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RESEARCH COMMUNICATION

Leptin and leptin-receptor polymorphisms in fertile and infertile men

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ABSTRACT

The association of leptin (*LEP*) -2548G/A and/or leptin receptor (*LEPR*) Gln223Arg polymorphisms with male infertility and plasma FSH, LH, and testosterone (T) levels was examined. The genotypes and allele frequency distributions of *LEP* -2548G/A and *LEPR* Gln223Arg polymorphisms were investigated in 150 fertile and 150 infertile men by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Also, plasma levels of FSH, LH, and T were measured using commercial ELISA kits. Frequencies of AA, AG and GG genotypes of LEP-2548G/A polymorphism were statistically different in fertile and infertile men (p=0.012). The AG genotype showed a protective effect which could decrease risk of male infertility about 3 fold ($p = 0.004$). We did not observe any differences in frequencies of *LEPR* Gln223Arg alleles and genotypes between groups ($p > 0.05$). Sperm counts from infertile men with the AG and GG genotypes of the LEP polymorphism were significantly higher than AA genotype ($p<0.05$). Moreover, infertile men who carried the RR genotype of *LEPR* showed a statistically higher percentage of sperm with progressive motility than individuals with other genotypes ($p = 0.004$). There was no correlation between different combinations of *LEP* and *LEPR* genotypes and LH, FSH, and T levels ($p > 0.05$). Our study suggests that the *LEP* -2548G/A polymorphism may play a role in male fertility and the AG genotype may have a protective effect through increasing sperm counts. The distribution of genotypes of *LEP* -2548G/A polymorphism are different in fertile and infertile males and may be a useful tool in evaluation of male infertility.

Abbreviations: *LEP*: leptin; *LEPR*: leptin receptor; T: testosterone; FSH: follicle-stimulating hormone; LH: luteinizing hormone

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Introduction

Leptin (LEP) is a 16 kDa protein that is produced by adipose tissue and plays important roles in the regulation of various physiological functions such as food intake, immune system, angiogenesis, and reproduction [Lado-Abeal and Norman 2002; Yiannakouris et al. 2001]. LEP acts through leptin receptors (LEPR) which belong to the interleukin-6 receptor family of class 1 cytokine receptors to exert its physiological effects [Tartaglia et al. 1995].

It was well documented that LEP has effects on the male reproductive system by regulating the hypothalamic-pituitary-gonadal (HPG) axis [Gonzalez-Añover et al. 2011] and sperm function [Aquila et al. 2008]. Stimulatory effects of LEP on gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were reported in various studies [Cunningham et al. 1999; Ponzo et al. 2004]. It was also documented that *LEP*-or *LEPR*-deficient mice were infertile and have low levels of gonadotropins [Clément et al. 1998; Strobel et al. 1998]. In addition, LEP and LEPR protein and mRNA were detected in seminiferous tubules, Sertoli, Leydig and testicular germ cells, cell membrane and tail of the sperm, seminal plasma, prostate gland, and seminal vesicle which signify possible paracrine and autocrine effects of LEP on male reproduction [Ishikawa et al. 2007; Jope et al. 2003; Dhanoa et al. 2015].

Moreover, some studies have reported the association between LEP and/or LEPR with testis volume, testosterone (T), spermatogenesis, sperm counts, motility, and capacitation [Aquila et al. 2008; Ishikawa et al. 2007; Lampiao and Du Plessis 2008]. However, some studies have demonstrated the presence of inverse correlations between LEP and male reproductive indicators such as T levels and sperm motility and count [Ishikawa et al. 2007;

