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RESEARCH

Dual effect of LEP -2548G>A (rs7799039) and LEPR Q223R (rs1137101) polymorphisms as a risk factor for azoospermic male infertility in Turkish population

Türk popülasyonunda azoospermik erkek infertilitesi için bir risk faktörü olarak LEP -2548G>A (rs7799039) ve LEPR Q223R (rs1137101) polimorfizmlerinin ikili etkisi

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Abstract

Purpose: Infertility is the situation in which pregnancy cannot be achieved despite unprotected sexual intercourse within at least one year. Male infertility can range from the entire absence of spermatozoa in the testicles (azoospermia) to noticeable variations in sperm quality. The patients with a mutation in the leptin (LEP) gene have been reported to be infertile and the patients with a mutation in the Leptin Receptor (LEPR) gene were shown to lack pubertal development. This study was performed to state if there is a relationship between azoospermic male infertility and LEP gene -2548G>A and LEPR gene Q223R polymorphisms.

Materials and Methods: One hundred thirty-seven azoospermic infertile men and a hundred fertile men were included in this study. DNAs obtained from peripheral blood of participants were analyzed by polymerase chain reaction (PCR) along with restriction fragment length polymorphism (RFLP) technics..

Results: In terms of LEP -2548G>A (rs7799039) and LEPR Q223R (rs1137101) polymorphisms, no statistically remarkable distinction was observed in the genotype and allele distributions of azoospermic infertile and fertile men. In the composite genotype analysis, it was determined that the GGQR composite genotype was approximately 9 times more common in azoospermic infertile men than in fertile men (8.8% vs. 1.0%).

Conclusion: It has been determined that LEP -2548G>A and LEPR Q223R polymorphisms may have a dual effect in azoospermic male infertility. We believe that more efficient and precise results can be obtained by conducting these studies in larger populations.

Keywords: Azoospermic male infertility, LEP, LEPR, rs7799039, rs1137101.

Öz

Amaç: İnfertilite, korunmasız cinsel ilişkiye rağmen en az bir yıl içerisinde gebelik elde edilememesi durumudur. Erkek kısırlığı, testislerde hiç sperm olmamasından (azospermi) sperm kalitesindeki gözle görülür değişikliklere kadar değişebilir. Leptin (LEP) geninde mutasyon olan hastaların infertil olduğu, Leptin Reseptör (LEPR) geninde mutasyon olan hastaların puberte gelişiminin olmadığı gösterilmiştir. Bu çalışma azospermik erkek infertilitesi ile LEP geni -2548G>A ve LEPR geni Q223R polimorfizmleri arasında bir ilişki olup olmadığını ortaya koymak amacıyla yapılmıştır.

Gereç ve Yöntem: Bu çalışmaya 137 azospermik infertil erkek ve 100 fertil erkek dahil edildi. Katılımcıların periferik kanlarından elde edilen DNA'lar, polimeraz zincir reaksiyonu (PCR) ile birlikte restriksiyon fragman uzunluk polimorfizmi (RFLP) teknikleri ile analiz edildi.

Bulgular: LEP -2548G>A (rs7799039) ve LEPR Q223R (rs1137101) polimorfizmleri açısından azospermik infertil ve fertil erkeklerin genotip ve alel dağılımlarında istatistiksel olarak dikkat çekici bir farklılık gözlenmedi. Kompozit genotip analizinde GGQR kompozit genotipinin azospermik infertil erkeklerde fertil erkeklere göre yaklaşık 9 kat daha fazla olduğu belirlendi (8.8% vs. 1.0%).

Sonuç: LEP -2548G>A ve LEPR Q223R polimorfizmlerinin azospermik erkek infertilitesinde ikili etkiye sahip olabileceği belirlendi. Bu çalışmaların daha geniş popülasyonlarda yapılmasıyla daha etkin ve kesin sonuçlara ulaşılabileceğine inanıyoruz.

Anahtar kelimeler: Azospermik erkek infetilitesi, LEP, LEPR, rs7799039, rs1137101.

