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Novel SNPs in heat shock protein 70 gene and their association with sperm quality traits of Boer goats and Boer crosses

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ABSTRACT

The semen quality of bucks affects the reproduction performance of the herd and is influenced by genetic and non-genetic factors. Heat shock protein 70 (*HSP70*) is considered as an important gene affecting semen quality traits. The objectives of this study are to find single nucleotide polymorphisms in *HSP70* coding region and their association with semen quality traits on Boer and Boer cross bucks. DNA isolated from 53 goats (36 pure South African Boer and 17 Boer crosses) was subjected to PCR amplification of the exon 1 region of the caprine *HSP70* gene. Single-strand conformation polymorphism (SSCP) was used to detect polymorphisms and the variant DNA fragments were sequenced. Two synonymous SNPs (74A > C (*ss836187517*) and 191C > G (*ss836187518*)) were detected. Qualities of fresh and post-thaw semen were evaluated for sperm concentration, semen volume, sperm motility and velocity traits, live sperm percentage, and abnormal sperm rate. The C allele of *ss836187517* and G allele of *ss836187518* were at higher frequencies in both the breeds. The C allele of *ss836187517* appeared to be the favorable allele for semen concentration, progressive motility of fresh semen, and motility and sperm lateral head displacement of post-thaw semen. A negative overdominance was observed for *ss836187517* alleles on velocity traits of post-thaw semen. The C allele of *ss836187518* was favorable for sperm concentration and progressive motility. Results herein suggest that the SNPs in *HSP70* may affect on semen quality in tropical regions and specially on the potential of semen for freezing.

1. Introduction

Heat shock proteins (HSPs) are a group of proteins that provide thermo tolerance in cell and protect cells against apoptosis during injury and oxidative stress (Beere and Green, 2001). Heat shock protein 70 (HSP70) is produced by the *HSP70* gene, and includes a family of HSPs which range in size from 68 to 73 kDa. The HSP70 gene is encoded by a single exon. The open reading frame the gene is 1926 bp and its protein includes 641 amino acids (Gade et al., 2010). HSP70 plays a protective role in reaction to hyperthermia as well as other stress conditions (Santoro, 2000) by providing a balance between synthesis and degradation of cellular proteins (Shi et al., 1998). It also acts as a molecular chaperone, which assists in the process of folding, transporting and assembling proteins in the cytoplasm, mitochondria and endoplasmic reticulum (Georgopoulos and Welch, 1993). Elliott et al. (2009) found that HSP70, as sperm-binding oviductal proteins, increase longevity and viability of sperm in bull and boar. Lack of the *HSP70* gene

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leads to a significant increase in apoptosis (Dix et al., 1996). It was reported that semen quality may be influenced by levels of HSP70 protein in boars (Huang et al., 2000). Govin et al. (2006) found association between HSP70 function and spermatid DNA-packaging proteins during spermatogenesis. Knockout *HSP70* mice showed structural abnormalities in spermatocytes, arrested evolution of primary spermatocytes, and increased apoptosis of these cells (Christians et al., 2003). Previous studies reported five SNPs in the 5′-flanking region of *HSP70* gene (Chen et al., 2000; Hess and Duncan, 1996; Huang et al., 2002). Polymorphism in this region showed association with sperm quality of boars (Huang et al., 2002), sperm characteristics in bull (Shrum et al., 2010) and calving traits (Rosenkrans et al., 2010). The present study attempted to detect SNPs in the exon regions of the *HSP70* gene and determine their effects on semen quality traits of pure Boer and Boer cross bucks.

