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ASSOCIATION OF *MTNR1A* AND *CYP19* GENES POLYMORPHISMS WITH SPERM QUALITY AND TESTICULAR SIZE IN SANJABI BREED RAMS*

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Abstract

The purpose of this study was to identify mutations in melatonin receptor 1A (*MTNR1A*) and aromatase cytochrome P450 (*CYP19*) genes using PCR-RFLP technique and their associations with sperm quality and testicular size traits in Sanjabi breed rams. The blood and sperm samples were collected from Sanjabi rams (n = 96). Genomic DNA was extracted from the blood. A 824bp fragment from exon II of *MTNR1A* gene and a 517bp fragment from promoter 2 (P2) of *CYP19* gene were amplified using two pairs of specific primers. The PCR products were separately digested by two restriction enzymes, *SsiI* for *MTNR1A* locus and *DraI* for *CYP19* locus. Digestion by *SsiI* restriction enzyme resulted in CC, CA and AA genotypes with frequency of 0.45, 0.41 and 0.14, respectively. However, digestion of 517bp fragment of *CYP19* gene by *DraI* endonuclease determined two AG and AA genotypes with frequency 0.89 and 0.11, respectively. The Chi-square test proved that the two loci were in Hardy-Weinberg equilibrium (HWE). The significant effect was observed between different genotypes of *MTNR1A* gene and morphological trait (P<0.05) and there was a significant association between different genotypes of *CYP19* gene and scrotal circumference trait (P<0.05). The results of this study indicated that polymorphisms of *MTNR1A* and *CYP19* genes were not associated with most traits of sperm quality and testicular sizes. Therefore, it seems that further studies are needed to identify mutations in other regions of these genes and other genes responsible to genomic regions for the sperm quality and testicular size in Sanjabi ram in order to improve fertility in these herds.

Key words*: CYP19* gene, *MTNR1A* gene, Sanjabi sheep, sperm quality, testicular size

Reproduction, as an important economic trait in sheep breeding, is a complex trait under effects of both genetic and environmental factors and has low (5–10%)

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heritability (Javanmard et al., 2011). Most of the researches on fertility in farm animals have cleared many inclinations on the female side. Fertility in the male is as important as that in the female (Davidson and Farver, 1980). Rams with superior reproductive traits are required in order to increase fertility in herds, to improve the genetic merit and also to decrease the number of breeding males (Mukasa-Mugerwa and Ezaz, 1992). Production of large amount of high-quality spermatozoa by genetically superior rams has led to increase conception rates and to reduce the percentage of non-pregnant ewes and in general to improve overall flock fertility (Rege et al., 2000).

Survey of semen characteristics is one of the most effective factors for selecting breeding rams. The quality of semen is usually determined by sperm motility, volume, concentration, livability and morphological features (Mia et al., 2015). Direct selection for semen quality traits is difficult due to low heritability (Mathevon et al., 1998). However, the major genes associated with these traits can be used in breeding programs through marker-assisted selection (MAS). Selection of rams in herds based on DNA markers at an early age can reduce generation interval, increase selection accuracy and reduce cost of rams breeding in herds. Recently, researchers have focused on genetic background of ram fertility and the IGF-1 and leptin have been known as candidate genes affecting sperm quality of ram (Bakhtiar et al., 2017 a; b).

Melatonin receptor 1A (*MTRN1A*) and Aromatase cytochrome P450 (*CYP19*) are known as candidate genes for reproduction. Melatonin is released by the pineal gland during night. In sheep, short photoperiods which correspond to the highest melatonin secretion, positively effect the secretion of gonadotropin-releasing hormone (GnRH) from hypothalamus and thus stimulate the secretion of luteinizing hormone (LH) (Malpaux et al., 1999). LH secretion is responsible for alternating presence or absence of ovulation in the female and for sperm production in the males (Malpaux et al., 1999). Melatonin stimulates testis for spermatogenic activity through increasing of Leydig cell sensitivity to LH (Langford et al., 1987). So far, in mammals, two specific melatonin receptors named MT_1 and MT_2 have been identified, but it seems that MT_1 receptor may be involved to control reproductive activity (Pelletier et al., 2000). In sheep, *MTRN1A* gene is located at chromosome 26 (Messer et al., 1997) and includes two exons divided by a large intron (Reppert et al., 1994). Moreover, the exon II of this gene has shown a high degree of polymorphism in sheep populations (Pelletier et al., 2000).