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INTRODUCTION

Infertility is the inability to achieve pregnancy despite unprotected sexual intercourse in at least one year[1]. Infertility is divided into primary and secondary infertility. The complete absence of pregnancy is primary infertility. Secondary infertility is the inability to get pregnant without any protection after at least one previous successful pregnancy. The source of infertility can be either gender. According to scientific evidence, the top causes of infertility include 30% male, 20% ovulatory, 20% tubal/peritoneal, 5% uterine/cervical, and 25% unexplained factors[2]. Male infertility, which affects about 7% of men, is a challenging, multifaceted pathological condition with a wide range of phenotypic variations, from the total lack of spermatozoa in the testicles (azoospermia) to observable deviations in sperm quality[3,4] Azolospermia can be detected in 1% of male cases and in 15%–20% of patients evaluated for infertility[5].

The leptin (*LEP*) gene encodes a protein excreted into the circulation by white adipocytes and this protein mainly arrange energy homeostasis. The *LEP* gene, that has 3 exons, is located on the human chromosome 7q32.1[6]. When circulating LEP binds to the leptin receptor (LEPR), signaling pathways that promote energy expenditure and discourage nutrient intake are activated. This protein also has a variety of endocrine functions, including reproduction. LEP has been shown to have influences on the reproductive system and sperm function of men by organizing the hypothalamic-pituitary-gonadal (HPG) axis[7]. Several studies have demonstrated that LEP stimulates the production of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and gonadotropin release hormone (GnRH).[8,9]. Infertile male patients have been shown to have high LEP concentrations[10]. Variants in the *LEP* gene's regulatory region may impact the level of LEP because *LEP* gene mutations have been linked to infertility[11]. The *LEP* gene promoter polymorphism, -2548G>A (rs7799039), is one of the frequent one. An association between high LEP levels in blood and this polymorphism was reported in previous studies[12,13]. Male infertility has previously been linked to the *LEP* gene -2548G>A polymorphism[14].

LEPR is a member of the gp130 cytokine receptor family, which is known for activating cytosolic signal transducer and activator of transcription (STAT) proteins to stimulate gene transcription[15]. *LEPR* gene, which has 24 exons, is located on human chromosome 1p31.3[16]. LEPR protein, which is a receptor for leptin, is included in the arrangement of fat metabolism. Some studies have reported a relationship between sperm capacitation, counts and motility, testicular volume, spermatogenesis, testosterone and *LEP* and *LEPR* genes[17,18]. It was shown that patients with mutations in the *LEPR* gene have deficient pubertal development[19]. One of the prevalent polymorphisms, Gln223Arg, is situated in the extracellular region of the LEPR (Q223R, rs1137101). It has been reported that this extracellular domain is associated with LEPR activity and correct folding, and Q223R polymorphism reduces the leptin-binding quality[20].

Because previous research has suggested a relationship between *LEP*/*LEPR* genes and sperm capacitation, counts and motility, testicular volume, spermatogenesis, testosterone, in this study we aimed to investigate the potential association between *LEP* gene -2548G>A (rs7799039) and *LEPR* gene Q223R (rs1137101) polymorphisms and azoospermic male infertility in a Turkish population.

MATERIALS AND METHODS

Participants

One hundred and thirty-seven infertile men who were examined and diagnosed with idiopathic azoospermia in Urology Clinic of Tokat Gaziosmanpasa University Research Hospital between 2009 and 2019, were included in this study. The DNA of these 137 azoospermic infertile males was routinely extracted from blood samples for Y chromosome microdeletion analysis, and no alterations were discovered in the Laboratory of Medical Biology Department. The patients' endocrine profiles (FSH, LH, testosterone), physical examination, in-depth medical history, semen analysis, and standard hematological and biochemical testing were all used to evaluate the patients. We excluded infertile men with chromosomal abnormalities, vas deferens agenesis, obstructive azoospermia, testicular trauma, testicular torsion, or a history of undescended testes from the study. The consent form was signed by 137 azoospermic infertile malesduring the routine examination. The control group includes 100 fertile males who had at least two children each willingly volunteered to participate in the study and completed the consent form. They had no known inherited or chronic illnesses. By the help of G*power 3.1.9.4 program, using chi-square test family, with 80% power, 5% toleration and 0.20 effect size, 237 samples were determined for the

study. All study participants were chosen from Turkey's interior Central Black Sea region. Tokat Gaziosmanpasa University Clinical Research Ethics Committee was approved the study (Approval no: 19-KAEK-179).

