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Polymorphism of the Melatonin Receptor 1A Gene and Its Association with Litter Size in Zel and Naeini Sheep Breeds

Research Article

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ABSTRACT

The influence of melatonin receptor 1A gene on litter size was studied in 150 ewes from Zel (n=100) and Naeini (n=50) sheep breeds. Two restriction fragment length polymorphism (RFLP) analyses were done to determine all genotypes occurring by variations at *MnlI* and *RsaI* restriction sites. The M and C were the most frequent alleles in both of these breeds. The MM genotype was predominant in Zel and Naeini breeds (0.52 and 0.60, respectively). The CT genotype (0.45) has the highest frequency in Zel and the CC genotype (0.44) was predominant in Naeini breed. The MMCT genotype was identified with the highest frequency of 0.25. There were no significant differences between melatonin receptor 1A (MTNR1A) genotypes and litter size in Naeini ewes. But it seems that selection of animals with mm and Mm genotypes can progress mean litter size in Zel breed. The positive effect of mC and mT alleles was significant on lambing rate in Zel and Naeini breeds. However, the mmCT genotype has considerably greater mean litter size than the other ones. Finally, the MTNR1A polymorphism can explain only a small part of the genetic variability on seasonal sexual activity between these breeds. Therefore, the implication of other genes must be noticed.

KEY WORDS litter size, melatonin receptor 1A, Naeini, polymorphism, Zel.

INTRODUCTION

Highly variation exists among ovise arise species in some physiological characteristics, such as ovulation rate, fecundity and efficiency of reproduction that can be caused by the action of single or closely linked group of genes (Davis *et al.* 2005). Investigation of polymorphic quantitative trait loci (QTL) or major genes in selection programs have distinguished advantages especially in genes that affected the control of the seasonal reproduction of sheep due to low heritability, expressed relatively late in life, expressed in only one sex or revealed only in some environmental conditions or management systems (Al Shorepy and Notter, 1996; Al Shorepy and Notter, 1997; Hernández *et al.* 2005).

It has been suggested that the efficiency of selection to decrease seasonality of breeding in sheep would be improved by identification of informative genetic markers (Malpaux *et al.* 1996). Melatonin is an important hormone that plays a key role in animal physiology. This hormone affects on regulation of circadian rhythms, seasonal reproduction, and inhibition of dopamine release from retina, vasoregulator activity, immune modulatory roles, cell growth and cytoprotective (Von Gall *et al.* 2002; Pandi Perumal *et al.* 2006). High level of blood concentration of melatonin has a positive effect on reproduction in small ruminants (Carcangui *et al.* 2005). Sheeps belong to mammals which have seasonal reproductive activity that is controlled by photoperiod. It thought to be due to melatonin

(Thiery *et al.* 2002). Melatonin secretion depends on circadian rhythm. By increasing of darkness peak the level of hormone occurrs in all vertebrate animals whether diurnally or nocturnally active (Bittman *et al.* 1983; Karsch *et al.* 1984; Von Gall *et al.* 2005). Light signal reached by the retina lead to melatonin being synthesized by the pineal gland (Goldman, 2001). In sheep, the high melatonin levels typically occur when the darkness increases exponentially (short photo period) which stimulates the pulsatile secretion of gonadotropin releasing hormone (GnRH) and as a consequence luteinizing hormone (LH) is secreted (Chabot *et al.* 1998; Malpaux *et al.* 1999; Carcangiu *et al.* 2005). Melatonin exerts its roles via link with specific two guanine nucleotide binding protein (G-Protein) coupled receptors named MT1 and MT2 (Ebisawa *et al.* 1994; Reppert *et al.* 1996; Hazlerigg and Loudon, 2008). Three subtypes Mel1A, Mel1b and Mel1c belong to MT1 which shows high affinity to this hormone (Reppert *et al.* 1996; Migaud *et al.* 2002) that it is located with higher density in the hypophyseal pars tuberalis rather than other areas in the central nervous system (CNS) or pituitary in most species (Lincoln and Clarke, 1994; Dubocovich, 1995; Dubocovich *et al.* 1998). Only in mammals, Mel1A and Mel1b have been identified after cloning and characterization (Reppert *et al.* 1994; Reppert *et al.* 1995). MT2 has a subtype named Mel2 which has low affinity to melatonin hormone (Reppert *et al.* 1996). It seems, only Mel1A gene is involved in the regulation of reproductive activity (Reppert *et al.* 1994; Weaver *et al.* 1996; Drew *et al.* 1998; Dubocovich *et al.* 1988). The melatonin receptor 1A (MTNR1A) genotypes are linked with seasonal reproduction in Merion d'Arles ewes (Pelletier *et al.* 2000). An association with seasonal reproduction was also reported in a composite line of 50% Dorset, 25% Rambauillet and 25% Finn sheep (Notter *et al.* 2003). The gene coding for the melatonin receptor protein 1A, mapped on chromosome 26 in ovine, consisted of two exons interrupted by an intron (Reppert *et al.* 1994; Barrett *et al.* 1997). The second exon encodes the main part of the aforesaid gene; the presence of variation can be evidenced by means of the *MnlI* and *RsaI* restrictive enzymes in this region (Messer *et al.* 1997). Notter *et al.* (2003) showed that the association between different allelic forms of the MT1 locus and reproductive activity of sheep, the genotype at this gene might become particularly a marker in investigation of the sexual activity in sheep.