In sheep, the *CYP19* gene codifies aromatase P450 enzyme which is a terminal enzyme responsible for estrogen biosynthesis via conversion of androgens to estrogens (Simpson et al., 1994). Estrogen is an important hormone for controlling reproduction in male and female animals, fat deposition (Jones et al., 2000) and growth (Simpson et al., 2000). The *CYP19* gene has been mapped on the ovine chromosome 7 (Goldammer et al., 1999). It is transcribed from four different promoter regions ($p_{1.1}$-$p_{1.4}$-$p_{1.5}$ and p_2) depending on organs-specific activities but P_2 is mainly active in granulosa cells (Vanselow et al., 2001). The purpose of this study was to identify polymorphisms of *MTRN1A* and *CYP19* genes and their associations with semen quality and testicular size in Sanjabi breed sheep.

Material and methods

Animals and sampling

Sanjabi sheep is the most important fat-tail meat sheep breed in the west region of Iran (Kermanshah province). In this study, blood and sperm samples were collected from 96 Sanjabi rams belonging to three large flocks (more than 400 heads of sheep) located in the province. At the same time, the dimensions of testis such as length, width and circumference were measured.

The flocks were under same management and housed in semi-extensive conditions with dry summer and cold winter. The grazing season generally lasted from April to October; animals were on pasture during the day and kept indoors at night. Supplemental feed, including alfalfa and barley grains, was offered to the animals. Annually, 30–40 rams were allocated randomly to mate with about 10–15 ewes in breeding pens.

Semen collection and evaluation

Semen samples were collected from rams (2–5 years old) using an artificial vagina in spring and autumn over two consecutive years. In order to increase accuracy of sperm evaluation, the rams were separated from flocks 24–48 hours before sampling. Immediately after sampling, semen samples were evaluated for color, volume (ml), mass motility (%), progressive motility (%) and sperm concentration ($\times 10^7$ sperm/ml) according to Evans and Maxwell (1987). Smears related to morphology (%), viability (%), HOS and water tests (%) were prepared and transferred to the laboratory for more evaluations. Sperm volume was directly recorded from the graduated collection tube and the concentration of sperm was determined using a hemocytometer. For assessing mass activity, 20µl of undiluted semen samples was transferred to a pre-warmed glass slide (37°C) and examined under light microscope (10X), using scales from 0 (no motility) to 5 (excellent motility). Diluted semen samples (1:10) were placed (10µl) on a pre-warmed slid (37°C) covered with cover slip to examine progressive motility under light microscope (40X). The eosin-nigrosine stained slides were evaluated for viability, where unstained spermatozoa were considered to be alive (Evans and Maxwell, 1987). The proportion of abnormal morphologically sperm was also determined by examining 200 spermatozoa in an eosin-nigrosine smear under microscope (40X). Total number of spermatozoa per ejaculate was calculated by multiplication of the seminal volume with the sperm density. Semen index (semen volume × sperm concentration × live sperm × progressive motility) was calculated as a semen quality indicator. The percentage of spermatozoa with plasma membrane integrity was determined using two methods including HOS (Revell and Mrode, 1994) and water test (Nur et al., 2005).

DNA extraction, PCR reactions and samples genotyping

Blood samples were taken from jugular vein of rams using sterile tubes containing EDTA as an anticoagulant. Genomic DNA was extracted from whole blood using DIAtom DNA prep100 kit (Gene Fanavaran Co, Iran), then quality and quantity of the extracted DNA samples were observed using 1% agarose gel electrophoreses and kept at –20°C until they were used. The sequences of the specific primers to amplify

MTNR1A and *CYP19* genes are given in Table 1. Primers were synthesized by Metabion Company, Germany.