Genotyping

To extract genomic DNA from peripheral blood cells, a commercial DNA isolation kit (Invitrogen Life Sciences, Carlsbad, CA) was used. To decide the genotypes of *LEP* gene -2548G>A (rs7799039) and *LEPR* gene Q223R (rs1137101) polymorphisms, polymerase chain reaction (PCR) along with restriction fragment length polymorphism (RFLP) techniques were applied. Forward 5´-TTT CCT GTA ATT TTC CCA TGA G-3´ and reverse 5´-AAA GCA AAG ACA GGC ATA AAA A-3´ primers were used for PCR analysis of the 242 bp region in the *LEP* gene. The following PCR conditions were used: at 94°C for 4 min; subsequent to 30 cycles at 94°C, 52°C, and 72°C for 45 s; and in the end at 72°C for 5 min. After digestion with *HhaI* (New England Biolabs) restriction endonuclease, 182 and 60 bp for GG genotype; 242, 182 and 60 bp for GA genotype were recorded (Figure 1). The presence of the AA genotype did not cause a difference in the size of the PCR product. Forward 5'-AAA CTC AAC GAC ACT CTC CTT-3' and reverse 5'-TGA ACT GAC ATT AGA GGT GAC-3' primers were used for PCR analysis of the 80 bp region in the *LEPR* gene. The PCR conditions were as follows; 3 min at 95°C; subsequent to 35 cycles of at 94°C for 30 s, at 55°C for 38 s, and at 72°C for 45 s; and finally 5 min at 72°C. After digestion with *Msp*I (Thermo Scientific) restriction endonuclease, 57 and 23 bp for RR genotype; 80, 57 and 23 bp for QR genotype were recorded (Figure 2). The existence of the QQ genotype did not affect the size of the PCR product. For results where we were in doubt, we performed second PCR and RFLP.

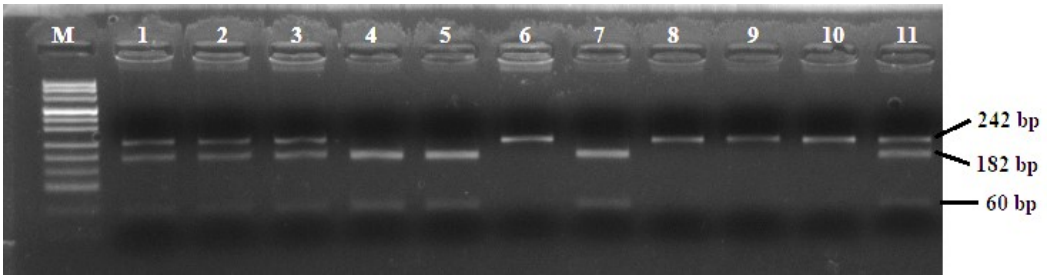

Figure 1. RFLP results of *LEP* gene -2548 G>A polymorphism. Wells 1, 2, 3, and 11 show GA heterozygous genotype, wells 6, 8, 9, and 10 show AA homozygous genotype, wells 4, 5, and 7 show GG homozygous genotype. M: pUC Mix Marker. The DNA Marker contains the following 13 fragments (in base pairs): 1118, 881, 692, 501/489, 404, 331, 242, 190, 147, 111/110, 67.

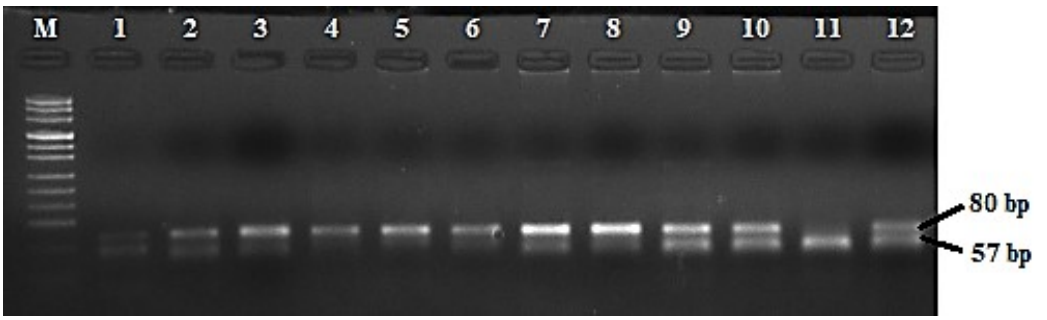

Figure 2. RFLP results for the *LEPR* gene Q223R polymorphism. Wells 1, 2, 3, 6,7, 8, 9, 10 and 12 show QR heterozygous genotype, wells 4 and 5 show QQ homozygous genotype, and well 11 shows RR homozygous genotype (23 bp is not observed). M: pUC Mix Marker. The DNA Marker contains the following 13 fragments (in base pairs): 1118, 881, 692, 501/489, 404, 331, 242, 190, 147, 111/110, 67.