MATERIALS AND METHODS

Animal and sample collection

Zel and Naeini sheep are known as two important indigenous breeds of Iran. Zel sheep is un-tail breed and adapted to humid climate in northern Iran. This breed has small body size between 29 and 33 kg in adult ewes and it was known as out-of-season mating breed with about 15% twining rate (Khaldari, 2004). Lambs are mated in autumn and spring seasons. Naeini sheep is a fat tail and one of the populated breed which is located in the central provinces of Iran. The one year old weight of this breed, that is large in size, is between 38 and 40 kg. Naeini ewes have seasonal reproduction activity. Individuals are mainly uni parous and twining rate increased up to 5% with supportive feeding (Flashing) (Khaldari, 2004). In this study a total of 150 ewes lamb (Zel (n=100) and Naeini (n=50)), which were chosen at random, were assessed. The blood samples were collected (10 mL per sheep) by jugular vein puncture into vacuum tube with EDTA as an anticoagulant along with data on litter size which encompassing 450 records from three parities in studied sheep breeds.

DNA extraction and PCR amplification

Genomic DNA was isolated from whole blood using salting out procedure according to Miller *et al.* (1988) with some modifications. The isolated DNA was submitted to PCR. A 824 bp PCR fragment of the main part of the exon 2 of the ovine MTNR1A gene was amplified with specific primers (synthesized by CinnaGen, Iran) as described by Messer *et al.* (1997). The PCR reaction was performed in 25 µL of total volume, containing 50-100 ng genomic DNA, 3 m*M* of $MgCl_2$, 1 µL each of sense and antisense primer (10 m*M* each), 0.5 µL of 10 m*M* dNTPs (0.2 m*M* each), 1 Unit of Taq DNA polymerase (CinnaGen, Iran) and 1.5 µL of 10X supplied PCR buffer. The amplification was conducted in ABI 2720 thermal cycler (Aplied Biosystem, USA) with following temperatures profile consisting of an initial denaturation at 94 ˚C for 5 min, followed by at 35 cycle program with denaturation at 94 ˚C for 1 min, annealing at 62 ˚C for 1 min, elongation at 72 ˚C for 1 min and final elongation at 72 ˚C for 10 min. PCR products were separated by electrophoresis on 1% agarose gel in 1X TBE buffer alongside with a 100 bp DNA size marker (Fermentase, Germany). After staining with ethidium bromide (200 ng/mL), the visualization was carried out under the Gel Documentation System (BioRad, USA).

Genotyping

Variation in melatonin receptor 1A gene was examined after enzymatic treatment of resulting amplicon with *MnlI* or / and *RsaI* (Messer *et al.* 1997) restriction enzymes. The digestion reaction was conducted in 10 µL final volume; containing 5 µL of each amplicon digested separately with the *MnlI* or *RsaI* enzymes (Fermentase, Germany) at 37 ˚C overnight. Deactivation of enzymes were conducted at 65 ˚C for 20 min. Digestion products were analyzed by electrophoresis on 9% polyacrylamid gel in the presence of the

Gene Ruller™ 50 bp DNA size marker (Fermentase, Germany). The results were visualized and documented using the Gel Documentation System (BioRad, USA).

