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Original Research Article

ASSOCIATION OF NUMBER OF ARTIFICIAL INSEMINATIONS PER PREGNANCY IN HOLSTEIN DAIRY COWS WITH POLYMORPHISM IN LUTEINIZING HORMONE RECEPTOR AND FOLLICLE STIMULATING HORMONE RECEPTOR GENES

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Abstract: Failure to become pregnant is the primary reason for a dairy cow to be culled from the production herd. A cow that is cycling normally, with no reproductive abnormalities, but has failed to conceive after at least three successive inseminations may cause economic losses in dairy farms. The present study aimed to examine the association between follicle-stimulating hormone receptor (*FSHR*) and luteinizing hormone receptor (*LHCGR*) genes polymorphisms and number of artificial inseminations in the Holstein cattle breed, raised in Turkey. A total of 264 Holstein cows were included in this study, consisting of 222 cows which had undergone a low number of artificial inseminations (two or less inseminations; LI) and 42 cows with a high number of artificial inseminations (three or more inseminations; HI). The polymerase chain reaction followed by restriction fragment length polymorphism method was used to determine the *FSHR-Alu*I and *LHCGR-Hha*I DNA variants. Three genotypes (CC, CG and GG) were observed for the *FSHR* gene in LI and HI cows. No statistical difference was found among LI and HI animals for the *FSHR* genotypes (P=0.934). However, only the CC genotype was detected in LI cows whereas the CC, CT and TT genotypes were detected in HI cows for the *LHCGR* gene. The genotype frequency of CC was found to be highest (93%) in the HI animals and an association between LHCGR genotypes and the number of artificial inseminations per pregnancy was identified (P<0.001). This is the first report to describes an association between *FSHR* and *LHCGR* polymorphisms and number of artificial inseminations in cows.

Key words: candidate genes; pregnancy; cow; number of artificial inseminations; polymorphism

Introduction

Many parameters such as calving interval, average days open, heat detection efficiency, number of inseminations per pregnancy, and pregnancy rates are important for determining the reproductive performance of dairy herds (1, 2, 3). Reproductive problems cause important economic losses due to reduced fertility as a result of prolonged calving intervals (CI) (4), increased artificial insemination (AI) costs, reduced number of calves and higher replacement costs (5). Reproductive problems in dairy cattle breeding are the most common cause for the culling of animals (6). In France, the culling of cows due to low fertility rate has been reported to account for 25% of disposed animals (7). Depending on the yield of offspring, the prolonged calving interval costs dairy farms about 1.8 million US dollars in Ireland, without accounting for the costs of higher selection due to failures to conceive (8). In different studies, 1.9 (1), 1.7-2.2 (2) and <2 (3) were given as the average number of inseminations required to

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maintain a successful pregnancy. In Turkey, cows that were inseminated more than twice, financial losses due to extra service and to extended calving interval were estimated to be 3-fold and approximately 2-fold higher than in cows which were inseminated only a few times (9).

Fertility traits such as calving interval and the number of inseminations per pregnancy have been neglected in dairy cattle genetic improvement studies globally, mainly because fertility traits are known to have low heritability (10). There are many reasons for fertility decline in dairy cows, such as genetics, physiology, nutrition and management (11). Furthermore, dairy cows that have high genetic merit for milk production traits were reported to have reduced fertility (12) and subsequently required a higher number of inseminations per pregnancy in recent years (13).

The degree of heritability for fertility traits in dairy cattle was estimated to be low (14). On the other hand, an association between fertility traits and some genes in different dairy cattle was reported (15). Due to their roles in the physiological and biochemical processes for reproductive traits, some candidate genes were identified in different studies (15, 16). The analysis of candidate genes from hormones and hormone receptors, which affect fertility, has been considered a favourable tools (17, 18) and, for this purpose, the genes encoding the follicle-stimulating hormone receptor gene (*FSHR)* and luteinizing hormone/choriogonadotropin receptor (*LHCGR)* have been selected as candidate reproductive markers for livestock.

FSHR encodes the transmembrane receptor that interacts with the follicle-stimulating hormone (FSH) (19). FSHR activation is necessary for the hormonal functioning of FSH and is found at high levels in the ovaries and testes of mammals (20). Mayorga *et al.* (21) showed that the *FSHR* gene is a main determinant of ovarian responsiveness to FSH for the induction of ovulation in female. The *FSHR* gene is located on bovine chromosome 11 (BTA 11) and consists of 10 exons (22).