Statistical analysis

By chi-square (χ^2) test, Hardy Weinberg equilibrium (HWE) was estimated for each polymorphism to analyze the discrepancy of the study groups. Statistical Package for the Social Sciences (IBM SPSS Statistics, version 20) and OpenEpi Info software package version 3.01 (www. openepi.com) were applied for statistical evaluation. The χ^2 test was used to evaluate the genotype and allele distributions for both polymorphisms in infertile and fertile males. For comparative analysis of combined genotypes of *LEP* and *LEPR* gene polymorphisms, χ^2 or Fischer's exact tests were performed. One-way analysis of variance (ANOVA) was used to link the genotypes of each polymorphism with the clinical features of infertile males. 95% confidence intervals (CI) and odds ratio (OR) were used to determine the risk factors. The p values of 2-tailed were used and values with $p<0.05$ were regarded as meaningful.

RESULTS

The average age of 137 azoospermic infertile men was 32,39±6,682 and the average age of the 100 fertile men was 41,52±7,826. The genotype and allele distributions of the *LEP* and *LEPR* gene polymorphisms in infertile and fertile men were identified, as shown in Table 1. When the genotype and allele distributions of the infertile and fertile groups were analyzed, no statistically significant association between infertility and LEP gene -2548 G>A polymorphism was found (p=0.246 and p=0.343, respectively). No statistically significant difference between infertile and fertile men was found when GG vs GA+AA or GG+GA vs. AA were evaluated (p=0.103 and p=0.870, respectively) (Table 1). Variant dispersion of the infertile and fertile groups were in agreement with HWE (p=0.256 and p=0.408, respectively). No statistically significant associaiton was observed between azoospermic male infertility and the *LEPR* gene Q223R polymorphism, after comparison of the genotype and allele distributions of infertile and fertile groups (p=0.616 and p=0.376, respectively). When QQ vs QR+RR and QQ+QR vs RR were evaluated, there was no statistically significant difference between the two groups (p=0.330 and 0.683, respectively) (Table 1). Variant distributions of the infertile and fertile groups were in agreement with the HWE (p=0.487 and p=0.175, respectively).

Table 1. Genotype and allele frequencies of *LEP* and *LEPR* gene polymorphisms in infertile and fertile men

Gene (Polymorphism)	Infertile men n=137 (%)	Fertile men (n=100)	p	OR (95% Cl)
LEP (-2548 G>A)	HWE p=0.408	HWE p=0.256		
Genotypes				
GG	24 (17.5)	10 (10.0)	0.246	
GA	61 (44.5)	51 (51.0)		
AA	52 (38.0)	39 (39.0)		
GG : GA+AA	24 : 113	10: 90	0.103	0.52 (0.24 - 1.15)
GG+GA : AA	85: 52	61: 39	0.870	0.96 (0.56 - 1.62)
Allel				
G	109 (39.8)	71 (35.5)	0.343	0.83 (0.57 - 1.21)
A	165 (60.2)	129 (64.5)		
LEPR (Q223R)	HWE p=0.175	HWE p=0.487		
Genotypes				
QQ	49 (35.8)	42 (42.0)	0.616	
QR	72 (52.6)	48 (48.0)		
RR	16 (11.7)	10 (10.0)		
QQ : QR+RR	49: 88	42: 58	0.330	1.3 (0.76 - 2.21)
QQ+QR : RR	121: 16	90: 10	0.683	1.19 (0.52 - 2.74)
Allel				
Q	170 (62.0)	132 (66.0)	0.376	1.19 (0.81 - 1.74)
R	104 (38.0)	68 (34.0)		

Data were analyzed by χ2 test. HWE, Hardy Weinberg Equilibrium; *LEP,* Leptin; *LEPR*, Leptin receptor

The frequencies of composite genotypes of *LEP* -2548 G>A / *LEPR* Q223R polymorphisms were also compared between infertile and fertile men (Table 2). Infertile males were found to have the GG/QR composite genotype approximately 9 times more frequently than fertile men. (8.8% vs. 1.0%, p=0.007).