Statistical analysis

Directly counting was used to calculate allelic (f_i) and genotypic (f_{ij}) frequencies, as:

$f_{ij} = n_{ij} / N$

$p_i = f_{ii} + ½ \Sigma f_{ij}$

Where:

n_{ij}: the number of the animals with the ij genotype.

N: the total number of the animals.

The Chi-Squre Test (χ^2) was used to assess both, whether there is a difference in various MTNR1A allele and genotype frequencies between two sheep breeds and whether the populations are in Hardy-Weinberg equilibrium or not.

Analysis of relationship between litter size and melatonin receptor 1A genotype using general liner model (GLM) procedure was done by Statistical Analysis System (SAS V. 9.1). The following model was employed in order to analysis of litter size in Zel and Naeini ewes:

$Y_{ijk} = \mu + P_i + G_j + e_{ijk}$

Where:

Y_{ijk}: phenotypic value of litter size.

μ: the average of favorable trait in stock.

P_i: the fixed effect of the i^{th} parity (i=1, 2 and 3).

G_j: the fixed effect of the j^{th} genotype.

e_{ijk}: the effect of the random error of each observation.

In this study, association analysis was carried out in three separate approaches with different classification of the MTNR1A genotypes. The first analysis was performed based on the association of the *MnlI* / MTNR1A and the *RsaI* / MTNR1A genotypes and litter size separately. In the second approach, the potential association of the number of the MTNR1A alleles with litter size was considered. Therefore, the lambs were classified into four MTNR1A genotype categories depend on whether they carried 2, 1 or 0 copies of each allele (i.e. MC/MC, MC/XX and XX/XX for MC allele, where XX represents non specified allele). The third analysis was carried out based on the association of litter size with different MTNR1A haplo genotypes.

RESULTS AND DISCUSSION

In the present study MTNR1A polymorphisms were identified in Zel and Naeini sheep breeds. In addition, association between the polymorphisms and the litter size were analyzed. A DNA fragment with the expected size of 824 bp corresponding to the important region of the exon 2 of the melatonin receptor 1A gene was obtained from sheep DNA using specific primers. These amplimers exhibited polymorphism upon PCR-RFLP analysis by *MnlI* and *RsaI* restriction enzymes (Figure 1 and Figure 2).

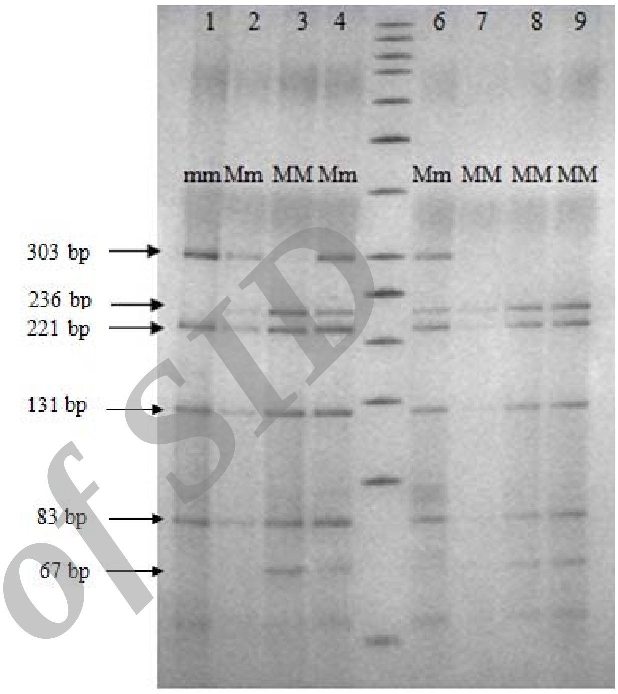

Figure 1 Electrophoresis of digestion with *MnlI* on 9% polyacrylamid gel in Zel and Naeini ewes lambs; lanes 3, 7, 8 and 9: MM; lanes 1: mm; lanes 2, 4 and 6: Mm; lane 5: 50 bp DNA size marker

