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MOLECULAR ASSOCIATIONS OF *GH* GENE GENOTYPES WITH SEMEN TRAITS IN RABBITS

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ABSTRACT

Five genetic groups of rabbits, APRI (A), Moshtohor (M), ½A½M, Sinai Gabali (G) and V-line (V) rabbits were genotyped for the growth hormone gene (*GH*) to detect the polymorphic associations of *GH* genotypes (TT, TC and CC) with semen traits. Semen traits included: ejaculate volume (VE), semen pH (pH), motility of spermatozoa (MS), sperm cell concentration (SC), live spermatozoa (LS) and normal spermatozoa (NS). Across all genetic groups, the TC genotype had the highest values for VE, MS, LS and NS traits compared to the other genotypes, while the CC genotype had the highest values for SC and pH. In V-line rabbits, the TT genotype showed higher values than CC genotype with an increase of 1.2% and $34x10^6$ in NS and SC, respectively and the TC genotype was higher than CC for MS and LS with an increase to be 2.0% and 3.6%, respectively. In Moshtohor line rabbits, the TC genotype showed higher values than TT genotype for MS and NS with an increase of 4.4% and 0.6%, respectively and the TT genotype was higher than CC genotype for SC with an increase of $37x10^6$. The TC genotype in APRI line rabbits was associated with an increase of 0.11 *ml*, 2.3% and 2.2% in VE, MS and NS, respectively compared with CC genotype and the CC genotype was the highest in SC with an increase of $178x10^6$, respectively over TT genotype. The TT genotype in ½A½M cross was the highest in MS and NS with an increase of 3.8% and 0.9%, respectively over CC genotype, while TC genotype had the highest value for SC compared with other genotypes. The TT genotype in Sinai Gabali rabbits was significantly associated with an increase of 7.4 % and $91x10^6$ in MS and SC over TC genotype. The TC genotype was the highest in VE with an increase of 0.17 *ml* over CC genotype and over TT genoytpe with an increase in LS.

Key words: Rabbits, Semen traits, Polymorphism associations, Candidate *GH* gene, PCR-RFLP.

INTRODUCTION

The candidate genes have shown successful approaches at identifying several DNA markers associated with production traits in rabbits (Fontanesi *et al.*, 2012; Zhang *et al.*, 2012; Peng *et al.*, 2013; Helal, 2019). However, growth hormone gene (GH) could be used as a candidate gene for assessing male reproductive performance particularly to seminal characteristics and fertility. The association of single nucleotide polymorphisms (SNPs) with different economical traits in rabbits was successfully explored (Peiro *et al*., 2008; Wu *et al*., 2015; El-Sabrout and Aggag, 2017; Migdal *et al*, 2019). However, assessments of the polymorphic association between GH gene and semen traits in rabbits are scarce. The main objective of the present study was to detect the molecular associations between *GH* gene genotypes and semen traits in five genetic groups of APRI (A), Moshtohor (M), ½A½M, Sinai Gabali (G) and V-line rabbits.

MATERIALS AND METHODS

Animals used, housing and feeding

This experiment was conducted in the rabbitry of the Faculty of Agriculture (Benha University, Egypt) during the period from September 2015 until December 2017. The animals used in this experiment were APRI line (A), Moshtohor line (M), ½A½M, Sinai Gabali and V-line as a reference population. A total number of 1050 ejaculates collected from 149 bucks (36 for V-line, 28 for M-line, 42 for APRI-line, 20 for Sinai Gabali and 23 for ½A½M) were used. The bucks were raised in one floor rabbitry. Rabbits received standard requirements of lighting, ventilation, as well as vaccination program. The animals were fed *ad libitum* all over the experimental period on a pelleted commercial ration. The ration was composed of 23% barley, 19% wheat bran, 24% soybean meal, 21% berseem hay, 13% yellow corn, 1% limestone,

0.5% table salt, 14 kg di-calcium phosphate/ton, 1 kg minerals mixture/ton, 1 kg anti-coccidian/ton, 1 kg anti-toxicity/ton, provided 18.01% crude protein, 13.7% crude fiber and 2.5% fat (digestible energy = 2500 to 2700 kc/kg feed).