Table 2. Comparative analysis of combined genotypes of infertile and fertile men

	Infertile men (n=137)		**Fertile men (n=100)**		**p**
Genotypes	n	%	n	%	
-2548G>A / Q223R					
GG/QQ	8	5.8	8	8.0	0.513
GG/QR	12	8.8	1	1.0	0.007
GG/RR	4	2.9	1	1.0	0.360
GA/QQ	25	18.2	21	21.0	0.597
GA/QR	31	22.6	22	22.0	0.909
GA/RR	5	3.6	8	8.0	0.146
AA/QQ	16	11.7	13	13.0	0.759
AA/QR	29	21.2	25	25.0	0.487
AA/RR	7	5.1	1	1.0	0.093

Data were analyzed by $\chi 2$ or Fischer's exact tests. Data that is statistically significant typed in bold

After corporation of age and clinical characteristics (FSH, LH, Prolactin, Estradiol, Total testosterone) of infertile men with *LEP* and *LEPR* gene polymorphisms, no statistically significant associations were observed as shown in Table 3 (p>0.05)

Table 3. Clinical characteristics of infertile men stratified according to *LEP* and *LEPR* gene polymorphisms

		***LEP* (-2548 G>A)**				***LEPR* (Q223R)**			
Characteristics	Total n=137	GG n=24	GA n=61	AA n=52	P value	QQ n=49	QR n=72	RR n=16	P value
Mean age (years)	32.34±6.68	33.13±5.22	32.36±7.48	32.08±6.35	0.819	31.73±6.43	32.90±7.15	32.06±5.25	0.630
FSH (IU / L)	15.57±17.54	20.41±17.48	14.56±17.11	14.48±18.16	0.400	19.25±22.08	12.90±13.15	15.89±17.87	0.208
LH (IU / L)	8.59±7.72	10.85±6.67	7.81±6.32	8.42±9.34	0.331	9.97±10.02	7.70±6.06	8.25±5.54	0.353
Prolaktin (ng/ml)	11.24±6.15	12.34±7.64	12.16±6.06	9.56±5.13	0.170	10.86±6.51	11.37±5.52	11.92±7.63	0.874
Östrodiol (pg / mL)	23.90±13.29	22.42±11.47	24.16±13.08	24.09±14.67	0.946	24.06±13.90	22.62±12.84	29.08±14.03	0.516
Total Testesteron (ng / mL)	3.64±2.25	3.33±1.19	3.68±2.54	3.75±2.31	0.787	3.59±2.76	3.57±2.00	4.14±1.53	0.704

Data were analyzed ANOVA. mean ± standard deviation values were given for all characteristics. FSH, Follicle stimulating hormone; *LEP,* Leptin; *LEPR*, Leptin receptor LH, Luteinizing hormone

DISCUSSION

Infertility is the situation where the couples do not get pregnant within 1 year despite having regular sexual intercourse. The cause of infertility is divided into three categories as female factor, male factor and unexplained infertility. Male infertility accounts for 30% of all infertility cases[2]. Azoospermia can be detected in 1% of male cases and in 15% to 20% of patients evaluated for infertility[5]. LEP affects men's sperm function and reproductive system by rearranging the HPG axis[7]. Infertile male patients was reported to have elevated levels of LEP[10]. LEP shows its effect by binding LEPR. Women were reportedly more sensitive to the loss of LEPR in gonadotropic cells than men[21].

This study investigated the relationship between *LEP* -2548G>A and *LEPR* gene Q223R polymorphisms and azoospermic male infertility. As a result of genotype and allele comparisons, it was found that these polymorphisms were not associated with azoospermic male infertility individually, but when evaluated together, they could be associated with azoospermic male infertility. The GG/QR composite genotype has been found to occur approximately 9 times more frequently in azoospermic infertile men than in fertile men (p=0.007) and the co-existence of these genotypes may predispose to azoospermic male infertility. While the GG genotype of *LEP* -2548G>A polymorphism causes a decrease in LEP expression, the QR genotype of *LEPR* Q223R polymorphism causes a decrease in LEP binding. This dual effect may cause infertility by affecting spermatogenesis.