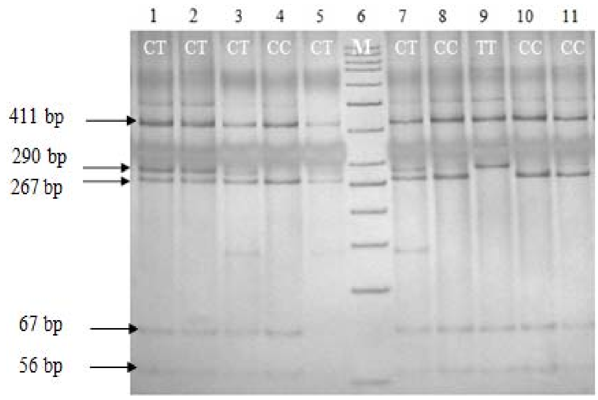

Figure 2 Electrophoresis of digestion with *RsaI* on 9% polyacrylamid gel in Zel and Naeini ewes lambs; lanes 1, 2, 3, 5 and 7: CT; lanes 4, 8, 10 and 11: CC; lane 9: TT: lane 6: 50 bp DNA size marker

Digestion of the amplimers with *MnlI* produced fragments of 221, 131, 83, 36, 28 and 22 bp and polymorphic fragments of 236 and 67 bp. When the cleavage site was present the M allele was detected. Whereas the loss of this restriction site, caused by substitution of Guanine-Adenine (G612A) (GenBank U14109), resulted in a 303 bp fragment in length (m allele) (Carcangiu *et al.* 2009). Digestion of the amplimers with *RsaI* resulted in 267 and 23 pb fragments when the restriction site (allele C) caused by substitution cytosine-thymine (C606T) was present, whereas the loss of cleavage site in the T allele led to a single 290 pb fragment in length.

Identified genotypes were shown as MM, Mm and mm according to *MnlI* restriction enzyme and CC, CT and TT according to *RsaI* restriction enzyme.

Allelic and genotypic frequencies

Allelic and genotypic frequencies of MTNR1A locus, when the two restriction sites were considered separately for *MnlI* and *RsaI* sites and the degree of heterozygosity presented in Table 1 and 2. Genetic evaluation of the MTNR1A gene in examined lambs showed that all six expected genotype in *MnlI* and *RsaI* sites have been identified.

In *MnlI*, site allelic frequencies for Zel and Naeini lambs were 0.65 and 0.71 for the M allele and 0.35 and 0.29 for m allele, respectively.

On the whole, the M allele showed high frequency (0.68). The M allele was predominant in either Zel or Naeini lambs. Genotypic frequencies for the MM, Mm and mm genotypes were 0.52, 0.25 and 0.23 in Zel breed and 0.60, 0.22 and 0.18 in Naeini breed (Table 1). In general, tested samples showed the following distribution in Zel and Naeini ewes (52 and 30) for MM, (25 and 11) for Mm and (23 and 9) for mm genotype, respectively. Frequencies of genotypes in the Zel and Naeini lambs were in accordance with Hardy-Weinberg equilibrium in *MnlI* site. In this study, two allelic forms C and T with the frequency of 0.62 and 0.38 in Zel and 0.62 and 0.38 in Naeini ewes were also observed, respectively (Table 2).

The C allele was predominant in both studied breeds. Overall, frequency of this allele was 0.62. Genotypic frequency for the CC, CT and TT genotypes were 0.39, 0.45 and 0.15 in Zel and 0.44, 0.36 and 0.20 in Naeini, respectively. In general, researched samples exhibited the following distribution 39 (CC), 45 (CT) and 15 (TT) in Zel and 22 (CC), 18 (CT) and 10 (TT) in Naeini ewes, respectively.

Genotypic frequencies for *RsaI* marker site in Zel and Naeini ewes were not conducted following the Hardy-Weinberg equilibrium.

Genetic variability, which was estimated by heterozygosity at two MTNR1A marker sites, differed between Zel and Naeini sheep populations.

Both Zel and Naeini ewes had a high value of heterozygosity for *MnlI* and *RsaI* of MTNR1A marker sites which were 0.3 and 0.46 (*MnlI*) and 0.47 and 0.49 (*RsaI*), respectively.

The two restriction sites were considered constantly in haplotype analysis. The most frequent haplo-genotype (here after called genotype) was MMCT (0.21) and MMCC with a frequency of 0.19 was the next frequent genotype in Zel ewes. On the contrary, the mmTT (0.01) and the MmTT (0.03) were the least frequent genotypes.