Semen collection and evaluation

Semen was collected in the morning once per week using an artificial vagina according to Khalil *et al.* (2007). Each ejaculate was evaluated manually and under the microscope. The ejaculate volume was measured per *ml* using a graduated tube directly after collection. The pH of semen samples was specified using pH indicator paper. Percentage of progressive motility of spermatozoa was estimated subjectively and immediately after each collection using microscopic screening and by placing a small drop of fresh semen on a cleanly warm glass slide (37º- 38º), then diluted with two drops of warm 0.9% NaCl and enveloped with a cover slip. Semen examination was made under the high power microscope (x400). Discrimination between live and dead spermatozoa was estimated by Eosin - Nigrosin stain technique. The percentage of normal sperms was assessed according to Khalil *et al.* (2007). Sperms cell concentration ($x10^6$/*ml*) was quantified by direct cell count using the improved Neubauer haemocytometer**.**

DNA extraction and genotyping

Blood samples from 149 bucks were used for the extraction of genomic DNA using the Gene Jet Whole Blood Genomic DNA purity Mini Kit (Cat No. #K0781, Thermo Scientific). On chromosome 19, PCR was done for amplification of part of the 5'untranslated region and part of exon 1 of the growth hormone gene (*GH*) with expected amplicon size of 231 bp. PCR reaction was carried out in 25 µl containing 5 µl of the DNA template, 10 pmol of each primer and 12.5µl of Dream Taq Green PCR master mix (Fermentas, # K1071) and nuclease free water up to 25µl. Thermal cycling was carried out by initial denaturation at 95ºC for 5 min, followed by 35 cycles each at 95ºC for 30 seconds, annealing temperature at 58ºC for 30 seconds, extremism temperature at 72ºC for 30 seconds and final extension at 72ºC for 10 min., then the samples were held at 4ºC. The amplified DNA fragments were separated on 2% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by gel documentation system (Alpha Imager M1220, Documentation and Analysis, System, Canada). The C>T SNP of the growth hormone gene was genotyped by PCR-RFLP using *Bsh1236I (BstUI)* restriction enzyme (Fermentas, Vilnius, Lithuania) according to Fontanesi *et al* (2012).

Model for detecting the molecular associations between *GH* gene genotypes and semen traits

For each genetic group of the buck, the following mixed linear model (in matrix notation) was used:

$$\mathbf{y = Xb + Z_p u_p + e}$$

where **y** = vector of observed semen parameter for the buck; **b** = vector of fixed effects of, year-season of semen collection (10 levels), C/T SNP genotype of GH gene (three genotypes of TT, CC and TC); $\mathbf{u_p}$ = vector of random permanent environmental effect of the buck; **X,** and $\mathbf{Z_p}$ = incidence matrices relating records to the fixed and random permanent effects, respectively; **e** = vector of random residual effects. The same previous linear model was used for analysing the pooled data of all genetic groups jointly after adding the fixed effect of genetic group in the model. The permanent and residual variances were estimated using REML methodology using VCE6 software (Groeneveld *et al.*, 2010). These variance components were used in the PEST software (Groeneveld, 2006) to obtain the estimates of the fixed effects, and the molecular associations between genotypes of GH gene and semen traits.

RESULTS AND DISCUSSION

Means and variations

Means, standard deviations (SD) and coefficients of variation (CV%) for semen quality traits across the five studied genetic groups are presented in Table 1. Values of most semen traits obtained in the present study were similar to those reported in literature (García-Tomás *et al.*, 2006; Khalil *et al*, 2007; El-Tarabany *et al.*, 2015). In Saudi Arabia, Khalil *et al.(*2007) estimated that the average semen traits to be 0.63 *ml*, 7.5, 65.7% and $434x10^6$ sperm/ml for VE, pH, MS and SC in the cross between V-line and Saudi Gabali rabbits, respectively. The ranges between minimum and maximum values in semen traits across all studied genetic groups of rabbits were high with coefficients of variability ranging from 7 to 43% (Table 1). Khalil *et al.*(2007) in Saudi Arabia stated that the range of values for semen traits were high, being 0.1 to 1.5 *ml*, 5.5 to 9.0, 5.0 to 95.0% and 5.0 to $1080x10^6$ spermatozoa for VE, pH, MS and SC, respectively, in V-line and Gabali Saudi rabbits and their crosses.