2. Materials and methods

2.1. Experimental animals and semen evaluation

Fifty-three unrelated mature bucks (36 pure South African Boer and 17 Boer crosses), aged 2–3-years, were used for the experiment. The bucks were fed and managed under the same conditions. Three samples of semen were collected from each buck at one week intervals into graduated collection tubes using artificial vagina. Each sample was divided into two parts after measuring the semen volume (VOL). One portion was used for fresh semen evaluation, while the other portion was frozen in at least 6 straws. Three straws were thawed for semen evaluation after one day, and the other three straws were thawed after six months of freezing. The fresh semen quality traits which included sperm concentration (SCON), sperm general motility (MOT), sperm progressive motility (PROG), live sperm percentage (LIVE) was evaluated using a light microscope (Maina et al., 2006). To evaluate the quality of the post-thaw semen, 10 μl of diluted samples (1:2), were placed in the Hamilton 2X-Cel sperm analysis Chamber (Hamilton-Thorne, Biosciences MA, USA) and analyzed using computer-assisted semen analysis system (CASA) (HTM-IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA). The CASA analysis yielded MOT, PROG, fast motile sperm (FAST), static sperm (STAT), average path velocity (VAP), curvilinear velocity (VCL), straight linear velocity (VSL), and lateral head displacement (ALH). Analysis was accomplished with the following settings: magnification 1.92, frame rate 60 Hz, frame acquisition 30, minimum contrast 60, minimum size 5. The definitions of the sperm parameters are given in WHO (1999).

2.2. DNA extraction, PCR and genotyping

Genomic DNA was isolated from either the blood or semen sample of each animal using a QIAGEN blood and tissue DNA extraction Kit. Primers for the specific amplification were designed using the Primer3 software, based on published sequence information (GenBank No.: JN604433.1). The forward and reverse primers were acctgggcaccacctactc and aaaggccagtgcttcatgtc, respectively. PCR was performed in a final volume of 25 μl containing 100 ng DNA template, 0.2 μM of each primer, 0.2 mM dNTP, 0.5 μl proofreading Taq polymerase (Fermentas, UK), 1.5 mM $MgCl_2$ and 1x PCR buffer. The PCR was programmed as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. A final extension step was performed at 72 °C for 10 min. Electrophoresis of the amplicons was carried out in 1.5% agarose gels containing ethidium bromide in 1x TBE buffer, and the gels were visualized under ultraviolet light.

For SSCP analysis, 5 μl of the PCR product was mixed with 10 μl of denaturing solution (98% formamide; 20 mM EDTA, pH 8.0; 0.05% bromophenol blue; 0.05% xylene cyanol), and the mixture was denatured at 95 °C for 8 min, and chilled on ice for 10 min. Electrophoresis was carried out in non-denaturing 12% polyacrylamide gels in 1x TBE buffer at 4 °C and 90 V for 12 h. The gels were subsequently stained using silver staining method (0.1% $AgNO_3$) and scanned using a densitometer (L 800, BIO-RAD). The DNA samples exhibiting different patterns on the SSCP gels were selected for sequencing. The PCR products were purified using PCR purification kits (Fermentas®, UK) and sequenced (1st BASE Sequencing Services, Singapore). Nucleotide sequence alignments and comparisons were accomplished using the BioEdit version 7.0.9.0 software.

2.3. Statistical analysis

The mixed model analysis was used to test the effects of breed, age and genotype on the traits of the fresh and post-thaw semen. Age referred to two groups: 2-year-old group with animals between 22 and 28 months of age and 3-year-old group with animals between 34 and 40 months of age. The effects of individual SNPs were evaluated.

The data was analyzed using the SAS v9.2 software. The following model was used for association analysis for post-thaw semen quality traits:

$$y_{ijklm} = \mu + b(P)_{i(k)} + A_j + B_k + G_l + C_m + e_{ijklm} \quad (1)$$

where μ was the mean for each trait, A_j, B_k, G_l and C_m were the effects of age, breed, semen genotype and cryopreservation duration, respectively, b was the random effect of the buck which was nested in the population, and e was the random error. The model for analysis of the fresh semen traits was the same except without the effect of cryopreservation duration.

The additive (a) and dominance (d) effects were estimated only for the SNPs which were significantly associated with the semen quality traits and where all three genotypes were observed. The following formula was used for the purpose (Lin et al., 2006):

$$a = \frac{1}{2}(\mathrm{BB} - \mathrm{AA}); \quad d = \mathrm{AB} - \frac{1}{2}(\mathrm{AA+BB}) \quad (2)$$

where AA, AB and BB were the means of the trait values for each genotype. The significance of the effects were tested using t-test ($\alpha = 0.05$).