Out of nine possible genotypes, eight genotypes were observed for Naeini ewes. The MMCT genotype was realized with the highest frequency of 0.28, followed by MMCC (0.18), mmCC (0.16) and MMTT (0.14). The mmCT, MmCT and MmTT had the lowest frequencies 0.02, 0.06 and 0.06, respectively. The mmTT genotype was not found in the present study in Naeini ewes. In general, the MMCT genotype (0.24) was the most frequent, followed by the MMCC genotype (0.19). In contrast, animal of genotypes mmTT, MmTT and mmCT occurred at low frequencies (Table 3).

Association of the *MnlI* / MTNR1A and *RsaI* / MTNR1A genotypes with litter size

Results of studied possible relationship between *MnlI* / *RsaI* and litter size are shown in Table 4. For *MnlI* site, there was a significant difference between genotypes for litter size only in Zel breed (P<0.05).

The MM genotype had significantly the lowest mean for litter size. In general, the highest mean measurements for litter size were from mm, followed by Mm, whereas minimum mean measurements were from MM in both assessed breeds. For *RsaI* site, no significant differences both in Zel and Naeini sheep were identified.

Table 1 Allelic and genotypic frequency of MTNR1A / *MnlI* in Zel and Naeini sheep breeds

Breed	n	Allelic frequency (%)		Genotypic frequency (%)			Obs-Het	χ^2
		M	m	MM	Mm	mm		
Zel	100	0.65	0.35	0.52	0.25	0.23	0.25	99.92^{ns}
Naini	50	0.71	0.29	0.60	0.22	0.18	0.22	22.44^{ns}

Obs-Het: observed heterozygosity; χ^2: test of Hardy-Weinberg equilibrium and NS: non significant.

Table 2 Allelic and genotypic frequency of MTNR1A / *RsaI* in Zel and Naeini sheep breeds

Breed	n	Allelic frequency (%)		Genotypic frequency (%)			Obs-Het	χ^2
		C	T	CC	CT	TT		
Zel	100	0.62	0.38	0.39	0.45	0.15	0.45	12.54^{ns}
Naini	50	0.62	0.38	0.44	0.36	0.20	0.36	2.60^{ns}

Obs-Het: observed heterozygosity; χ^2: test of Hardy-Weinberg equilibrium and NS: non significant.

However, the CT genotype had the highest mean and adversely the TT genotype had the lowest mean measurements for litter size in Zel and Naeini breed.

Table 3 Haplotype frequencies for *MnlI* / *RsaI* polymorphisms[a] in Zel and Naeini ewes

MnlI genotype	RsaI genotype			
	CC	CT	TT	Combined
MM	0.19	0.24	0.13	0.56
Mm	0.10	0.09	0.04	0.23
mm	0.13	0.07	0.01	0.21
Combined	0.42	0.40	0.18	1

[a] Total number of animals= 150.

Table 4 Association of the *MnlI* / MTNR1A and *RsaI* / MTNR1A genotypes with litter size in Zel and Naeini sheeps

Polymorphism site	Genotype	Litter size	
		Zel*	Naeini[ns]
		LSM±SE	LSM±SE
MnlI site	MM	1.23±0.03	1.31±0.05
	Mm	1.52±0.05	1.33±0.08
	mm	1.54±0.05	1.43±0.09
RsaI site	CC	1.32±0.04	1.32±0.06
	CT	1.44±0.04	1.37±0.06
	TT	1.29±0.06	1.30±0.08

LSM: least square mean and SE: standard error.
NS: non significant.
* P<0.05.

Association of the MTNR1A alleles with litter size

There were no significant differences between mT, MT, MC and mC allele categories and litter size in Naeini lambs (Table 5).

Table 5 Means and standard deviation for litter size in Zel and Naeini sheep by number of alleles

Allele	Genotype	Litter size	
		Zel*	Naeini[ns]
		LSM±SE	LSM±SE
mT	mT / XX	1.61±0.05	1.42±0.10
	XX / XX	1.28±0.03	1.32±0.04
mC	mC / mC	1.45±0.08	1.43±0.09
	mC / XX	1.56±0.04	1.25±0.09
	XX / XX	1.25±0.03	1.33±0.04
MC	MC / MC	1.34±0.06	1.27±0.08
	MC / XX	1.33±0.04	1.34±0.05
	XX / XX	1.30±0.04	1.35±0.06
MT	MT / MT	1.25±0.09	1.19±0.10
	MT / XX	1.36±0.04	1.42±0.06
	XX / XX	1.40±0.03	1.30±0.05

XX denote any allele except specified allele.
Estimated means and standard deviation for litter size for different categorized based on number of specified allele.
LSM: least square mean and SE: standard error.
NS: non significant.
* P<0.05.

