116 bp were yielded by the PCR reaction using the following primers synthesized by Shanghai Sangon Biotech Co., Ltd.:

GH1: 5’-CTCTGCCTGCCCTGGACT-3’ and 5’-GGAGAAGCAGAAGGCAACC-3’

GH2: 5’-TCAGCAGAGTCTTCACCAAC-3’ and 5’-CAACAACGCCATCCTCAC-3’

The Forced PCR-RFLP method was used to detect the A 781 G and A 1575 G mutations. The PCR products were digested by *Hae*III restriction endonuclease (TaKaRa, Tokyo, Japan) at 37 °C for 12-14 h with the following reaction system: a final volume of 10 μL containing 4 μL of the PCR product, 4 U of the *Hae*III enzyme and 1 x buffer R. In order to improve resolving power and accuracy, 8% polyacrylamide gel electrophoresis (PAGE), and, subsequently,

silver staining, were used for detecting the reaction products. *PBR322/MspI* (HuaMei Bioengineering Co., Ltd., China) was employed as a size marker for defining restriction-fragment sizes.

Statistical analysis

The allelic and genotypic frequencies in the *GH* gene in both goat breeds were analyzed. A Bonferroni correction (derivative-free restricted maximum likelihood, DFREML) was used to analyze the relationship between *GH* gene and litter size with animal models (Meyer and Kirkpatrick, 2005). Pedigrees of base population animals were traced back three (Matou) or four (Boer) generations, in order to create the numerator relationship matrix.

All the analyses were carried out in two steps, first using a full animal model and then using a reduced animal model. The full-animal model included all the factors, viz., genotypes, parity (1, 2, 3, 4 and ≥ 5), age of dam (1, 2, 3 and ≥ 4 years), kidding year (2002-2009), four kidding seasons – Spring (March and April), Summer (May to September), Autumn (October and November), and Winter (December to February), as well as the interactions of year-season, year-parity, parity-season and parity-age. Data of litter size from consecutive parities in the same individual were considered as repeated measures, and included in the statistical model. The reduced-model included only those fixed effects (genotype, kidding year, parity and age of dam) that exerted a significant influence on LS ($p < 0.05$), and was only used in the final analysis. The reduced-animal model was:

$$y = Xb+Za+Zp+e$$

where y is a vector of observations for litter size, b a vector of fixed effects for genotype, kidding year, parity and age of the dam, a a vector of animal genetic effects, p a vector of permanent environmental effects for parities of each dam, and e a vector of random residual effects. When analyzing the phenotypic data of primiparity and the third parity, the parity effect was excluded.

Data on superovulation response were analyzed statistically by applying the one-way ANOVA of SAS procedures (SAS Institute, Cary, NC, USA). The significance of the difference was determined by F-test at the significance level of 0.05.

Results

PCR amplification and RFLP analysis

As expected, two fragments of the *GH* gene (422 and 116 bp) were amplified from caprine genomic DNA by the *GH*1 and *GH*2 primers. Both fragments, when digested by endonuclease *Hae*III, resulted in four genotypes named: AA (366 and 56 bp) and AB (422, 366 and 56 bp) for *GH*1, and CC (88 and 28 bp) and CD (116, 88 and 28 bp) for *GH*2 (Figure 1). Thus, in the 534 individuals from the 2 differently prolific breeds, four genotypes of the two fragments of the *GH* gene were detected in both the Matou and Boer breeds, but no homozygotes of either BB or DD individuals were found (Figure 1).

Allele genotypic and haplotypic frequencies of GH gene in the two goat breeds

The genotypic and haplotypic frequencies of sequence polymorphisms are given in Table 1. The frequencies of AB and CC genotypes were much higher than those of AA and CD in both goat breeds. On comparing the same genotypic frequency between the two breeds, that of AB and CC genotypes in the Matou breed was much higher than in the Boer. When combining the two loci, the frequency of the ABCC haplotype proved to be the highest and that of the AACD the lowest.