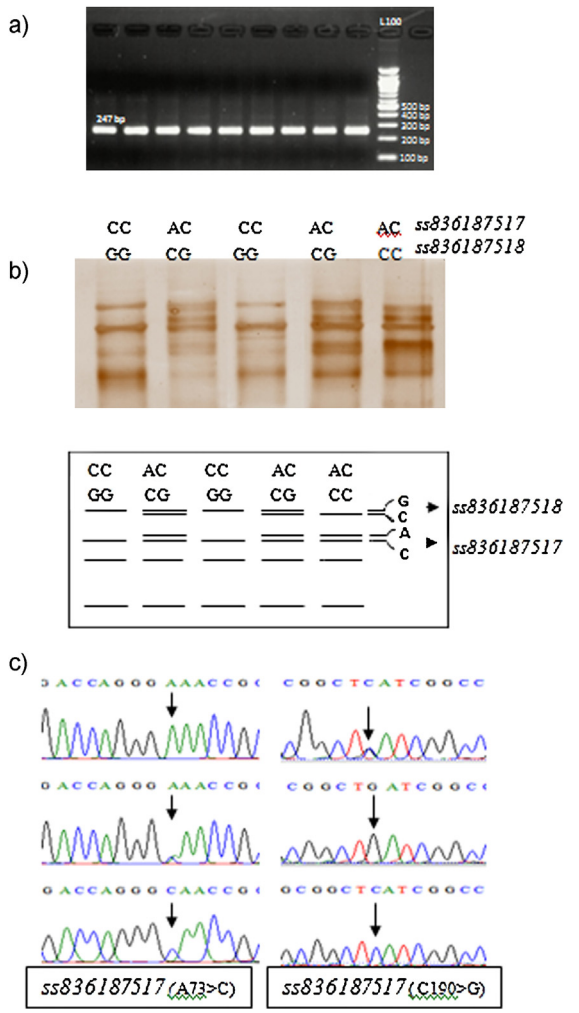

Fig. 1. PCR amplicon and genotypes of caprine HSP70 detected by PCR-SSCP and sequencing. (a) Gel electrophoresis image of PCR product of HSP70. (b) Electrophoresis image showing genotypes of caprine HSP70 detected by PCR-SSCP. (c) Sequence electropherograms for HSP70 loci showing SNP sites. The SNPs symbolized by arrows, the 3 genotypes are shown.

3. Results

A 247-bp fragment of the caprine *HSP70* gene (exon1) (base positions 29–276) was amplified for all 53 bucks (Fig. 1a) . PCR-SSCP analysis of the PCR amplified and nucleotide sequencing revealed two transversion mutations to be present in the *HSP70* gene at positions 74A>C (*ss836187517)* and 191C>G (*ss836187518)* (Fig. 1b) (GenBank: KC731565.1). The C allele of *ss836187517* and the G allele of *ss836187518* showed higher frequencies in both the breeds. Both the SNPs were synonymous and no amino acid replacement is expected in the products of the respective genotypes. The results of the effects of the SNPs are displayed in Table 1. Association analysis with the fresh semen quality traits showed bucks with the AC genotype at *ss836187517* had SCON and lower PROG for fresh semen ($P<0.01$). On the other hand, VOL, SCON, MOT, PROG and LIVE of fresh semen were the lowest for the

Table 1
LSmeans (±SE) of fresh and frozen semen quality traits showing the effects of SNPs at exon 1 of the *HSP70* gene.