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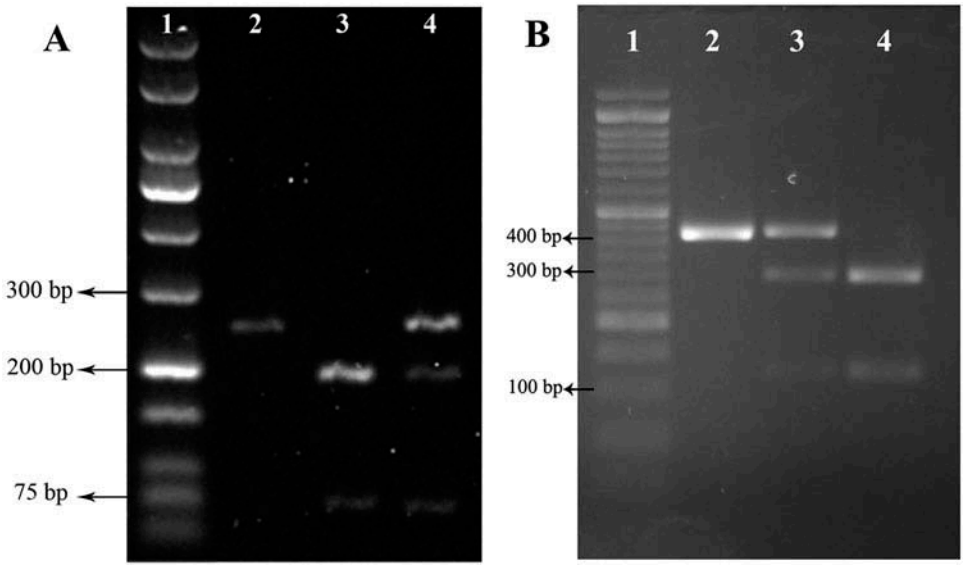

Figure 1. Leptin -2548G/A and Leptin receptor Gln223Arg polymorphisms were examined by restriction fragment length polymorphism technique using Hha1 (Cfo1) and HpaII (Fermentas, USA) as restriction enzyme digestion, respectively. Digested products have been run on 2% agarose gel and visualized under UV light. (A) Leptin -2548G/A: Lane 1 is DNA Molecular Weight Marker) Fermentas, USA); Lane 2 is AA genotype (242bp); Lane 3 is GG genotype (181 and 61bp); Lane 4 is AG genotype (242, 181, and 61bp); (B) leptin receptor Gln223Arg: Lane 1 is 50 bp DNA Molecular Weight Marker (SinaClon, Iran); Lane 2 is QQ genotype (416 bp); Lane 3 is QR genotype (416, 291, and 125 bp); Lane 4 is RR genotype (291 and 125 bp).

Glander et al. 2002; Dhanoa et al. 2015; Karimova 1982]. Also, a high concentration of LEP was found in infertile male patients [Jahan et al. 2011].

Genetic variation in the regulatory region of the *LEP* gene might affect the LEP level and also male fertility. Strobel et al. [1998] reported that individuals with a mutation in the *LEP* gene were infertile, where pubertal development was absent in patients with the *LEPR* gene mutation [Clément et al. 1998]. One of the common promoter region polymorphisms of the *LEP* gene is -2548G/A (rs7799039). Previous studies have reported significant associations between this polymorphism, and circulating LEP and LH concentrations, adipose tissue *LEP* mRNA, and secretion levels [Yiannakouris et al. 2003; Su et al. 2012; Hoffstedt et al. 2002]. Moreover, 668A/G is the *LEPR* gene polymorphism in which adenine (A) at position 668 is substituted by guanine (G). This polymorphism leads to a change in the extracellular domain of the receptor CRH1, whereby glutamine (Q) at position 223 (Gln223Arg, rs1137101) is replaced with arginine (R). The CRH1 domain is involved in LEPR activity and its correct folding, therefore, any changes in this domain such as Gln223Arg polymorphism may affect LEP signaling [Saukko et al. 2010; Peelman et al. 2006]. Studies have shown that this polymorphism is associated with decreased binding affinity for LEP which causes LEP resistance [Hoffstedt et al. 2002].

Based on our knowledge, there are no studies investigating the association between *LEP* -2548G/A and/or *LEPR* Gln223Arg polymorphisms with male infertility. Therefore, this study was conducted to examine the association between these polymorphisms and male infertility and to evaluate the effects of these polymorphisms on plasma FSH, LH, and T levels.