Influence of genotype on litter size

The association of independent genotypes in *GH* gene with litter size, in Boer and Matou dams, is given in Table 2. In various parity groups, Matou dams with AB or CC genotypes had larger litter sizes than those with AA or CD ($p < 0.05$), although no significant difference appeared at

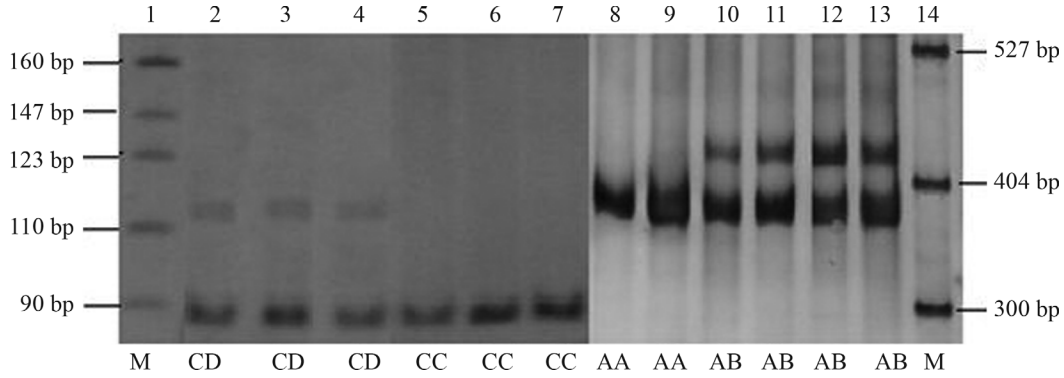

Figure 1 - Polyacrylamide gel electrophoresis (PAGE) profile for *Hae*III digestion products of *GH1* (AA and AB) and *GH2* (CC and CD). Lanes 2 to 4 represent digestion products from samples with the CD genotype (116, 88 and 28 bp). Lanes 5 to 7 represent the CC genotype (88 and 28 bp). Lanes 8 and 9 represent the AA genotype (366 and 56 bp). Lanes 10 to 13 represent the AB genotype (422, 366 and 56 bp). Small fragments of 28 bp and 56 bp were invisible in the gel. Lanes 1 and 14 show the PBR322/MspI DNA marker.

Table 1 - Sample size, and genotypic and haplotypic frequencies of *GH* polymorphisms in Boer and Matou goat breeds.

Breeds	N	A 781 G		A 1575 G		Haplotype			
		AA	AB	CC	CD	ABCC	ABCD	AACC	AACD
Boer	352	0.233 (82)	0.767 (270)	0.776 (273)	0.224 (79)	0.648 (228)	0.119 (42)	0.128 (45)	0.105 (37)
Matou	182	0.165 (30)	0.835 (152)	0.846 (154)	0.154 (28)	0.735 (134)	0.100 (18)	0.110 (20)	0.055 (10)
Total	534	0.210 (112)	0.790 (422)	0.799 (427)	0.201 (107)	0.678 (362)	0.112 (60)	0.122 (65)	0.088 (47)

Numbers in parentheses indicate sample size.

primiparity. At third parity, the performance in Matou dams with either genotype AB or CC was significantly superior to those with BB or CD by 0.58 and 0.63 kids born ($p < 0.05$). The same tendency was observed in Boer dams, although the difference was not significant.

The effects of combined genotypes in *GH* genes on litter size in Boer and Matou dams are given in Table 3. Combined genotype analysis of the two loci showed that LS was the lowest in the AACD genotype and the highest in ABCD. A significant difference was observed in various parity groups of the Matou breed, whereas, in the Boer breed this only appeared in the third parity ($p < 0.05$).

Influence of the genotype on superovulation response

The effects of the different *GH* genotypes on superovulation response are given in Table 4. The two SNPs of the *GH* gene had significant effects on the superovulation response. On undergoing identical treatments, corpora lutea and harvested ova were significantly more numerous in dams with AB and CC genotypes than in those with AA and CD. The numbers of small, medium and large follicles in dams with genotypes AB and CC were also higher than those carrying the genotypes AA and CD, although these differences were not significant. The incidence of ovarian cysts in dams with AB and CC genotypes was lower than in those with AA and CD.

Discussion

Alleles, genotypes and haplotypes diversity

In the present study, two polymorphisms of the goat *GH* gene at the loci A 781 G and A 1575 G were detected in 534 dams of the Boer and Matou breeds. The frequencies of AB and CC genotypes were higher in the relatively highly prolific Matou breed than in the relatively lowly prolific Boer breed. On the other hand, neither BB nor DD homozy-

Table 2 - Effects of separate *GH* gene genotypes on litter size in primiparity, the third parity and all the parities in Boer and Matou dams (means ± SD).