Table 1. Summary of general characteristics of primers and genes studied in this research

Genes	Primer sequence	Amplified region	Product size (bp)	Mutation	Annealing (°C)	Restriction enzyme
MTNR1A	F:5`-TGTGTTTGTGGTGAGCCTGG-3` R:5`- ATGGAGAGGGTTTGCGTTTA-3`	exon II	824	C893A	62	*SsiI*
CYP19	F:5`-ACAATGGGAGGCTCTGAGAATG-3` R:5`-GAAAAATTAGAAAATCCCCAAAA-3`	promoter2 (P_2)	517	A113G	59	*DraI*

PCR reaction was carried out in 25µl of final volume, containing 50–100ng of DNA used as a template, 0.25µM of each primer (Table 1), 2.5µM PCR buffer 1X, 0.2mM dNTPs, 2.5mM $MgCl_2$ and 1U of Taq DNA polymerase. The amplification conditions for primers of the *MTNR1A* and *CYP19* genes were as follows: an initial denaturation of 3 min at 94°C, followed by 35 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing (62°C for *MTNR1A* locus, 59°C for *CYP19* locus), 1 min of extension at 72°C and final extension of 10 min at 72°C. PCR products were electrophoresed on 1% agarose gel and DNA bands were visualized by ethidium bromide staining. The PCR products including of exon II of *MTNR1A* gene and promoter 2 (P2) of *CYP19* gene were digested with *SsiI* and *DraI* restriction enzymes (Thermo Scientific, Germany), respectively. The digestion reactions were separately carried out in 15µl volume, containing 8µl of PCR products and 5U of *SsiI* or *DraI* enzymes. The reaction mixture was incubated at 37°C for 16 h. Sample genotyping was conducted based on fragments separated by electrophoresis on 3% agarose and stained with ethidium bromide.

Statistical analysis

PopGene (Version 32) software (Yeh et al., 1999) was used to estimate allelic and genotype frequencies and heterozygosity. The Hardy-Weinberg equilibrium (HWE) was tested based on Chi-square test (χ^2). The association between different genotypes and traits studied was analyzed using GLM procedure of SAS package (Version 9.1) software (SAS Institute, 2004).

The linear model with the fixed effects was:

$$y_{ijkl}=\mu+HYS_i+W_j+Age_k+G_l+e_{ijkl}$$

where: y_{ijkl} is the trait measured on each animal (semen quality and testicular size); μ is overall mean, HYS_i is the fixed effect of herd-year-season, W_j is the effect of ram weight (as covariate effect), Age_k is the fixed effect of ram age, G_l is the effect of the l^{th} genotypes, e_{ijkl} is the residual effect.

Least square means of the different genotypes were calculated by LSMEANS option in SAS software (Version 9.1) to determine the effects of these genes on semen quality and testicular size traits. The significant level of 0.05 was considered to show statistically significant differences for all comparisons.

Results

PCR-RFLP and animal genotyping

Genomic DNA quality of all samples was evaluated on 1% agarose gels (Figure 1). The 824bp fragment for exon II of *MTNR1A* gene (GenBank U14109) was successfully amplified in all ram samples (Figure 2). The A>C single nucleotide polymorphism (C893A) in the exon II of *MTNR1A* gene was detected by *SsiI* restriction enzyme, where the mutation led to Alanine by Aspartic acid substitution. Digestion of the amplified fragments by *SsiI* enzyme revealed three fragments (422, 218 and 184bp) for C allele (wild type) and two fragments (640 and 184bp) for A allele (mutant) (Figure 3). The allele and genotype frequencies of *MTNR1A* gene and Chi-square (χ^2) value have been presented in Table 2. The frequencies of CC, CA and AA genotypes were 0.45, 0.41 and 0.14 and allelic frequencies of C and A alleles were 0.65 and 0.35, respectively.