Genotypes		Number of individuals	Fresh semen traits					Post-thaw semen traits						
			VOL (ml)	SCON ($\times 10^6$)	MOT (%)	PROG (%)	LIVE (%)	MOT (%)	PROG (%)	STAT (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)
ss836187517	AA	3	0.68^{a} (0.15)	5025.44^{a} (1273.3)	74.93^{a} (8.69)	72.44^{a} (8.26)	90.33^{a} (6.09)	29.69^{a} (5.09)	11.91^{a} (3.11)	59.08^{a} (5.87)	106.32^{a} (5.93)	96.20^{a} (5.87)	157.14^{a,b} (9.95)	4.76^{a} (0.26)
	AC	28	0.61^{a} (0.04)	8359.48^{b} (413.21)	70.81^{a} (2.49)	57.07^{b} (2.43)	85.05^{a} (1.76)	38.64^{a,b} (1.60)	15.89^{a} (1.05)	46.79^{b} (1.96)	91.67^{b} (1.67)	79.99^{b} (1.72)	146.75^{a} (2.80)	5.57^{b} (0.09)
	CC	22	0.64^{a} (0.06)	6578.70^{a} (502.53)	77.32^{a} (3.34)	64.64^{a} (3.25)	89.28^{a} (2.27)	41.99^{b} (2.12)	17.42^{a} (1.30)	43.83^{b} (2.43)	98.27^{a} (2.22)	85.81^{a} (2.30)	157.65^{b} (3.73)	5.80^{c} (0.11)
P-value			0.470	0.006	0.413	0.004	0.146	0.021	0.119	0.017	0.004	0.004	0.022	<.0001
ss836187518	CC	6	0.80^{a} (0.11)	7694.10^{a} (927.69)	79.51^{a} (6.17)	70.86^{a} (5.97)	91.17^{a} (4.23)	34.97^{a} (3.86)	13.83^{a} (2.38)	52.66^{a} (4.47)	100.78^{a,b} (4.14)	89.68^{a} (4.20)	156.38^{a} (6.95)	5.25^{a} (0.20)
	CG	24	0.51^{b} (0.07)	4985.00^{b} (624.95)	66.12^{b} (4.25)	58.46^{b} (4.05)	83.34^{b} (3.11)	39.18^{a} (2.70)	16.47^{a} (1.65)	48.02^{a} (3.11)	101.26^{a} (3.16)	89.33^{a} (3.12)	162.09^{a} (5.30)	5.63^{b} (0.14)
	GG	23	0.63^{a,b} (0.05)	7284.52^{a} (459.03)	77.43^{a} (3.04)	64.83^{a} (2.89)	90.16^{a} (2.12)	36.18^{a} (1.72)	14.93^{a} (1.09)	49.03^{a} (2.05)	94.22^{b} (2.00)	82.98^{a} (1.99)	143.07^{b} (3.36)	5.26^{a} (0.09)
P-value			0.001	0.477	0.001	0.022	0.018	0.333	0.276	0.276	0.042	0.063	0.001	0.002

^{a,b,c} Means for a particular trait (column) not sharing any superscripts were significantly ($P<0.05$) different. VOL: semen volume, SCON: sperm concentration, MOT: sperm motility, PROG: Progressive motility, LIVE: live sperm rate, VAP: average path velocity, VSL: straight-line velocity, VCL: curvilinear velocity, ALH: lateral head displacement.

heterozygous genotype at *ss836187518* ($P < 0.05$); VOL was not significantly different ($P > 0.05$) when compared to the GG genotype. Association analysis of post-thaw semen showed that the CC genotype at *ss836187517 displayed* significant ($P < 0.05$) higher MOT and ALH than AA genotype. The velocity traits (VAP, VCL and VSL), were lowest for the AC genotype. There was no difference in MOT among the three genotypes at *ss836187518* for post-thaw semen was associated with lower velocity traits.

The additive (a) and dominance (d) effects of the loci estimated for the fresh and post-thaw semen traits associated with the *ss836187517* and *ss836187518* genotypes are shown in Table 2. The statistical analyses revealed that allele A of *ss836187517* had negative influence on SCON and PROG of fresh semen, and post-thaw MOT and ALH ($P < 0.01$). The additive effect of *ss836187517* on percentage of STAT was also significant and negative. The means of the sperm velocity traits for the heterozygote of *ss836187517* were lower ($P < 0.05$) than the means of the two homozygotes, indicating a significant, negative overdominance effect of the alleles at this locus. For the *ss836187518* locus additive effect on SCON and PROG of fresh semen was highly significant ($P < 0.01$), while a significant ($P < 0.01$) dominance effect was observed on VCL and ALH for post-thaw semen. The linkage disequilibrium measure for two SNPs on HSP70, *ss836187517* and *ss836187518*, is $D' = 0.275$ and seven haplotypes of the two SNPs were detected in the samples.

4. Discussion

Two SNPs were detected in the *HSP70* gene at positions 74A>C (*ss836187517*) and 191C>G (*ss836187518*). These SNPs demonstrated significant association with quality traits of fresh and post-thaw semen. The *ss836187517* locus showed significant association with many of the fresh and post-thaw semen quality traits. However, allele C was identified as the favorable allele for the motility traits and ALH of post-thaw semen. In addition, the analysis of the additive effects showed allele C to have a positive effect on SCON and PROG of fresh semen ($P < 0.01$). The allele C of *ss836187518* also could be considered as the favorable allele for many of the important fresh semen quality traits such as SCON, VOL and PROG, as well as for velocity traits of post-thaw semen.