Parity groups	Breeds	A 781 G			A 1575 G		
		AA	AB	p-value	CC	CD	p-value
Primiparity	Boer (352)	1.42 ± 0.51 (82)	1.54 ± 0.71 (270)	0.6160	1.58 ± 0.69 (273)	1.51 ± 0.75 (79)	0.6646
	Matou (182)	1.69 ± 0.60 (30)	1.88 ± 0.77 (152)	0.6029	1.75 ± 0.74 (154)	1.73 ± 0.65 (28)	0.9632
Third parity	Boer (290)	1.81 ± 0.81 (62)	1.87 ± 0.63 (228)	0.8196	1.94 ± 0.64 (235)	1.77 ± 0.71 (55)	0.5227
	Matou (156)	2.04 ± 0.76 (25)	2.62 ± 0.89 (131)	0.0158	2.65 ± 0.87 (133)	2.02 ± 0.83 (23)	0.0294
All parities	Boer (352)	1.80 ± 0.73 (82)	1.81 ± 0.65 (270)	0.9341	1.89 ± 0.65 (273)	1.65 ± 0.74 (79)	0.2394
	Matou (182)	1.93 ± 0.65 (30)	2.37 ± 0.91 (152)	0.0036	2.32 ± 0.86 (154)	1.80 ± 0.76 (28)	0.0135

Numbers in parentheses indicate sample size.

Table 3 - Effects of combined *GH* gene genotypes on litter size in primiparity, third parity and all the parities in Boer and Matou dams (means ± SD).

Parity groups	Breeds	AACD	AACC	ABCC	ABCD
Primiparity	Boer (352)	1.33 ± 0.57 (37)	1.44 ± 0.53 (45)	1.53 ± 0.70 (228)	1.61 ± 0.78 (42)
	Matou (182)	1.50 ± 0.55^{a} (10)	1.62 ± 0.57^{ab} (20)	1.80 ± 0.79^{ab} (134)	2.00 ± 0.71^{b} (18)
Third parity	Boer (290)	1.50 ± 1.00^{a} (23)	1.88 ± 0.78^{ab} (39)	1.84 ± 0.62^{ab} (196)	2.04 ± 0.65^{b} (32)
	Matou (156)	2.00 ± 0.81^{a} (8)	2.17 ± 0.00^{ab} (17)	2.25 ± 0.87^{ab} (116)	2.55 ± 1.03^{b} (15)
All parities	Boer (352)	1.45 ± 0.71 (37)	1.76 ± 0.73 (45)	1.80 ± 0.64 (228)	1.88 ± 0.74 (42)
	Matou (182)	1.73 ± 0.47^{a} (10)	1.97 ± 0.68^{ab} (20)	2.02 ± 0.90^{ab} (134)	2.37 ± 0.90^{b} (18)

Numbers in parentheses indicate sample size.
Values marked in different superscripts on the same row (small letters) were significantly different ($p < 0.05$).

Table 4 - Effects of *GH* gene genotypes on the numbers of corpora lutea (NCL), follicles of different size and ova harvested, as well as the incidence of ovarian cysts after superovulation (means ± SD).

Genotype	Follicle size in diameter (mm)			NCL	Ova harvested	Incidence of ovarian cysts (%)
	Small (< 2.0)	Medium (2.0~4.0)	Large (4.0~8.0)			
AA (20)	8.3 ± 2.9	5.1 ± 1.8	5.7 ± 3.4	6.6 ± 2.6	4.4 ± 2.1	50.0 (10/20)
AB (37)	12.4 ± 2.3	8.4 ± 2.9	6.5 ± 2.5	11.7 ± 3.1	10.6 ± 3.3	27.0 (10/37)
P-value	0.2069	0.1105	0.4798	0.0379	0.0488	-
CC(49)	12.1 ± 3.8	9.3 ± 2.1	6.4 ± 2.6	11.7 ± 3.9	9.8 ± 3.4	22.4 (11/49)
CD(8)	9.0 ± 2.5	5.8 ± 2.3	5.9 ± 3.5	5.0 ± 2.1	5.5 ± 2.0	37.5 (3/8)
p-value	0.5611	0.4508	0.4191	0.0349	0.0782	-