Table 1: Summary statistics for semen quality traits in rabbits

Semen traits +	Symbol	Mean	SD	CV%	Minimum	Maximum
Volume of ejaculate (*ml*)	VE	0.65	0.24	36	0.1	1.4
Semen pH	PH	7.53	0.51	7	6.2	8.7
Motility of sperms, %	MS	49.91	15.1	30	10	90
Live sperms, %	LS	80.58	8.19	10	18	96
Normal sperms, %	NS	86.81	5.7	7	10	98
Concentration of sperms $x10^6$	SC	445.9	191	43	60	965

[+]Number of records=1050.

Molecular associations between *GH* gene genotypes and semen traits

The GH gene genotypes of showed significant effects on most semen traits in each line and across the five rabbit lines (Table 2). The heterozygous GH genotype (TC) gave the highest VE, MS, NS and LS, while the homozygous CC genotype gave the highest SC and pH of semen. The TC genotype had the highest values for MS compared to the other genotypes across all genetic groups and in each separate line of A, M, ½A½M, and V rabbits. The TT genotype had the highest values for SC in M, V and G rabbits. The TC genotype had the highest values for NS across all the genetic groups and in M and A lines. Across all the genetic groups, the TC genotype had the highest values for VE, MS, LS and NS traits, while CC genotype had the highest values for SC and pH. In V-line rabbits, the molecular associations of TT, TC and CC genotypes of GH gene were significant for some semen traits ($P<0.05$) and TT genotype showed higher values than CC genotype with an increase of 1.2% and 34 $x10^6$ in NS and SC, respectively. While, the TC genotype was higher than CC genotype for MS and LS with an increase to be 2% and 3.6%, respectively . In Moshtohor rabbits, the TC genotype showed higher values over TT genotype for MS, NS and VE with an increase of 4.4%, 0.6% and 0.03 *ml*, respectively. The TT genotype was higher than CC genotype with an increase of $37x10^6$ in SC, while the CC genotype was higher than TC with an increase of 2.1% in LS. In APRI rabbits, the TC genotype was higher than CC genotype in VE, MS and NS with an increase of 0.11 *ml*, 2.3% and 2.2%, respectively. The CC genotype was higher tha TT genotype in SC with an increase of 178 $x10^6$, contrarily, the TT genotype was higher than CC with an increase of 2.9% in LS. In ½A½M cross rabbits, the TT genotype was higher than CC in pH, MS, NS and SC with an increase of 0.3, 3.8%, 0.9% and $105x10^6$, respectively. On the contrary, the CC genotype was higher than TT genotype for LS with an increase of 5.5%. In Sinai Gabali rabbits, the TT genotype was higher than TC genotype for MS and SC with an increase of 7.4 and $91x10^6$, respectively. The TC genotype was higher than CC genotype in VE with an increase of 0.17 *ml*, and higher than TT genotype with an increase of 1.4% in LS.

CONCLUSION

The TC genotype was favorably associated with most semen traits. The sperm cell concentration showed the most relevant differences among different GH genotypes. Therefore, the T>C mutation of the GH gene constitutes a good candidate gene for increasing selection efficiency for semen traits in rabbits.