Results and discussion

Evaluation of the *LEP* -2548G/A polymorphism as illustrated in Figure 1 with results summarized in Table 1 showed the frequency of *LEP* -2548G/A genotypes were statistically different in fertile and infertile men (p=0.012). We observed that the frequency of the AA genotype was higher in the infertile group compared to fertile individuals. In addition, a lower frequency of AG carriers were observed in the infertile group in comparison with the fertile group. Based on logistic regression analysis, the AG genotype could have a protective effect and possibly decrease the risk of male infertility about 3 (1/0.326) fold. How -2548G/A polymorphism may impact fertility is not clear. The location of this polymorphism is at the 5'end of the promoter region of *LEP* that contains transcriptional inhibitory elements. In addition, the binding site for SP-1 transcription factor and repetitive sequences of MER11 are very close to the location of this polymorphism (-2538 vs. -2548 and -2514 vs. -2548, respectively). The role of the SP-1 transcription factor and repetitive sequences of MER11 in the regulation of several genes was reported [Gong et al. 1996]. Therefore, it is likely that the G to A substitution at -2548 nucleotide

affects the expression of *LEP* and may reconcile the effect of the *LEP* polymorphism on fertilityAs shown in Table 1, in the *LEPR* Gln223Arg region, the frequencies of QQ, QR, and RR genotypes in fertile (41.4%, 49.3%, and 9.3%, respectively) and infertile groups (44.7%, 46.7%, and 8.6%, respectively) were not statistically different ($p>0.05$). Also, there was no significant difference in the distribution of the polymorphic alleles between the two groups ($p=0.602$). Moreover, logistic regression analysis showed no significant association between male infertility and *LEPR* Gln223Arg polymorphism. These results have suggested that the *LEPR* Gln223Arg polymorphism had no significant effect on male fertility. While the null effect of this mutation in the *LEPR* gene on fertility has previously been shown in mice [Akhter et al. 2014], there is evidence that *LEPR* deficient mice are infertile and have lower gonadotropic functions [Strobel et al. 1998; Mann and Plant 2002]. It seems that further investigation is needed to clearly delineate the impact of *LEPR* polymorphism on male fertility.

The population studied was in Hardy-Weinberg equilibrium for the *LEPR* Gln223Arg polymorphism but inconsistency of the equilibrium was seen for *LEP* -2548G/A polymorphism in the subgroups. This departure may reflect genotyping errors, small sample size, or biological selection [Nielsen et al. 1998]. We confirmed that data collection bias was minimal and confirmed the genotyping method by re-genotyping 20% of the samples. In the case of sample size, there were studies on *LEP* -2548G/A polymorphism in Iran and other countries with lower sample size which reported no deviation from Hardy-Weinberg equilibrium [Mohammadzadeh et al. 2015; Yiannakouris et al. 2003; Li et al. 2016; Hoffstedt et al. 2002]. Deviation from the equilibrium can be seen in the only study that examined this polymorphism in the same region as the study presented here, which showed the possibility of ethnic bias [Hassanzadeh et al. 2013]. However, more studies with larger sample sizes are required for clarification.

The semen profile of the infertile group and its association with various genotypes are presented in Table 2. Sperm counts in infertile men with the AG

Table 1. The frequency of Leptin -2548G/A and Leptin receptor Gln223Arg genotypes, alleles, and odds ratios in fertile (n = 150) and infertile (n = 150) males using χ2 test and regression logistics analysis.