Another candidate gene is *LHCGR* which is located on BTA 11 and encodes the transmembrane receptor. *LHCGR is* found predominantly in the ovaries and testes and the receptor interacts with luteinizing hormone (LH). LH is critical for follicular development, ovulation, corpus luteum formation, and preimplantation embryonic development (23). Previous studies showed that *LHCGR* gene variants are associated with polycystic ovary syndrome in humans (24) and superovulation traits in cattle (23).

Although the association between *FSHR* and *LHCGR* gene variants with different fertility traits was studied in various mammal species, no study has been conducted to investigate the effects of these gene variants with the number of artificial inseminations per pregnancy in dairy cattle. Therefore, the objective of this study was to investigate the effects of two single nucleotide polymorphisms (SNPs) in the *FSHR* and *LHCGR* genes in dairy cattle requiring either low or high numbers of artificial-inseminations per pregnancy.

Material and methods

The study was conducted in a large commercial dairy herd in Kayseri province of Turkey. HI and LI cows were housed in semi-covered sheds and fed according to (25) requirements with appropriate amount of forage and concentrated feed. The voluntary waiting period was 60 days in the herd. Cows were observed for estrus activity. Estrous signs were comfirmed by rectal and ultrasonographic examinations (such as preovulatory follicle, tonic uterus, echogen endometrium). In this study 264 Holstein cows, varying in age of 4 to 7 years and having birth twice, were examined for *FSHR* and *LHCGR* genes. All of animals have no problems had affected reproductive performance such as cystic ovaries, anoestrous, suboestrus, endometritis and pyometra. Artificial insemination is only the method applied for reproduction and the average milk yield was 27 kg in LI and 26.2 kg in HI in studied herd. The body condition score was 3.25-3.5 among all animals in the herd. No animals were culled for reproductive failure due to nutritional problem in the herd but culling rate for reproductive failures were 8% among all breeders. The blood samples were collected in heparinized tubes from Vena coccygea A total of 264 Holstein cows were examined for *FSHR* and *LHCGR* genes. The cows were divided into two groups according to their number of inseminations to become pregnant. The first group (low insemination number-LI) represented pregnant cows (n= 222) that were inseminated once or twice (average 1.6), The average age was 5.5 years. In the second group (high insemination number-HI) (n= 42), three or more inseminations (average 3.1) were

Table 1: Primer sequence and PCR product size

Gene	Accession number	Restriction Enzymes		Primers	PCR product size
FSHR	L22319.1	*Alu*I	F	5'- CTG CCT CCC TCA AGG TGC CCC TC-3'	306 bp
			R	5'- AGT TCT TGG CTA AAT GTC TTA GGG GG-3'	
LHCGR	U20504	*Hha*I	F	5'- CAA ACT GAC AGT CCC CCG CTT T- 3'	303 bp
			R	5'-CCT CCG AGC ATG ACT GGA ATG GC- 3'	

F: Forward; R: Reverse

necessary for two previous pregnancies. The postpartum periods ranged from 45 to 60 days in this group and the average age was 5.8 years. Average intercalving period was 405 and 480 days in LI and HI groups, respectively.

Genomic DNA was isolated from blood using a phenol chloroform protocol (26). Genotyping for the *FSHR-Alu*I and *LHCGR-Hha*I polymorphism were performed by PCR-RFLP according to the method proposed by Houde *et al.* (20). PCR analysis of *FSHR* and *LHCGR* genes were performed in thermal cycler (Bio-Rad-T100, USA). The nucleotide sequences and accession numbers of the specific PCR primers used for standard PCR amplification are shown in Table 1.