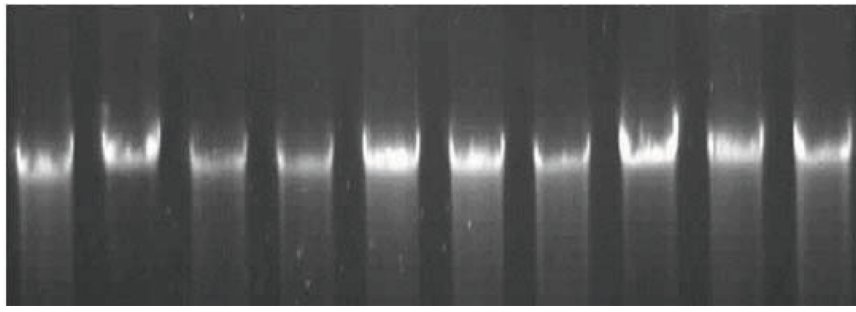

Figure 1. DNA extracted from blood of Sanjabi ram on 1% agarose gel

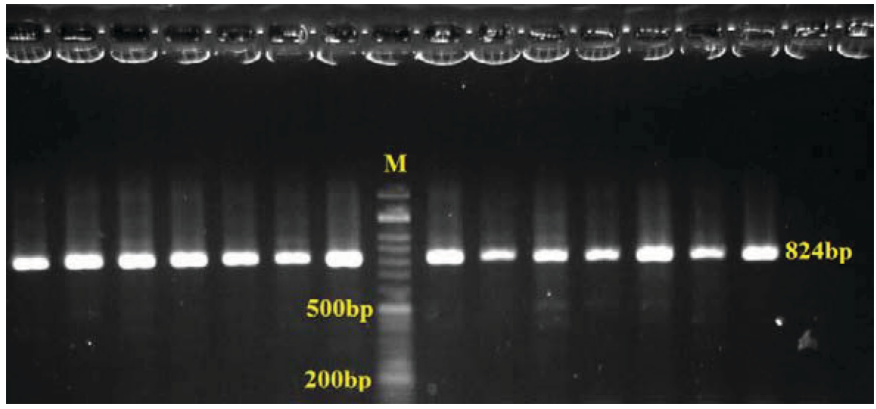

Figure 2. Amplification of exon II of *MTNR1A* gene (824bp) on 1% agarose gel. M: 50bp Ladder marker

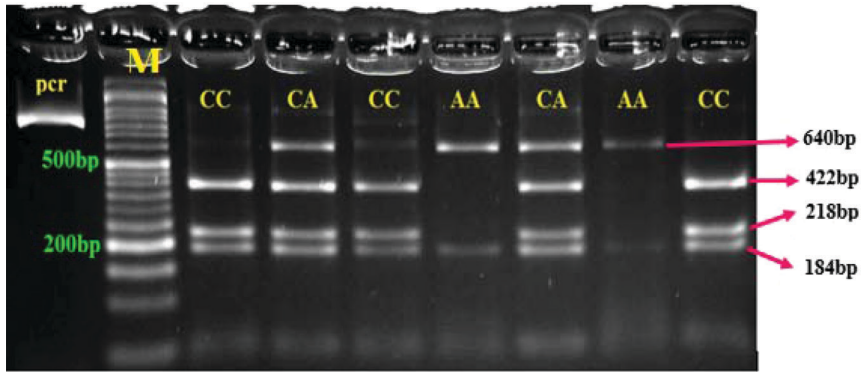

Figure 3. Digestion of 824bp fragment of *MTNR1A* gene using *SsiI* enzyme on 3% agarose gel. AA (640 and 184bp), CA (640, 422, 218 and 184bp), CC (422, 218 and 184bp) genotypes

A 517 bp fragment including promoter 2 (P2) at CYP19 gene was amplified for all samples (Figure 4). Digestion of amplicons by *DraI* restriction enzyme showed presence of A to G mutation located in position 113 of the sequence (GenBank AJ012144). PCR-RFLP results detected two alleles (A and G) and two genotypes (AA: 401, 116 bp and AG: 517, 116, 401 bp) in the studied population (Figure 5). The estimated frequencies of the A and G alleles were 0.95 and 0.05 and those for the AA and AG genotypes were 0.89 and 0.11, respectively (Table 2).

Table 2. The genotypes and allele frequencies of *MTNR1A* and *CYP19* genes polymorphisms and Chi-square values in Sanjabi sheep rams

Gene	Allele frequency		Genotype frequency	Chi-square (P-value)
MTNR1A	C=0.65	A=0.35	CC(43)= 0.45 CA(39)= 0.41 AA(14)= 0.14	1.18 (0.27)
CYP19	A= 0.95	G = 0.05	AA(85)= 0.89 AG(11)= 0.11	0.32 (0.57)