	Fertile *n* (%)	Infertile *n* (%)	*p*-Value (χ^2, df)	Odd ratio, 95% CI (Lower-Upper, *p*)
***LEP*-2548G/A** *Genotypes*				
AA	10(6.6%)	27(18%)		Reference group
AG	135(90 %)	119(79.3%)	0.012 (χ^2=8.930, df=2)	0.326 (1.278-4.916, p=0.004)
GG	5(3.4%)	4(2.7%)		0.357 (0.256-4.240, p=0.197)
AG+GG	140(93.4%)	123(82%)	0.003 (χ2=8.910, df=1)	0.327 (1.431-6.554, p=0.004)
Alleles				
A	155(51.6%)	173(57.6%)	0.140 (χ2=2.179, df=1)	Reference Allele
G	145(48.4%)	127(42.4%)		0.794(0.577-1.093, p=0.157)
***LEPR* Gln223Arg** *Genotypes*				
QQ	62(41.4%)	67(44.7%)		Reference group
QR	74(49.3%)	70(46.7%)	0.843 (χ^2=0.342, df=2)	1.105 (0.747-1.637, p=0.617)
RR	14(9.3%)	13(8.6%)		1.061 (0.539-2.088, p=0.863)
QR+RR	88(58.6%)	83(55.3%)	0.560 (χ^2=0.340, df=1)	0.885 (0.595-1.315, p=0.545)
Alleles				
Q	198(66%)	204(68%)	0.602 (χ^2=0.271, df=1)	Reference Allele
R	102(34%)	96(32%)		0.924(0.688-1.241, p=0.599)

df: degrees of freedom; CI: confidence interval.

Table 2. Semen parameters of infertile group regarding genotypes of Leptin -2548G/A and Leptin receptor Gln223Arg polymorphisms.

	Sperm motility[1] (%)				
Genotypes	Progressively motile sperm	Non-progressively motile sperm	Immotile sperm	Sperm counts (million/ml)	Sperm normal morphology (%)
Semen parameters	15 (5.67-25)	19.25 (10-25)	64.45 (50-80)	50 (30-70)	25 (15-25)
LEP -2548G/A					
AA (n=27)	15 (5.45-25)	16.2 (10-25)	63.9 (50-80)	43 (25-55)	20 (15-25)
AG (n=119)	15 (5-21)	19.95 (10-25)	69.55 (50.4-80)	50 (35-60.7)[a]	25 (15-25)
GG (n=4)	20 (17.5-38)	20 (15-25)	55 (50-67.4)	65 (40-75)[b]	25 (20-30)
LEPR Gln223Arg					
QQ (n=67)	15 (6.12-25)	16.9 (10-25)	64.45 (50-80)	56 (30-70)	22.5 (15-25)
QR (n=70)	15 (5-21.2)	20 (10-25)	67 (50.6-80)	50 (30-75)	25 (15-25)
RR (n=13)	26 (17.5-30)[b,c]	20 (15-25)	55 (50-67.4)	45 (35-60)	25 (20-30)

[1]Grade of sperm movement according to WHO [2010] criteria.
[a,b,c]p-value <0.05. [a]Significant difference between AG and AA or QR and QQ; [b]between GG and AA or RR and QQ; [c]between GG and AG or RR and QR. Results are presented as Median (Interquartile Range).

and GG genotypes of *LEP* polymorphism were significantly higher than that of subjects with the AA genotype (p=0.009 and p=0.026, respectively). Considering these results, part of the protective effect of the AG genotype on male infertility may be related to effect on semen parameters because we observed that those carrying the AG genotype had higher sperm count compared to AA genotype carriers. Previous studies have shown that the LEP level in men with the AA genotype was higher than that of men carrying the AG genotype [Mammes et al. 2000]. Moreover, negative correlation of LEP expression with sperm counts have been reported [Ishikawa et al. 2007]. Therefore, it is likely that the reduced leptin level in men with the AG genotype leads to an increase in sperm counts as observed in the present study. However, we did not measure LEP levels in this study and cannot comment on the possible association between the genotypes and circulating level of LEP.