The PCR amplification reaction was carried out in a total volume of 25 µl consisting of ddH_2O, 1.5 mM $MgCl_2$ 50 µM dNTP, primers (5 pmol), 1X buffer, Taq DNA polymerase 1U/ µL and DNA 100 ng. The PCR protocol started with an initial denaturing step of 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 57°C for 45 sec for *FSHR* and 60°C for 45 sec for *LHCGR*, 72°C for 45 sec and a final cycle at 72°C for 10 min. The PCR product of *FSHR* and *LHCGR* genes were digested with the enzymes *Alu*I (Fermentas, Vilnius, Lithuania) and *Hha*I (Fermentas, Vilnius, Lithuania) respectively. Restriction products were electrophoresed on 3% agarose gel and then displayed under a UV-transilluminator (Kodak-Gel Logic 200, USA).

Direct counting was used to estimate the genotype and allele frequencies of the *FSHR* and *LHCGR* genes. Differences among the genotype groups were examined by simple logistic regression. Significance was considered to be $P<0.05$. Goodness-of-fit of genotype distribution to Hardy–Weinberg equilibrium was examined by Pearson's x2-test. All calculations were performed using the computer program SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

Results

The amplified PCR product of *FSHR* and *LHCGR* genes produced 306 bp and 303 bp fragments using *Alu*I and *Hha*I restriction enzymes, respectively. After the digestion of *Alu*I, two fragments were expected to be seen for the CC genotype (243 and 63 bp), four fragments were expected to be seen for the CG genotype (243, 193, 63 and 50 bp) and three fragments were expected to be seen for the GG genotype (193, 63 and 50 bp). However, since 243 and 193 bp bands were clearly seen together and separately, it can be assumed that genotyping was successfully fulfilled without observing the 63 and 50 bp bands. Three genotypes (CC, CG and GG) were identified for the *FSHR* gene both of LI and HI cows (Figure 1).

After the digestion of the PCR products of the *LHCGR* gene with *Hha*I restriction enzyme, a polymorphism with two alleles was detected. The *LHCGR* site had three genotypes: TT (303 bp), TC (303 bp, 155 and 148 bp) and CC (155 and 148 bp) (Figure 2).

The allelic and genotypic frequencies of the *FSHR* and *LHCGR* genes and polymorphisms for the LI and HI Holstein cows are given in Table 2. Deviation between observed genotypic frequencies and this expected under HWE was significant in LI and HI cows for the *FSHR* gene (Hardy-Weinberg Equilibrium-HWE, P=0.029). Also, a significant deviation from HWE was observed in the HI cows on the *LHCGR* gene (HWE, P=0.06). For the *LHCGR* gene, the CC genotype was found in LI and HI cows; additionally, the CC genotype was found to be highest in the HI cows (92.86%). The CC genotype frequency (69.23%) was observed to be higher than CG and GG frequency in the HI and LI (61.26%) cows for the *FSHR* gene (Table 2). 7

No statistical differences between groups (LI and HI) were observed for genotypes of the *FSHR*

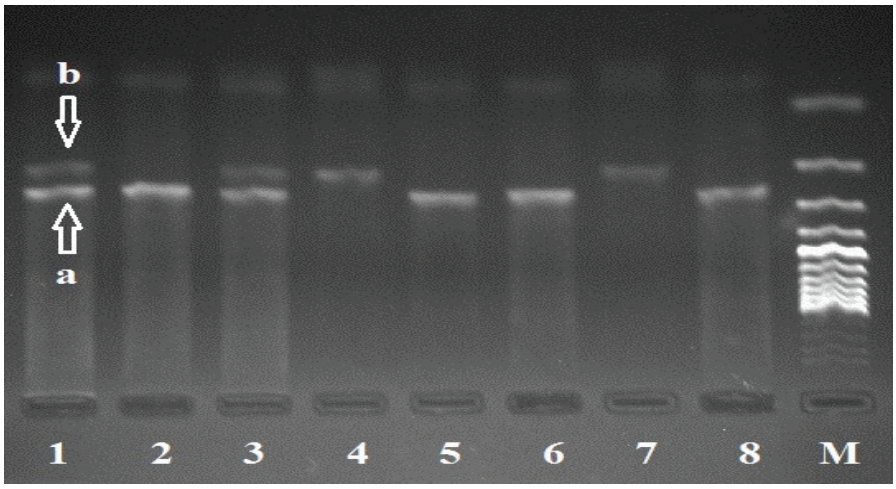

Figure 1: *AluI* enzyme digestion products of different *FSHR* genotypes. a: 243bp, b:193bp, M: 100 bp DNA ladder; 2, 6 and 8 CC individuals genotyped; 1 and 3 CG individuals genotyped; 4, 7 GG individuals genotyped