Lambs carrying only one copy of the mT allele had slightly heavier mean measurements (1.42) than those with zero and two copies (1.30 and 1.19, respectively) (P=0.1). Also, there were no significant differences between MC and MT allelic classes and litter size in Zel lambs. For mC allele category, there were significant differences between mC / mC and mC / XX with XX / XX (P<0.05). Lambs carrying one or two copies of mC allele had significantly heavier mean than those with zero copies (1.56 and 1.45 *vs.* 1.25, respectively). For mT allele category, there was highly significant variation between mT / XX and XX / XX (P<0.0001). The mT hetrozygote genotype had heavier mean (1.61) than another genotype with no copies of the mT allele (1.28).

Association of MTNR1A genotypes with litter size

Overall, nine and eight genotypes were identified in studied Zel and Naeini ewes, respectively. Effects of these genotypes on litter size were evaluated.

The least square mean and standard error for litter size of various MTNR1A genotypes in Zel and Naeini sheep are summarized in Table 6. As it is shown differences were statistically small and there were no significant differences between genotypes for litter size in Naeini ewes. However, the highest Ls mean measurements for litter size were from MmTT (1.55) genotype followed by mmCC (1.45) genotype in Naeini lambs. The MMTT genotype had lighter Lsmean than other genotypes.

There were highly significant differences between MTNR1A genotypes and litter size in Zel ewes. The mmCT genotype had heavier Lsmean (1.64) than other genotypes. There were significant differences between MMTT, MMCC and MMCT genotypes with mmCT genotype (1.22, 1.23 and 1.24 *vs.* 1.64, respectively).

The present study focused on lambing rate and its association with MTNR1A gene in Zel and Naeini breeds. A DNA fragment with the expected size of 824 bp was amplified from exon II of MTNR1A gene. Analysis of this region, using restriction endonuclease *MnlI* and *RsaI* treatment, confirmed presence of polymorphism in Zel and Naeini breeds as in other sheep breeds (Messer *et al.* 1997; Chu *et al.* 2003). Both alleles, in *MnlI* and *RsaI* sites and three possible genotypes in each site were identified in two analyzed sheep breeds. Consistent with Pelletier *et al.* (2000), Chu *et al.* (2003), Chu *et al.* (2006), Carcangiu *et al.* (2009) and Seker *et al.* (2011) two polymorphic fragment with 303 bp and 236 bp in length were identified for MTNR1A / *MnlI* site in the present study.

The results showed that there was low frequency for mm genotype in both mentioned breeds, Zel (0.23) and Naeini (0.18). Also, these breeds had small proportion of the m allele (0.35 and 0.25, respectively). Similar results were observed in Sarda (Mura *et al.* 2010), Greyface XB and Soay (Barrett *et al.* 1997), Han (Chu *et al.* 2003), Dorset (Mateescu *et al.* 2009), Suffolk and CoopWorth sheeps (Messer *et al.* 1997). The frequencies of the m allele in these sheep were 0.29, 0.25, 0.25, 0.33 and 0.25, respectively.

A very notable role was demonstrated by the M allele, which had very high frequencies among the ewes of the two studied breeds (0.65 for Zel and 0.71 for Naeini).

Table 6 Analysis of litter size based on MTNR1A genotypes

Genotype	Litter size	
	Zel*	Naeini[ns]
	LSM±SE	LSM±SE
mmCT	1.64±0.07	1.33±0.27
MmCT	1.61±0.07	1.33±0.15
MmTT	1.55±0.14	1.55±0.15
mmCC	1.43±0.08	1.45±0.10
MmCC	1.40±0.08	1.20±0.12
mmTT	1.33±0.25	-
MMCT	1.24±0.05	1.38±0.07
MMCC	1.23±0.05	1.29±0.09
MMTT	1.22±0.07	1.19±0.10

LSM: least square mean and SE: standard error.
NS: non significant.
* P<0.05.