The SNPs were synonymous in their effects and do not alter the amino acids of the relevant protein. It has been reported that synonymous SNPs may affect the relevant protein via change in transcription and may also influence the accuracy or efficiency of splicing of mRNA or transcriptional control (Cartegni et al., 2002; Komar, 2007). There are many reviews that described the potential effect of pre-mRNA splicing on phenotype of traits (Faustino and Cooper, 2003; Ho et al., 2011; Nissim-Rafinia and Kerem, 2002). Kimchi-Sarfaty et al. (2007) stated that silent mutation might influence the rate of translation via change in codon usage during production of nascent protein. Therefore, the observed effects of the SNPs in this study may be due to their influence on level of expression of the *HSP70* which consequently change the level of HSP70 protein.

Association between the SNPs of *HSP70* and SCON in current study may be due to the role of HSP70 on spermatogenesis. Similarly, Huang et al. (2002) reported an association between total sperm number and SNP of 5′-flanking region of *HSP70* gene in boar. HSP70 as a chaperon protein involves in the formation of protein complexes (Connell et al., 2001; Bozidis et al., 2002). Since spermatogenesis is a thermosensitive process (Bitto et al., 2008), normal spermatogenesis requires the testis temperature to be 4–5 °C lower than the body temperature. The functions of HSP70 may influence semen quality traits of goats in a tropical area such as Malaysia. HSP70 is associated with the nascent polypeptides and assists in the folding and assembly of proteins into complexes during spermatogenesis (Dun et al., 2012). Formation CDC2/cyclin B1 (CDC2: cell division cycle protein 2) complex during the spermatocyte pachytene phase depends to proper function of HSP70 (Dix et al., 1997). This complex is required for synaptonemal complex desynapsis. On the other hand, lack of spermatids and mature spermatozoa in knockout *HSP70* mice (Christians et al., 2003) is another evidence of the essential role of HSP70 in spermatogenesis and consequently on SCON.

LIVE showed an association with *ss836187518*, in this study. Viability of sperm can be varied due to DNA integrity of spermatozoa and the factors which may prevent the cells from oxidation and other stresses (Aitken et al., 2014). Association between the SNP of *HSP70* and LIVE in this study may be explained by relation of HSP70 with post-meiosis major spermatid DNA-packaging proteins. Govin et al. (2006) detected abundant HSP70 within the nucleus prior to histone removal and the formation of the new structure of spermatid. The increased expression of *HSP70* during spermatogenesis implied the importance of *HSP70* during meiosis (Dix et al., 1996). HSP70 also has association with transition proteins forms the specific acid resistant complexes in the spermatozoa cell (Govin et al., 2006). Exposition and relocation of HSP70 in spermatozoa after chemical and mechanical stress preserve sperm membrane and maintain sperm vitality (Spinaci et al., 2006). Therefore, regarding to the assumption of effect of the SNP on level of HSP70 and these functions of HSP70, it may influence sperm viability before and after ejaculation and consequently alter the LIVE trait among different genotypes.

In current study, both of the SNPs showed association with velocity traits. Similarly, Shrum et al. (2010) reported associations between a deletion in *HSP70* promoter and sperm motility and velocity traits in bulls. This may be due to influence of HSP70 on protection of proteins related to respiration activity and level of energy in spermatozoa. Yeung et al. (1996) and Nascimento et al. (2008) reported that the sperm velocity parameters are influenced by energy level and enzyme activity. On the other hand, cooling and freezing cause a fast drop in enzymatic respiration activity of the mitochondria which consequently causes depletion in sperm ATP supply and decrease in spermatozoa motility (Peña et al., 2003). HSP70 as a protein chaperon acts to prevent protein aggregation, help proteins maintain their conformation and assist restructure damaged protein (Gasch et al., 2000; Lewandowska et al., 2006; Daugaard et al., 2007). Thereby, effect of HSP70

Table 2
LSmeans (±SE) of Additive and dominance effects of the *ss836187517* and *ss836187518* alleles on fresh and post-thaw semen quality traits.