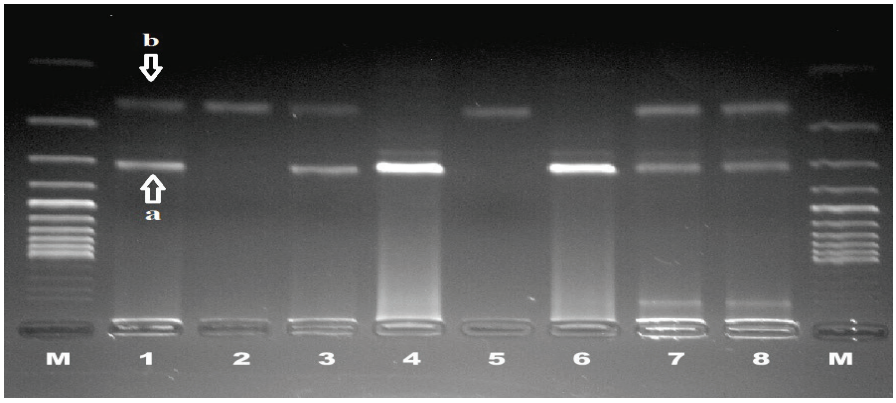

Figure 2: *HhaI* enzyme digestion products of different *LHCGR* genotypes. a: 303 bp, b: 155, 148 bp, M: 100 bp DNA ladder; 4, 6 TT individuals genotyped; 1 and 3,7,8 TC individuals genotyped; 2, 5 CC individuals genotyped

Table 2: Allelic and genotypic frequencies of *FSHR* and *LHR* genes in LI and HI cows

Gene	Groups	Frequency	Genotypes Frequency (%)			Allele Frequency (%)		Statistical Significant (Chi-squared HWE)
			CC	CG	GG	C	G	
FSHR	LI	Observed	61.26	30.63	8.11	76.6	23.4	x2 = 4.74
		Expected	58.64	35.87	5.49			P=0.029 (df=1)
	HI	Observed	69.23	23.08	7.69	80.8	19.2	x2 = 3.44
		Expected	65.24	31.07	3.70			P=0.06(df=1)
			CC	CT	TT	C	T	
LHCGR	LI	Observed	100	0	0	100	0	-
		Expected	100	0	0			
	HI	Observed	92.86	4.76	2.38	95.2	4.8	x2 = 9.48
		Expected	90.70	9.07	0.23			P=0.002(df=1)

HWE: Hardy-Weinberg Equilibrium; x2: Chi-Square value; df: Degree of freedom

Table 3: Relative distribution of *FSHR* and *LHCGR* genotypes on LI and HI cows and test of statistical significancy

Gene	Genotypes	Groups		Total	Statistical Significant (Chi-Square Tests)
		LI	HI		
FSHR	CC	136 (84.0%)	26 (16.0%)	162	$x^2 = 0.137$ P=0.934
	CG	68 (85.0%)	12 (15.0%)	80	
	GG	18 (81.8%)	4(18.2%)	22	
LHCGR	CC	222 (85.1%)	39 (14.9%)$_a$	261	$x^2 = 16.039$ P<0.001
	CT	0 (0%)	2(100%)$_b$	2	
	TT	0 (0%)	1 (100%)$_b$	1	
Total		222 (84.1%)	42 (15.9%)	264	

a,b : Different subscripts within the same column demonstrate significant differences at 0.05 level for each loci

gene (P=0.934). For the *LHCGR* gene,-only the CC genotype was seen in LI cows. Additionally, the CC genotype was found quite often in HI cows (39/42; 93%). Therefore, the genotypes between LI and HI cows were significantly different (P<0.001). The lack of CT and TT genotypes was observed in LI (Table 3).

Discussion

The selection of animals, which is aimed at improving quantitative traits, is a complex process, which may cause adverse effects on other traits. Genetic selection for milk yield has enabled increase in the amount of milk obtained per dairy cow. However, due to the antagonistic genetic correlation between milk yield and fertility, dairy cows with high milk yield have lower fertility (24). In dairy cattle, the production of one calf a year is a prerequisite for both the maintenance of the herd size and its profitability. Previous studies have demonstrated that candidate gene analysis is a good option for increasing the reproductive performance of cattle (16, 28).