However, many previous investigations found that the m allele had higher frequency in ewes with high degree of seasonality than animals with less seasonal sexual activity (Pelletier *et al.* 2000; Notter *et al.* 2003; Chu *et al.* 2006; Carcangiu *et al.* 2009; Mateescu *et al.* 2009). But our study contrasts with the result of these examinations and it shows that there are no distinguished differences between two survived breeds. A high frequency of the M allele was identified in sheep with seasonal sexual activity: Prolific Olkuska sheep (0.643), Polish Mountain sheep (0.684) and Suffolk (0.6). In contrast, in a seasonal Merino-Ramnov sheep, the m allele with high frequency (0.795) was observed (Kaczor *et al.* 2006). The occurrence of MTNR1A / *MnlI* loci variants in the study was in accordance with other sheep breeds investigated previously, but there is a difference with respect to gene frequencies. Similar genotype distribution in Zel and Naeini breeds for MM, Mm and mm (0.52/0.60, 0.25/0.22 and 0.23/0.18, respectively) were observed. There is a functional difference in melatonin express between MM and mm animals (Trecherel *et al.* 2010). Being in accordance with Hardy-Weinberg equilibrium in *MnlI* site in both zel and Naeini ewes may be because of no effect of selection in this site. However not being in Hardy-Weinberg equilibrium in *RsaI* marker site in Zel and Naeini ewes may be due to low number of samples or any selection in twining trait.

The influence of MM genotype on reproduction traits has already been obvious in many sheep breeds (Pelletier *et al.* 2000; Notter, 2008). Pelletier *et al.* (2000) reported that genetic variations in *MnlI* site have a notable influence on the incidence of spontaneous ovulation in sheep, outside the reproductive season. Increasing fertility (about 11.2%) in the early spring period in sheep with the MM genotype was observed (Notter *et al.* 2003). A significant positive relationship between the MM genotype and a high conception rate following artificial insemination was identified (Carcangu *et al.* 2011). This may be due to decreased sensitivity to the photoperiod. In many breeds, it was documented that there is no association between photoperiod and *MnlI* / MTNR1A gene, in both homozygous and heterozygous sheeps (Notter *et al.* 2003; Mateescu *et al.* 2009).

In investigations carried out with Merino d'Arles breed, the MM animals with spring ovarian activity were observed (Pelletier *et al.* 2000). While, all year round oestrus cycles were found in Chinese breeds (Chu *et al.* 2006). Also, Notter and Cockett (2005) reported that the presence of one M allele is adequate to determine a sexual activity less seasonal in several studied sheep breeds. Mura *et al.* (2010) concluded that among three possible genotypes, the MM genotype is able to influence reproductive response to melatonin treatment. Zel ewes homozygous for M allele had significantly lower mean (P<0.05) than the lambs with one or zero copy of this allele (1.23 *vs.* 1.52±0.05 and 1.54±0.05) which is in contrast with studies that mentioned above. There were no differences regarding MTNR1A genotypes on mean litter size in Naeini sheep. However, lambs with mm genotype had higher mean than the MM and Mm carriers. Dorset ewes carrying the M allele are able to become pregnant at younger ages (Mateescu *et al.* 2009). It is documented that there are small effects of the M allele on sheep's litter size. In spite of this, it means that genetic factors have no significant impacts on this trait (Notter *et al.* 2003). Studies with prolific Olkuska ewes showed that lambs with MM homozygote genotype had a greater number of lambs born in the first three lambing. However, significant effect of genotype on litter size was not found (Kaczor *et al.* 2006).

Digestion of MTNR1A marker site with *RsaI* in Zel and Naeini breeds showed that the C allele was predominant with frequency of 0.62 in both of them. Our results were similar to these recorded in the Cornell Dorset, Tisdale's Polypay, Hampshire and Han sheep (Wright, 2000; Chu *et al.* 2003; Notter and Cockett, 2005). The CT and CC genotypes had highly frequencies in Zel and Naeini, respectively. The TT genotype had the lowest frequency in these breeds. Also, Mura *et al.* (2010) found that the CC genotype had high frequencies in studied breeds while the TT genotype frequency was predominant (0.44) in Dorset ewes (Mateescu *et al.* 2009). The statistical analysis showed that there is no significant effect of MTNR1A / *RsaI* genotypes on litter size, both in Zel and Naeini lambs. However, ewes with heterozygote genotype had a slightly higher mean litter size in survived breeds.