Numbers in parentheses indicate sample size.
p-values for F- test.

gous were observed in any individual whatsoever. Our previous research on the *GH* gene also revealed this to be the case in 154 Boer bucks (Hua *et al.*, 2008), as was also reported in Chengdu-Ma (n = 37) and Boer (n = 29) goats (Bai *et al.*, 2005), and in a Gannan Yak population (n = 202) (Bai *et al.*, 2009). The A 781 G polymorphism was also detected in other goat flocks, such as the LuBei white goat (n = 50), and in the first filial generation of LuBei white and Boer (n = 105) goats (Li *et al.*, 2004).

All told, BB and DD genotypes were absent in both females and males in seven goat breeds, this including purebreds and crossbreds, as well as in Yaks, as mentioned above. It has been amply confirmed in the literature that the *GH* gene is essential for normal reproductive functions including oogenesis, follicular development and embryogenesis (Sirotkin *et al.*, 1998, 2003; Schams *et al.*, 1999; Hull and Harvey, 2000; Ola *et al.*, 2008; Silva *et al.*, 2009). Thus, it can be presumed that A 781 G (BB) and A 1575 G (DD) homozygous mutations in the *GH* gene may give rise to reproductive disturbance, even to the point of infertility.

Influence of the genotype on litter size and superovulation response

The association analysis showed that the different genotypes or haplotypes have significant effects on litter size and superovulation response. This is the first time that the effects of *GH* gene polymorphism on goat reproduction have been studied. To date, more than 10 goat *GH* variants have been detected, most of which involving growth traits (Gupta *et al.*, 2007; Hua *et al.*, 2008), and a few milk production (Malveiro *et al.*, 2001; Marques *et al.*, 2003). Nevertheless, no report has focused on reproduction traits. Our previous research has already confirmed that these two mutations of the *GH* gene exerted a highly additive effect on growth traits in Boer bucks (Hua *et al.*, 2008). The crucial role of *GH* in oogenesis and follicular development has been amply confirmed (Schams *et al.*, 1999; Sirotkin *et al.*, 2003; Silva *et al.*, 2009). Hence, the present study was designed to be a continuing step in evaluating the effect of the *GH* gene on litter-size and superovulation, with a mind to eventually providing further useful and detailed information for molecular marker-associated selection (MAS) programs.

The separation of AB and CC genotypes has been associated with larger litter sizes and a higher superovulation response. This effect is most likely due to allelic interaction and the important biological effects of *GH* on reproduction processes, this including oogenesis, follicular development and embryogenesis. It has been reported that, on initiating cattle superstimulation protocols, pre-treatment with growth hormone can increase the population of antral follicles (Bols *et al.*, 1998; Gong, 2002). Knockout experiments have demonstrated that *GH* enhances the development of small antral follicles up to the gonadotrophin-dependent stage, besides stimulating oocyte maturation (Silva *et al.*, 2009). The addition of bGH during *in vitro* maturation (IVM) of bovine oocytes has been found to induce cumulus expansion, besides accelerating nuclear maturation reflected by the accelerated extrusion of the 1st polar body, and promoting subsequent fertilization, cleavage and early embryonic development (Izadyar *et al.*, 1996, 1998). Kölle *et al.* (2001) reported that the *GH* gene is also involved in activating cellular functions in blastocysts, thereby stimulating glucose uptake and protein synthesis. Joudrey *et al.* (2003) reported that during bovine embryogenesis, bovine growth hormone contributes to proliferation, differentiation, and modulation of embryonic metabolism. The absence of BB and DD genotypes, as observed in the present and previous research, further confirmed the significance of *GH* in goat reproduction and growth.

When combined, the two SNPs displayed more profound impacts on LS than separately in both goat breeds. Both Boer and Matou dams with the ABCD genotype had the largest litter sizes compared to the others, and the combined genotype AACD was associated with the lowest litter sizes. This is consistent with previous research on goat growth traits by Hua *et al.* (2008), who reported that body weight and growth rate from birth to weaning was the lowest in Boer bucks bearing the AACD genotype,

whereby it can be concluded that goats with this genotype should be avoided in selection programs.

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