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Table 2: Least square means (LSM) and their standard errors (SE) for semen traits as affected by SNP genotypes of *GH* gene (TT, TC and CC) in each genetic group separately and across all the genetic groups

Semen trait	Genetic group+	TT		TC		CC	
		LSM	SE	LSM	SE	LSM	SE
Volume of ejaculate (VE), *ml*	V-Line	0.67^{b}	0.02	0.66^{b}	0.03	0.70^{a}	0.05
	Moshtohor line	0.70^{a}	0.07	0.73^{a}	0.06	0.69^{c}	0.04
	APRI line	0.58^{b}	0.04	0.67^{a}	0.02	0.56^{b}	0.08
	½A½M	0.72^{a}	0.05	0.68^{a}	0.04	0.73^{a}	0.09
	Sinai Gabali	0.50^{b}	0.04	0.59^{a}	0.03	0.42^{c}	0.10
	All genetic groups	0.65^{b}	0.02	0.68^{a}	0.01	0.62^{b}	0.03
Semen pH (pH)	V-Line	7.5^{a}	0.06	7.6^{a}	0.06	7.8^{a}	0.12
	Moshtohor line	7.7^{a}	0.02	7.6^{a}	0.03	7.5^{a}	0.05
	APRI line	7.3^{b}	0.06	7.4^{b}	0.04	7.8^{a}	0.14
	½A½M	7.4^{a}	0.09	7.4^{a}	0.08	7.1^{a}	0.19
	Sinai Gabali	7.5^{a}	0.07	7.5^{a}	0.19	7.7^{a}	0.06
	All genetic groups	7.5^{a}	0.03	7.5^{a}	0.02	7.6^{a}	0.06
Motility of sperms (MS), %	V-Line	47.1^{b}	1.73	49.1^{a}	1.81	47.1^{b}	3.60
	Moshtohor line	50.8^{b}	1.75	55.2^{a}	1.68	52.1^{b}	2.89
	APRI line	48.7^{a}	2.01	49.2^{a}	1.44	46.9^{b}	4.80
	½A½M	50.8^{a}	2.38	50.3^{a}	2.25	47.0^{b}	5.02
	Sinai Gabali	51.9^{a}	2.70	44.5^{c}	2.26	50.0^{b}	7.15
	All genetic groups	49.3^{a}	1.13	50.2^{a}	1.02	47.8^{b}	2.02
Live sperms (LS), %	V-Line	80.5^{a}	0.99	82.4^{a}	1.03	78.8^{b}	2.11
	Moshtohor line	81.1^{a}	1.02	79.7^{b}	0.98	81.8^{a}	1.64
	APRI line	80.2^{a}	0.82	79.6^{b}	1.15	77.3^{c}	2.74
	½A½M	81.1^{b}	1.60	81.7^{b}	1.51	86.6^{a}	3.38
	Sinai Gabali	79.9^{b}	1.11	81.3^{a}	0.93	80.7^{a}	2.96
	All genetic groups	80.5^{a}	0.16	80.8^{a}	1.56	80.7^{a}	1.13
Normal sperms (NS), %	V-Line	86.9^{a}	0.71	86.4^{a}	0.73	85.7^{b}	1.52
	Moshtohor line	86.2^{b}	0.87	86.8^{a}	0.84	86.6^{a}	1.44
	APRI line	86.0^{b}	0.59	87.4^{a}	0.42	85.2^{c}	1.41
	½A½M	87.4^{a}	0.82	87.0^{a}	0.77	86.5^{b}	1.72
	Sinai Gabali	87.9^{a}	0.83	87.0^{b}	0.74	88.2^{a}	2.08
	All genetic groups	86.7^{a}	0.41	86.9^{a}	0.37	86.2^{a}	0.75
Concentration of sperms (SC), x10^{6}	V-Line	472^{a}	22	462^{b}	22	438^{c}	46
	Moshtohor line	479^{a}	24	443^{b}	23	442^{b}	40
	APRI line	393^{c}	25	423^{b}	18	571^{a}	61
	½A½M	490^{a}	41	423^{b}	39	385^{c}	86
	Sinai Gabali	454^{a}	41	363^{c}	36	386^{b}	36
	All genetic groups	449^{b}	15	445^{b}	13	458^{a}	27

+Number of records = 275, 239, 312, 144 and 80 for V-line, Moshtohor line, APRI line, ½A½M and Sinai Gabali, respectively; different letters in the same row indicate significant differences at P<0.05