A considerable effect of the MTNR1A / *RsaI* polymorphism on litter size in seasonal and highly prolific Han sheep was observed at second lambing (Chu *et al.* 2003).

The study of Chu *et al.* (2006) demonstrated that the CC genotype was slightly correlated with seasonal reproduction. On the other hand, the effect of *RsaI* genotypes on selected reproductive traits in Dorset ewes was assessed and there were no statistically significant differences between genotypes and selected traits (Mateescu *et al.* 2009). Notter *et al.* (2005) observed that ewes with TT genotype had greater litters at second parity than ewes that were homozygous for the presence of the restriction site (3.19±0.13 *vs.* 2.25±0.12 lambs/litter) and larger litters than CT genotypes at both first and second parities.

The investigation of Carcangiu *et al.* (2009) revealed that the animals carrying one of the MM and CC genotypes did not show seasonal reproductive activity in Sarda ewes. Then, the allele MC may have high impact on seasonality of reproductive activity.

In the study of Notter *et al.* (2003) the MC, MT, mC and mT alleles with frequencies of 0.036, 0.385, 0.385, 0.302 and 0.277 were identified. In the mentioned study, genotypic effects on litter size were small and not significant (P=0.07). Ewes carrying the MMTT genotype were superior in mean litter size (2.00±0.09), whereas ewes of mmCT genotype were notably inferior (1.64±0.14). Adult ewes that carried at least one copy of M had slightly larger litters than those that did not (1.87±0.05 *vs.* 1.76±0.08; P<0.3). Overall, the MMCT (0.24) genotype was the most frequent genotype followed by MMCC (0.19), mmCC (0.13), MmCC (0.10), MmCT (0.09), mmCT (0.07), MmTT (0.04) and mmTT (0.01). Ewes with mmCT were significantly superior in mean litter size (1.64±0.07), whereas ewes with MMCT, MMCC and MMTT genotypes were distinguished inferior (1.24, 1.23 and 1.22, respectively). But, significant differences between genotype and mean litter size in Naeini lambs were not shown. The MMTT and MmCT were the most frequent genotypes with frequencies of 39.66 and were followed by mmCC genotype in Dorset ewes (Mateescu *et al.* 2009). Whereas, the other genotypes were absent or had a low frequency. The frequencies of MT, MC, mC and mT were relatively similar in Zel and Naeini ewes. Carcangiu *et al.* (2009) reported that out of four possible alleles, the MC allele was the most frequent followed by the MT, mC and mT alleles. Zel ewes that were heterozygous for mT allele had higher mean litter size (1.61±0.05) than the other genotypes. These results indicate that in Zel sheep, selecting of ewes with mT allele may improve the mean of litter size. Ewes with the mC / mC and mC / XX genotypes had also statistically meaningful greater mean litter size in the mC allele category than ewes with no copy of mC allele. While, there were no significant differences between various alleles and litter size in Naeini ewes. However, the mC / mC, mT / XX and MT / XX genotypes had slightly larger litters (1.43±0.09, 1.42±0.1 and 1.42±0.06) than others in Naeini lambs.

However, part of these differences between Zel and Naeini sheep breeds may be related to the different rearing geographical area. Zel is located in northern of Iran, southern part of Caspian coast which has Mediterranean climate and on the other hand, Naeini breed is located in the center of Iran which has relatively harsh environmental conditions. In general, three statistical approaches were consistent with each other.

CONCLUSION

Results from this study pointed out any significant relationship between MTNR1A locus and mean litter size in Naeini breed as breed showed seasonal sexual activity. But it seems that m allele of MTNR1A / *MnlI* site has a positive effect on mean litter size in Zel breed as an out-of-season breed. Reduction of seasonality in reproduction activity and lambing rate are very important economic factors in sheep rearing industry. To date numerous studies have been considered in order to improve out of season breeding and increasing lambing rate. However, low heritability and remarkable impact of epigenetically factors on these traits because reduced achievements of phenotypic differences in sheep breeds. Probably, melatonin receptor 1A gene exerts its effect by regulatory sequences. However, the MTNR1A polymorphism can explain only a small part of the genetic variability of seasonal sexual activity and the implication of other genes must be investigated.

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