We observed a higher percentage of progressively motile sperm in men carrying the RR genotype compared with QQ and QR genotype carriers of *LEPR* (Table 2). In accordance with these observations, Jope et al. [2003] reported the immunocytochemical localization of LEPR on the tail of human spermatozoa. Since the functions of the tail segment are critical for sperm motility, it is likely that the *LEPR* polymorphism could affect sperm cell movement. There are two possibilities for this polymorphism to influence motility. Perhaps, the Gln223Arg polymorphism of *LEPR* is in the segment encoding of the extracellular domain of this receptor. Therefore, replacing glutamine (Q, neutral charge) at position 223 with arginine (R, positive charge) changes the function of LEPR, alters signaling capacity [Yiannakouris et al. 2001], and increases motility. Alternativley, this polymorphism is perhaps located in the extracellular segment of LEPR which contains a leptin-binding site and is associated with decreased binding affinity for LEP which causes LEP resistance [Yiannakouris et al. 2001]. In addition, an inverse correlation between LEP and sperm motility was reported. It is reported that leptin resistance can be created by LEPR-mediated disruption [Gonzalez-Bulnes et al. 2012]. In addition, the relationship between reproductive disorders of human and leptin resistance has been shown [Gonzalez-Añover et al. 2011]. Thus, individuals with RR genotype may be less influenced by LEP and, therefore, have higher sperm motility.

Previously studies have examined the functional sites of interaction of LEP on the reproductive system. The hypothalamus is the main target for LEP action on the reproductive axis [Hausman et al. 2012]. However, direct action sites in reproductive tissues have been suggested according to distribution of *LEPR* in these tissues. Seminal plasma LEP is detected and the direct role of LEP on sperm is confirmed. [Martins et al. 2015]. The metabolic effects of LEP on the male reproductive system have also been examined. Martins et al. [2015] reported the modulatory role of LEP on acetate production and glycolytic activity of human sertoli cells. It is shown that the glycolytic activity of sertoli cells is necessary for normal spermatogenesis. They reported that LEP and LEPR can directly modulate the metabolic condition of sertoli cells, thereby affecting spermatogenesis [Martins et al. 2015]. This new role for LEP and its receptor may explain the link between male infertility and obesity. Particularly, hormonal dysregulation which is induced by obesity, has been associated with decrease male fertility [Alves et al. 2016].

As shown in Table 3, the plasma concentrations of LH and FSH were significantly higher in fertile men compared with the infertile group ($p < 0.001$). Gonadotropins play various physiological roles among the testis and the pituitary gland. Failure of FSH and LH secretion results in disorder of testicular function and male infertility. Serum concentration of FSH is an important index for the seminiferous epithelium status. The normal ranges for FSH and LH in serum in adult male are 1.4-15.4 and 1.24-7.8 IU/L, respectively. Our findings of LH and FSH concentration of fertile and infertile groups were in the normal range. However, in infertile males, medians of LH or FSH were closer to the lower limit of normal ranges. In addition, there were reports that suggest the possible effect of LEP on gonadotropic cells and secretion of FSH and LH which should be verified by other studies [Akhter et al. 2014]. There was no significant difference in T levels between the two groups (p=0.448). The results of the comparison of LH, FSH, and T levels among males with various genotypes of *LEP* and *LEPR* polymorphisms or combinations of *LEP* and *LEPR* polymorphisms can be compared in Tables 3 and 4. Based on these results, there are no significant associations between *LEP* and *LEPR* polymorphism or combination of two polymorphisms with plasma levels of LH, FSH, and T in the population studied. These findings indicate that *LEP* -2548G/A and *LEPR* Gln223Arg polymorphisms may not be considered as genetic factors for hormonal changes of LH, FSH, and T in fertile or infertile males. The impact of LEP on gonadotropic cells of the pituitary is less clear. It has been reported that gonadotropes respond to LEP with alteration in secretion of LH and FSH. However, the direct interactions between LEP and gonadotropic cells are still unclear [Akhter et al. 2014]. Akhter et al. [2014] have reported that males compared to females are less sensitive to the deletion of *LEPR* in gonadotropic cells and *LEPR* is less significant for fertility in males with mutant gonadotropes. These

Table 3. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) levels in genotypes of leptin -2548G/A and leptin receptor Gln223Arg in studied population.