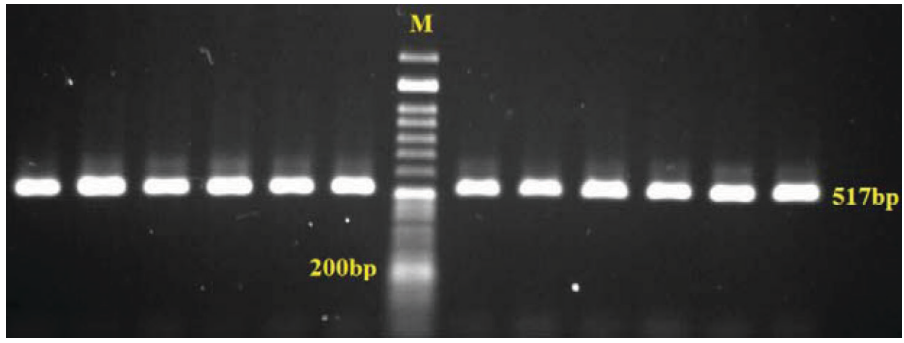

Figure 4. Amplification of p2 of *CYP19* gene (517bp) on 1% agarose gel. M: 50bp Ladder marker

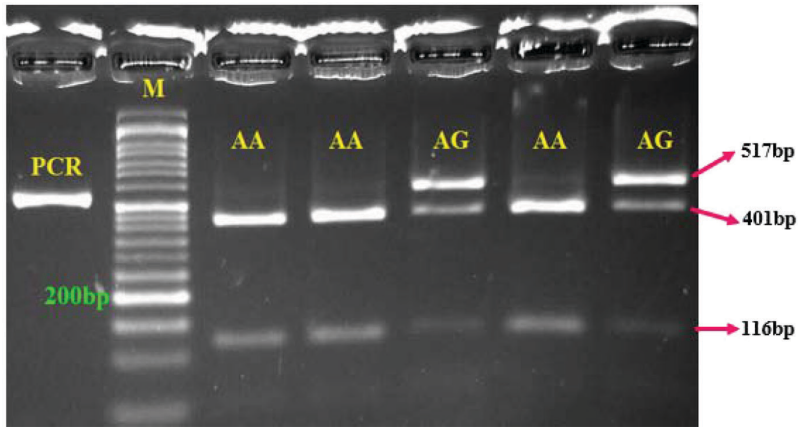

Figure 5. Digestion of 517bp PCR product of *CYP19* gene using *DraI* enzyme on 3% agarose gel. AA (401 and 116bp), AG (517, 116 and 401bp) genotypes

The result of Chi-square test (Table 2) indicated that the population of Sanjabi native sheep for these genes was in Hardy-Weinberg equilibrium (P>0.05), which can be a sign of absence of selection and immigration in the local herds. Population diversity indexes for these positions were shown in Table 3. For *MTNR1A* gene, Shannon and Nei index amounts were calculated (0.646 and 0.454, respectively), which show high genetic variation in this population. However, these indexes for *CYP19* gene were estimated to be 0.108 and 0.219, respectively (Table 3), which reveal moderate genetic diversity of *CYP19* gene in the present population.

Table 3. The estimation of population diversity indexes in Sanjabi breed rams

Gene	Ob-Hom	Ob-Het	Exp-Hom	Exp-Het	Ne	Nei	I
MTNR1A	0.593	0.406	0.543	0.456	1.832	0.454	0.646
CYP19	0.885	0.114	0.891	0.108	1.121	0.108	0.219

Ob-Hom = Observed Homozygosity, Exp-Hom = Expected Homozygosity, Ob-Het = Observed Heterozygosity, Exp-Het = Expected Heterozygosity, Ne = Effective number of alleles, Nei = Nei's genetic diversity, I = Shannon's Information Index.

Association of *MTNR1A* and *CYP19* genes polymorphisms with testicular size traits

Results of relationship between polymorphisms observed in exon II of *MTNR1A* gene and in promoter 2 (P_2) of *CYP19* gene with testicular size traits are presented in Table 4. Statistical analysis indicated no significant effect of different genotypes at *MTNR1A* gene on the testicular size traits (Table 4) but the AA genotype had the highest average of the testicular length, scrotal circumference and testicular volume.

The results showed a significant association between *CYP19* gene polymorphism and scrotal circumference (P<0.05), as AA genotype had the higher scrotal circumference than that of AG genotype. More significant associations were not observed between this polymorphism and other testicular size traits.