When we searched the literature, two studies addressing the connection between male infertility and *LEP* and *LEPR* polymorphisms were found[14,22]. One of them was carried out in Iranian population and the other in Slovanian population.

In the study conducted with 150 infertile and 150 fertile male individuals in the Iranian population, no relationship was found between *LEPR* gene Q223R polymorphism and male infertility, but a significant relation was determined between male infertility and *LEP* gene -2548G>A polymorphism[14]. AG genotype frequency was fewer in infertile group than in fertile group (p=0.004). For this reason, the AG genotype suggested to have a protecting impact and maybe approximately 3 (1/0.326) times reduce the risk of male infertility. Previous investigations have demonstrated that males with the AG genotype have lower LEP levels than men with the AA genotype[23]. In the study conducted in the Iranian population, it was found that male infertiles with the genotypes AG and GG had greater sperm counts than those with the genotype AA (p<0.05). In line with this result, there were also studies reporting a reverse association between sperm counts and *LEP* gene expression[17]. The results of the study conducted in the Iranian population is similar to the results of our study in terms of *LEPR* polymorphism, but differs in terms of *LEP* polymorphism. Khosropoura et al. did not compare fertile and infertile male individuals in terms of composite genotype in their study[14]. Maybe if they had made such a comparison, they would have found a significant relationship like ours. In the study conducted in the Iranian population, the clinical values (LH, FSH, and T levels) of infertile male individuals were compared with both *LEP* and *LEPR* gene polymorphisms and no correlation was found between them, in line with our findings.

In another case-control study conducted in the Slovenian population, the association between male infertility and eight polymorphisms, including *LEP* -2548G>A (rs7799039) and *LEPR* Q223R (rs1137101) polymorphisms, were studied in 317 infertile men and 241 fertile men[22]. Similar to our findings, they did not find a relationship between *LEP* -2548G>A and *LEPR* Q223R polymorphisms and male infertility. Once more, there was no connection between the clinical characteristics of infertile patients and polymorphisms in the *LEP* and *LEPR* genes that were identical to ours.

There were other studies investigating the association between female infertility and *LEP* -2548G>A and *LEPR* Q223R polymorphisms. These polymorphisms were associated with infertility in Iranian women with polycystic ovary syndrome[24].

Studies have reported that there are significant associations between increased expression of leptin and plasma secretion from adipocytes and *LEP* gene promoter -2548G>A polymorphism[12,20]. The exonic Q223R polymorphism of *LEPR* gene causes an alteration in the extracellular area of protein. It has been noted that any modification in this domain can impact LEP signaling since it is necessary for LEPR proper folding and activity[25,26]. This polymorphism was associated with decreased connection of LEP and consequently caused LEP resistance[12].

It has been reported that LEP shows a modulatory act on glycolytic activity and acetate production of human Sertoli cells by showing metabolic impacts on male reproductive system. It has been reported that Sertoli cells' glycolytic activity is necessary for normal spermatogenesis, and LEP and LEPR may clearly arrange Sertoli cells' metabolic state to have an impact on spermatogenesis[27]. The act of LEP and its receptor can provide light on the relationship between male infertility and obesity. In particular, obesity-induced hormonal irregularity was linked to decreased male fertility[28]. Polymorphisms in the *LEPR* gene could cause decrease in intracellular signaling because of functional and structural instability of the receptor which shows aberrant reproductive roles in human[29].

According to the findings of the literature assessment, this study, which examines *LEP* and

LEPR gene variants in azoospermic male infertility, is the third in the world and the sole study in Türkiye. The main limitation of our study is the relatively small sample size of azoospermic infertile men and fertile controls.

In conclusion, we observed an association between composite genotypes of *LEP* -2548G>A and *LEPR* Q223R polymorphisms and azoospermic male infertility, in this research. GGQR composite genotype may be a risk factor for azoospermic male infertility. The action of LEP and its receptor can shed light on how male infertility and obesity are related. In our opinion, these studies should be carried out in larger populations to get more accurate and efficient results.

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Ethical Approval: Our study was approved by the local Ethics Committee of Tokat Gaziosmanpasa University, Turkey on 11 October 2019 (Number: 19-KAEK-179).
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