Loci	ss836187517		ss836187517	
Effects	Additive	Dominance	Additive	Dominance
Fresh semen traits				
VOL (ml)	–	–	−0.15^{ns} (0.17)	0.18^{ns} (0.15)
SCON ($\times 10^6$)	−6867.13*** (1293.14)	2690.83** (1109.60)	5336.62** (1863.40)	−1474.21^{ns} (1603.57)
PROG (%)	−15.73** (5.99)	8.29 ^{ns} (5.04)	26.91** (8.66)	9.29 ^{ns} (7.43)
MOT (%)	–	–	−8.12 ^{ns} (10.20)	−1.66 ^{ns} (8.61)
LIVE (%)	–	–	−10.07 ^{ns} (7.01)	0.26 ^{ns} (5.89)
Post-thaw semen traits				
MOT (%)	−12.98** (4.78)	8.16^{ns} (5.86)	–	–
STAT (%)	15.25** (5.47)	−9.33^{ns} (6.71)	–	–
VAP (μm/s)	7.29^{ns} (5.57)	−21.79*** (7.02)	6.57^{ns} (3.89)	7.89^{ns} (5.08)
VSL (μm/s)	9.81^{ns} (5.53)	−22.45*** (6.87)	–	–
VCL (μm/s)	−2.78^{ns} (9.43)	−21.44* (11.68)	11.41^{ns} (6.69)	26.62** (8.50)
ALH (μm/s)	−1.04*** (0.25)	0.58^{ns} (0.30)	−0.01^{ns} (0.18)	0.75*** (0.22)

ns = not significant. VOL: semen volume, SCON: sperm concentration, MOT: sperm motility, PROG: Progressive motility, LIVE: live sperm rate, VAP: average path velocity, VSL: straight-line velocity, VCL: curvilinear velocity. ALH: lateral head displacement. -: the additive and dominance effects only estimated for the traits that was significantly associated with *HSP70* loci.

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

on refolding proteins may prevent mitochondrial enzymes denaturation and consequently alter sperm motility and velocity traits.

The observed differences in the effects of the SNPs on motility traits in fresh and frozen semen in this study may be due to the role of the HSP70 on semen quality after ejaculation and during storage, as reported by Elliott et al. (2009) and Lloyd et al. (2009). It was found that adding recombinant HSP70 to semen increased longevity and viability of spermatozoa during cooling and after freezing (Lloyd et al., 2012). The processes of cooling and freeze thawing cause physical and chemical stresses on sperms which can decrease semen quality and its fertility capacity (Stradaioli et al., 2007; Dorado et al., 2010). It was found that, peroxidation phenomenon on spermatozoa membrane occurs due to oxidative stress reaction on phospholipids polyunsaturated fatty acids (Maldjian et al., 2005; Yildiz et al., 2007; Kim et al., 2011), and leads to formation of toxic fatty acids and hence structural damage to the sperm cell and damages to DNA (Baumber et al., 2003) and sperm membrane (Aziz et al., 2007), which consequently reduces sperm motility. On the other hand, presence and level of HSP70 is required to protect the proteins involved in DNA repair or recombination (Jeong et al., 2009).

To the best of the authors' knowledge, the SNPs detected in the coding region of *HSP70* in the present study have not been reported, making them novel SNPs. Therefore, the association results could not be compared with those of other researchers. However, Huang et al. (2000) reported that the levels of heat-shock protein 70 influenced semen quality traits of boar. They detected SNPs in the 5′-flanking region of *HSP70* which were associated with boar semen quality traits in the hot season. Based on their findings, it may be assumed that *HSP70* would also influence semen quality traits of goats in tropical regions such as Malaysia.

5. Conclusion

This study detected two novel SNPs (*ss836187517* and *ss836187518*) in exon 1 *FSHB* that may create new binding sites. Statistical analyses revealed that these SNPs in caprine *HSP70* with were associated with most of the fresh and post-thaw semen quality traits, especially velocity traits. However, if the results could be further validated in even larger buck populations, these SNPs could be considered as early selection markers for semen quality of goats to be used in artificial insemination programs. The effect of the SNPs on the expression level of the HSP70 gene and protein in goats requires further investigation.

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