			LEP -2548G/A			
			AA (*n*=37)	AG (*n*=254)	GG (*n*=9)	*p*-value
All (*n*=300)	LH (IU/L)		2.3 (1.57-4.16)	2.1 (1.50-4.00)	1.95 (1.43-3.58)	0.841
	FSH (IU/L)		2.87 (2.13-4.12)	3.07 (2.27-4.59)	3.1 (2.13-3.86)	0.602
	T (ng/mL)		3.9 (3.08-6.32)	3.8 (3.06-6.3)	3.4 (3.03-3.85)	0.548
		Concentration	**AA (n=10)**	**AG (n=135)**	**GG (n=5)**	***p*-value**
Fertile (*n*=150)	LH (IU/L)	3.02 (1.70-5.04)*	2.2 (1.6-2.9)	3.05 (1.7-5.23)	3.15 (1.7-4.01)	0.086
	FSH (IU/L)	3.80 (3.01-5.36)*	3.54 (2.52-4.66)	3.70 (2.83-5.04)	3.85 (3.22-4.04)	0.744
	T (ng/mL)	4 (2.72-6.04)	3.5 (3.08-4.05)	4.03 (2.7-6.10)	3.18 (3.03-7.05)	0.631
		Concentration	**AA (n=27)**	**AG (n=119)**	**GG (n=4)**	***p*-value**
Infertile (*n*=150)	LH (IU/L)	1.9 (1.4-3)	1.9 (1.62-4.95)	1.80 (1.4-2.65)	1.55 (1.4-2.07)	0.066
	FSH (IU/L)	2.41 (2.06-3.4)	2.41 (1.93-3.96)	2.33 (2.0-3.77)	2.25 (1.55-3.02)	0.722
	T (ng/mL)	3.7 (3.12-5.97)	3.7 (3.32-7.17)	3.80 (3.32-6.47)	3.6 (2.07-3.85)	0.478
			LEPR Gin223Arg			
			QQ (*n*=129)	QR (*n*=144)	RR (*n*=27)	*p*-value
All (*n*=300)	LH (IU/L)		2.2 (1.5-3.97)	2.2 (1.5-4.05)	2.5 (1.65-4.87)	0.661
	FSH (IU/L)		2.3 (2.3-4.29)	3.35 (2.4-5.23)	2.97 (2.11-4.07)	0.058
	T (ng/mL)		3.7 (2.92-5.39)	3.7 (3.02-6.1)	3.8 (3.14-6.77)	0.462
		Concentration	**QQ (n=62)**	**QR (n=74)**	**RR (n=14)**	***p*-value**
Fertile (*n*=150)	LH (IU/L)	3.02 (1.70-5.04)*	2.7 (1.5-4.3)	3.02 (1.77-5.24)	3.7 (2.4-5.3)	0.131
	FSH (IU/L)	3.80 (3.01-5.36)*	3.78 (2.91-4.97)	3.84 (3.09-5.6)	3.62 (2.84-5.1)	0.395
	T (ng/mL)	4 (2.72-6.04)	3.8 (2.5-6.05)	4.03 (3.01-6.05)	4 (2.5-7.05)	0.811
		Concentration	**QQ (n=67)**	**QR (n=70)**	**RR (n=13)**	***p*-value**
Infertile (*n*=150)	LH (IU/L)	1.9 (1.4-3)	2.02 (1.5-3.25)	1.9 (1.35-2.85)	1.8 (1.4-2.57)	0.455
	FSH (IU/L)	2.41 (2.06-3.4)	2.4 (2.1-3.18)	2.47 (2.11-4.16)	2.18 (1.3-3.04)	0.078
	T (ng/mL)	3.7 (3.12-5.97)	3.7 (3.1-4.2)	3.7 (3.05-6.3)	3.8 (3.5-6.65)	0.322

Results are presented as Median (Interquartile Range). *$p < 0.001$ compared with infertile group.

Table 4. Comparison of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) levels in combination genotypes of Leptin -2548G/A and Leptin receptor Gln223Arg polymorphism.