Table 4. Least square means (± standard error) of *MTNR1A* and *CYP19* genotypes for testicular size in Sanjabi rams

Gene	Genotype	TL (cm)	TW (cm)	TV (cm)	SC (cm)
MTNR1A	CC	18.69±0.65	7.47±0.27	123.54±8.31	31.20±1.06
	CA	19.21±0.57	7.5±0.25	122.97±7.7	31.86±0.98
	AA	19.30±0.97	7.38±0.4	129.04±12.39	31.84±1.48
CYP19	AA	18.64±0.38	7.66±0.19	131.64±5.89	32.98±0.86 a
	AG	19.49±0.96	7.24±0.4	118.73±12.32	30.35±1.47 b

TL: Testicular Length, TW: Testicular Width, TV: Testicular Volume, SC: Scrotal circumference.
a, b – means in the column of each parameter with different letters differ significantly (P<0.05).

Association of *MTNR1A* and *CYP19* genes polymorphisms with sperm quality traits

In the present study, the effect of different genotypes at C893A position of *MTNR1A* gene on sperm quality traits in Sanjabi sheep breed was evaluated for the first time. Our results showed a significant association between *MTNR1A* genotypes and morphological traits (P<0.05), as the CA and CC genotypes had the highest and lowest rate of normal morphology, respectively, however there was no significant association between these genotypes and other traits of sperm quality (Table 5).

Table 5. Least square means (± standard error) of *MTNR1A* genotypes for sperm quality in Sanjabi rams

Genotype	SV (ml)	GM (0-5)	PM (%)	SCON (X×107)	TSE (X×107)
CC	1.31±0.12	1.52±0.1	4.44±0.045	265.12±32.12	337.83±56.8
CA	1.32±0.1	1.56±0.09	4.4 ±0.042	270.62±27.86	371.67±48.06
AA	1.12±0.17	1.56±0.14	4.45±0.06	231.88±44.81	256.82±87.11
Genotype	SMO (%)	SI (ml^{-1})	SVIA (%)	WT (%)	HOS (%)
CC	88.25±1.99 b	2489000.08	85.86±2.43	89.08±1.95	94.48±1.41
CA	92.75±1.78 a	2796146.40	82.93±2.24	89.00±1.79	93.40±1.31
AA	90.30 ±2.56 ab	1698477.88	83.68±3.41	89.98±2.7	92.85±2.03

SV: Sperm Volume, GM: Gross Motility, PM: Progressive Motility, SCON: Sperm Concentration, TSE: Total spermatozoa per ejaculate, SMO: Sperm Morphology, SI: Sperm Index, SVIA: Sperm Viability, WT: Water Test, HOS: Hypo Osmotic Swelling Test.
a, b, c – means in the column of each parameter with different letters differ significantly (P<0.05).

Table 6. Least square means (± standard error) of *CYP19* genotypes for sperm quality in Sanjabi rams

Genotype	SV (ml)	GM (0-5)	PM (%)	SCON (X×107)	TSE (X×107)
AA	1.26±0.07	1.52±0.06	4.42±0.03	262.00±23.8	318.48±35.1
AG	1.25±0.17	1.58±0.14	4.46±0.06	249.75±42.67	325.73±80.61
Genotype	SMO (%)	SI (ml^{-1})	SVIA (%)	WT (%)	HOS (%)
AA	90.81±1.63	2510651.65	84.92±1.67	87.80±1.34	93.85±0.97
AG	90.06±2.55	2145097.92	83.40±3.46	90.91±2.77	93.30±2.03

SV: Sperm Volume, GM: Gross Motility, PM: Progressive Motility, SCON: Sperm Concentration, TSE: Total spermatozoa per ejaculate, SMO: Sperm Morphology, SI: Sperm Index, SVIA: Sperm Viability, WT: Water Test, HOS: Hypo Osmotic Swelling Test.

The effect of *CYP19* promoter 2 (P_2) polymorphism on sperm quality has been shown in Table 6. No significant correlation was observed between *CYP19* gene genotypes and sperm quality traits in Sanjabi rams (P>0.05).

Discussion

In the present research, recessive homozygous genotype (GG) was not observed in this position of *CYP19* gene, which may be due to low frequency of the G allele (0.05) and also small sample size in this study. Based on our results, the frequency of A allele in this breed was higher than G allele for *CYP19* gene, which is in agreement with previous studies in eight breeds from Spanish and Hungarian sheep (Zsolnai et al., 2002) and eight native breeds from Turkish sheep (Elmaci et al., 2013).