The *FSHR* gene has been reported to be polymorphic in various cattle breeds (29, 30). In Zebu × *Bos taurus* hybrids raised in Brazil, the *FSHR* gene was reported to have three genotypes, including GG, CG and CC, and it was found that the CG genotype had the highest frequency and that the frequencies of the C and G alleles were similar (29). In the same study it was reported that in purebred Nellore cattle (*Bos indicus*) raised in Brazil, unlike in hybrids, the GG genotype displayed the highest frequency (0.490), the genotype CC did not exist, and the frequency of the G allele (0.745) was higher than that of the C allele (0.255) (30). In an investigation on European dairy cattle breeds, (Holstein Jersey) and European beef breeds (Angus and Charolais), the frequency of the *FSHR*-G allele was found to be higher in both beef breeds (Angus:0.53,Charolais:0.41) than in dairy cows (Holstein:0.28, Jersey:0.17) (31). Similarly, in the present study carried out in Holstein cattle, in both group LI and group HI, the frequency of the C allele was found to be higher than that of the G allele (Table 2).

Although the correlation between the *FSHR* gene and fertility has been investigated extensively in humans showing significant dependencies (32, 33), research on this topic in livestock is scarce. Only a few studies have focussed on the correlation between the *FSHR* gene and fertility in cattle. In a study carried out in Zebu × *Bos taurus* hybrids raised in Brazil, the investigation of the correlation between *FSHR* genotypes and pregnancy rate demonstrated that, although no significant difference existed between the genotypes for pregnancy rate, the pregnancy rate of the heifers with a CG genotype (66%) was higher than that of heifers with a CC genotype (64%) and GG genotype (58%) (30). In another study conducted in Holstein cattle raised in China, a new SNP of G-278A was detected in the 5′-upstream region of the *FSHR* gene. The number of ova obtained for this SNP from cattle with a CC genotype was found to be higher than that obtained from cattle with a genotype of CD and DD. Thus, it was reported that a higher number of transferable embryos were produced

by heifers with a CC genotype, in comparison to heifers with a CD and DD genotype (28). Cory *et al.* (31) reported that the c.337C>G, c.871A>G and c.1973C>G mutations were correlated with the percentages of viable embryos and unfertilized ova obtained after superovulation.

It was also found that the LH peak was delayed in "repeat breeder syndrome" cases. This delay caused prolonged lifespan of the preovulatory follicle, and a late postovulatory rise of plasma progesterone (34). Considering the central role of LH in ovulation, common genetic variation in the luteinizing hormone/choriogonadotropin receptor (*LHCGR*) may have functional consequences in reproduction performance (28). The *LHCGR* gene has been investigated to a limited extent in livestock. In one of these few studies, carried out in the *Bos indicus* × European *Bos taurus* beef hybrids of six different breed compositions, the digestion of the *LHCGR* gene with *Hha*I enzyme produced three genotypes, namely, TT, CT and CC. The frequency of the TT genotype was low and ranged from 0 to 0.091, whilst the frequencies of the CT and CC genotypes ranged between 0.366-0.849 and 0.151-0.574, respectively. The frequency of the C allele was higher than that of the T allele (29). Furthermore, it was reported that in purebred Nellore cattle (*Bos indicus*) raised in Brazil, the frequency of the TT genotype (0.540) was high, the frequency of the CC genotype (0.030) was low and the frequency of the T allele (0.755) was higher than that of the C allele (0.245) (35). In the present study only LI cows carried the *LHCGR*-CC genotype. HI cows included animals of all three genotypes and the frequency of the CC genotype was the highest among those tested (92.86%). These findings are in agreement with the reports of Marson *et al.* (29) and Milazzotto (35). While Milazzotto (35) reported that the frequency of the *LHCGR*-TT genotype (0.540) was high in purebred Nellore cattle (*Bos indicus*), Marson *et al.* (29) reported that the frequency of the *LHCGR*-TT genotype was lower than that of the other genotypes in *Bos indicus* × *Bos taurus* hybrids. In the present study, it was ascertained that in Holstein cattle, the *LHCGR*-TT genotype did not exist in group LI and had the lowest frequency in HI cows (1; 2.38%). On the other hand, while Milazzotto (35) reported that the frequency of the *LHCGR*-CC genotype was lowest in purebred *Bos indicus* cattle, in the present study, the frequency of the *LHCGR*-CC genotype was determined to be the highest in Holstein cattle (*Bos taurus*).