		AA/QQ (*n*=5)	AA/QR (*n*=5)	AG/QQ (*n*=57)	AG/QR (*n*=64)	AG/RR (*n*=14)	*p*-value
Fertile (*n*=150)	LH (IU/L)	1.550 (1.25-2.7)	2.3 (1.47-2.72)	2.45 (1.5-4.7)	3.3 (1.7-6.07)	3.6 (2.3-5.3)	0.100
	FSH (IU/L)	2.53 (2.1-497)	3.94 (3.28-5.3)	3.56 (2.81-4.7)	3.78 (2.88-5.59)	3.43 (2.68-4.7)	0.371
	T (ng/mL)	3.04 (1.93-4.16)	3.5 (3.08-4.26)	3.76 (2.5-6.08)	4.04 (2.85-6.08)	4.5 (3.05-7.09)	0.667
		AA/QQ (*n*=13)	AA/QR (*n*=14)	AG/QQ (*n*=50)	AG/QR (*n*=56)	AG/RR (*n*=13)	*p*-value
Infertile (*n*=150)	LH (IU/L)	2.6 (1.67-5.13)	2.3 (1.45-4.8)	1.75 (1.4-2.3)	1.9 (1.3-2.9)	1.7 (1.4-2.65)	0.190
	FSH (IU/L)	2.4 (2.23-3.2)	2.95 (1.77-7.03)	2.3 (1.77-3.8)	2.45 (2.01-4.3)	2.2 (1.41-2.92)	0.460
	T (ng/mL)	3.8 (3.12-4.86)	6.35 (3.12-8.35)	3.7 (3.22-6.85)	3.8 (3.3-6.3)	3.8 (3.6-6.8)	0.692

Results are presented as Median (Interquartile Range).

observations are in line with our results confirming that the genetic polymorphisms of *LEP* or *LEPR* has no effect on LH, FSH, and T levels.

The limitations of this study are many, highlighted by the small sample size. This includes our inability to obtain semen samples from fertile men preventing any conclusions to be drawn between polymorphisms and semen parameters. The study was further compromised by our lack of life style data including weight and BMI of the studied population. We therefore cannot evaluate the possible relation among the polymorphisms, BMI, and male infertility. This is rather unfortunate since BMI is an important index in the evaluation of overweight and obese individuals. Increasing BMI is associated with the deposition of excess fat in adipose tissue, this condition may affect levels of LEP which are mostly secreted by adipose tissue as previously reported by other [Alves et al. 2016]. The results of other studies indicated that the average BMI in the *LEP-2548G/A* polymorphism was 23.6±0.6 for GG+GA and 22.9±1 for AA genotypes [Yiannakouris et al. 2003; Su et al. 2012; Hoffstedt et al. 2002]. In addition, the average BMI in the *LEPR* Gln223Arg polymorphism was 22.3±0.5 for QQ, 21.8 ±0.5 for QR and 24.8±1.1 for RR genotypes [Yiannakouris et al. 2001].

Nevertheless, the data reported in this research communication suggests that the *LEP* -2548G/A polymorphism may have a role in fertility and the AG genotype may have a protective effect through increasing sperm counts. Based on our results, frequency of *LEPR* Gln223Arg genotypes in fertile and infertile males was not statistically different but a higher percentage of motile sperm was observed in RR genotype. Perhaps the AG genotype of *LEP* -2548G/A polymorphism may have a prominent role in male fertility and be envisioned as a key factor in the evaluation of male infertility. This awaits independent confirmation.

Materials and methods

Study population and sample collection

One hundred and fifty Iranian fertile men (having at least one child) and 150 infertile men who had no child after at least a year of unprotected intercourse and had been referred to the Fatemieh Fertility Clinic of Hamadan University of Medical Sciences were enrolled in this study. Subjects were aged 29-40 years and there was not a significant difference between the mean ages of the two groups. Patients with certain infertility causes such as inflammation and infectious disease, varicocele, and abnormal karyotype were excluded. In addition, having diseases such as chronic disease, thyroid disorder, liver and cardiovascular diseases, diabetes mellitus, and hypertension were also set as exclusion criteria. Written informed consents were obtained from the study population according to the Ethical Committee of Hamadan University of Medical Sciences criteria.