Measurement of testis is used as an indicator to determine reproductive status and spermatogenesis capacity of small ruminants such as sheep and goats (Roca et al., 1991). The testicular size parameter is important because it is highly heritable, therefore it can easily be improved by selection (Hafez et al., 1955). A good measurement of testis scrotal circumference (SC), testicular weight (TW), testicular length (TL) and testicular volume (TV) can reliably predict sperm production capacity (Ugwu, 2009). Among these parameters, SC is mostly used because it is easy to be measured and it shows significant positive correlation with TW and sperm concentration (Brito et al., 2004; Al-Ghalban et al., 2004). Generally, a positive correlation exists between SC, TW, percentage of seminiferous tubules and elongated spermatids (Al-Ghalban et al., 2004). Coe (1999) found a significant correlation between SC and semen quality, however, Fernandez et al. (2004) observed no significant relationship between testicular size and sperm production.

Results of the present research showed that different genotypes of *CYP19* gene could significantly affect the scrotal circumference trait (P<0.05), while significant effects were not observed for different genotypes of *MTNR1A* gene on the testicular size traits (P>0.05). However, according to previous studies, treatment of rams with implanted melatonin by effecting on hypothalamic pulse producers caused an increase in LH pulse frequency and testicular development (Webster et al., 1991).

Sperm concentration, motility and morphology are used for male infertility realization but sperm morphology alone has been a strict criterion for assessing fertile and infertile men and pregnancy rate in women (Guzick et al., 2001). Nayak and Gunasheela (2016) reported that less than 10 percent normal form of sperm has led to decrease pregnancy rates. Recent data has shown that planted melatonin in non-reproductive season has a significant effect on sperm motility and causes an increase in normal morphology rate in rams (Kaya et al., 2000). Furthermore, Casao et al. (2010) reported that planted melatonin or short-day exposure due to an increase in sperm motility can improve direct effect of melatonin on ram spermatozoa and change in secretion of gonadotrophins (increased secretion of FSH, LH and testosterone), because of an increase in GnRH secretion.

Our results showed a significant association between genotypes of *MTNR1A* gene and sperm morphological trait ($P<0.05$). To our best knowledge, no study to date has been conducted in order to investigate effects of *MTNR1A* gene polymorphisms on sperm quality, so there is no possibility to compare our results with previous studies. However, the *MTNR1A* gene can be one of the possible candidate genes for controlling ovine reproduction. In several studies, two SNPs in sheep *MTNR1A* gene, at 606 and 612 positions of exon II, had been detected to be associated with seasonal reproduction activity, as reported in different breeds including: Merino d`Arles (Pelletier et al., 2000), Small tail Han (Chu et al., 2006), Sarda (Carcangiu et al., 2009), Chokla (Saxena et al., 2014), and local Greek sheep breed (Giantsis et al., 2016). However, no significant correlation has been found between polymorphism in this gene and seasonal reproduction in Alpine and Creole goat breeds and Ile de France sheep (Migaud et al., 2002; Hernandez et al., 2005).

In the current study, no significant correlation was observed between *CYP19* gene genotypes and sperm quality traits ($P>0.05$) while the previous researchers have shown presence of that aromatase mRNA in Leydig, Sertoli, spermatocytes and spermatids cells (Carreau and Galeraud-Denis, 2008) and also in human ejaculated spermatozoa (Aquila et al., 2002). Furthermore, previous studies reported that aromatase deficiency in human reduces sperm number and motility (Carreau and Galeraud-Denis, 2008). The results of another study proved a significant association between *CYP19* $(TTTA)_7$ polymorphism and low sperm concentration and motility in normozoospermic men (Lazaros et al., 2011). Additionally, some mutations in *CYP19* gene led to estrogen (Maffei et al., 2004) and ERα (Smith et al., 1995) hormone deficiency which caused a reduction in sperm motility and viability.

Conclusions

In the present research, polymorphisms studied in *MTNR1A* and *CYP19* genes were not associated with most traits of sperm quality and testicular size. The results showed that these polymorphisms cannot be responsible for controlling spermatogenesis. Regarding the role of melatonin and aromatase in reproductive process, it seems that further studies are necessary in order to investigate association of other polymorphism regions in these genes with reproductive traits in the rams.

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