Very few studies are available on the correlation of the *LHCGR* gene with reproductive traits in different cattle breeds. A significant QTL was identified for inseminations per conception trait between 32.6-32.7 Mbp where the *LHCGR* gene is found on BTA 11 according to *Bos taurus* genome assembly (36). Since no QTL for male fertility, non-return rate and sire conception rate have been identified on BTA 11 in this region (37), this mutation may be responsible for female reproduction, especially inseminations per conception. Marson *et al.* (30) reported that no statistically significant correlation exists between the *LHCGR* genotype and conception rate in *Bos indicus* x *Bos taurus* hybrids. However, the authors indicated that, although not significant, the conception rate was higher in heifers with a CT genotype (67%), in comparison to those with a TT genotype (65%) and CC genotype (58%). Furthermore, in another study, it was suggested that the *LHCGR* genotype was not associated with early development (before 14 months) or prolonged development, in purebred Nellore cows (*Bos indicus*) raised in Brazil (35). In the present study, the heifers included in group LI were only of the CC genotype, whilst heifers included in group HI were of all three genotypes. In group HI, the frequency of the CC genotype (92.86%) was the highest.

In this report we investigated the relations between the *FSHR* and *LHCGR* gene polymorphisms and insemination number to obtain pregnancies in Holstein cows. No difference between LI and HI groups was found for the *FSHR* gene. However, it is noteworthy that all LI cows were of the CC genotype in the *LHCGR* gene. Further association studies with larger numbers of samples and artificial insemination number on these polymorphisms is required.

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POVEZAVA MED ŠTEVILOM UMETNIH OSEMENITEV IN POLIMORFIZMOM GENOV ZA RECEPTOR ZA LUTEINIZIRAJOČI HORMON IN RECEPTOR ZA FOLIKLE STIMULIRAJOČI HORMON PRI KRAVAH MOLZNICAH PASME HOLSTEIN

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Povzetek: Težave z obrejitvijo so glavni razlog za izločitev krav molznic iz proizvodne črede. Krave z normalnim ciklusom, brez reproduktivnih motenj, ki se ne obrejijo po vsaj treh zaporednih osemenitvah, so vzrok za velike ekonomske izgube na mlečnih farmah. V opisani raziskavi smo proučevali povezavo med genskimi polimorfizmi v genih za receptorje za folikle stimulirajoči hormon (FSH-R), genih za receptorje za luteinizirajoči hormon (LH-R) ter številom umetnih osemenitev pri kravah pasme hlstein, vzrejenih v Turčiji. V raziskavo je bilo vključeno skupno 264 krav holstein, od katerih jih je bilo 22, osemenjenih največ dvakrat do obrejitve (skupina LI), in 42 krav, ki so bile osemenjene trikrat ali večkrat (skupina HI). Preiskovane gene smo pomnožili v verižni reakciji s polimerazo in nato izvedli pregled dolžine razrezanih odsekov DNK (metoda RFLP) z namenom, da bi določili prisotnost različic genov *FSHR*-AluI in *LHR*-HhaI pri preiskovanih živalih. Tri različice genotipa (CC, CG in GG) so bile ugotovljene pri genu za FHSR pri kravah v skupinah LI in HI, med skupinama pa ni bilo statistično značilnih razlik v pogostnosti posameznih genotipov ($p = 0,934$). Pri genu za LHR je bil ugotovljen genotip CC le pri kravah iz skupine LI, ostale tri variante genotipa (CC, CT in TT) pa so bile ugotovljene pri kravah iz skupine HI. Pogostnost genotipa CC je bila najvišja (93 %) pri živalih iz skupine HI, pri statistični analizi pa smo ugotovili povezavo med genotipi *LH-R* in številom umetnih osemenitev ($p < 0,001$).

Ključne besede: FSH-R, LH-R; obrejitev; krava; število umetnih osemenitev; polimorfizem