Ten milliliters of blood sample was taken from each subject and collected in a tube containing heparin as an anticoagulant. After centrifugation, plasma aliquots stored at −20°C until FSH, LH, and T measurements and blood leukocytes were used for DNA extraction and genotype analysis. In the infertile group, semen samples were collected by masturbation considering three days of sexual abstinence and semen related parameters were determined after liquefaction according the World Health Organization [WHO 2010] procedures. Semen volume, concentration (haemocytometer), and morphology (Papanicolaou staining method) as well as motility grades of sperm (progressively motile sperm, non-progressively motile sperm, immotile sperm) were determined using WHO [2010] standard procedures.

DNA extraction and genotyping of LEP-2548G/A (rs7799039) and LEPR Gln223Arg (rs1137101) polymorphisms

The phenol-chloroform extraction method was used for DNA extraction from peripheral blood and its quality was determined as a ratio of A260/A280 absorbance. Determination of *LEP* -2548G/A and *LEPR* Gln223Arg variants was performed by amplification of 242 bp and 416bp fragments (PCR PreMix; BIONEER kit, Korea) using forward (5′-TTTCCTGTAATTTT CCCTGAG-3′ and 5′-ACCCTTTAAGCTGGGTGT CCCAAATAG-3′, respectively) and reverse (5′-AAAGCAAAGACAGGCATAAAAA-3′ and 5′-AGCT AGCAAATATTTTTGTAAGCAATT-3′, respectively) primers, as previously described [Li et al. 2016; An et al. 2016].

After confirming the size of PCR products (242 bp) of *LEP* -2548G/A on 1% agarose gel, genotypes were examined using restriction fragment length polymorphism technique. *Hha1* (Cfo1) (Fermentas, USA) enzyme were used for digestion of the *LEP* PCR products (8 Unit of Hha1 at 37°C for 2 h). Digested samples were run on a 2% agarose gel and visualized under UV light. In the *LEP* -2548G/A, the AA genotype resulted in a 242 bp fragment (absence of restriction site), the GG genotype was identified with two bands (181 and 61 bp), and the AG genotype yielded three 242, 181, and 61 bp fragments (Figure 1A). For *LEPR* Gln223Arg polymorphism, the PCR product (416 bp) was digested by *HpaII* (Fermentas, USA) enzyme (10 unit at 37°C for 3 h) and fragments were visualized on a 2% agarose gel. The QQ genotype (absence of restriction site) was identified by 416 bp fragment, the RR genotype exhibited two 291 and 125 bp fragments, while the QR genotypes produced three 416, 291, and 125bp fragments (Figure 1B). Genotyping accuracy was checked by random analysis of 20% of samples.

Hormones assay

Plasma FSH and LH levels were measured using commercial sandwich ELISA kits (Catalog No. PT-FSH-96 and PT-LH-96 respectively, PishtazTeb, Iran,), according to the manufacturer's instructions. FSH and LH quantitative tests were carried out based on solid phase enzyme-linked immunosorbent assay. These ELISA kits used one anti-FSH or LH antibody for solid phase immobilization and another mouse monoclonal anti-FSH or LH antibody in the antibody-enzyme conjugate solution. Also, for evaluating T concentrations in plasma samples, we used DiaMetra assay kit (DiaMetra Ltd., Milano-Italy, Catalog No. DKO002). In this kit a competitive immunoenzymatic colorimetric method was used for determination of testosterone.

Statistical analysis

Data were analyzed using SPSS V.16 software and the results were expressed as median with inter-quartile range. Based on One-Sample Kolmogorov-Smirnov test results, the distribution of data was not normal and therefore an equivalent non-parametric analysis was used. Differences in the mean of hormone levels between groups were tested using Mann-Whitney U test. The distribution of genotypes for Hardy-Weinberg equilibrium was checked in the studied groups. Chi-Square test was used for comparison of alleles and genotypes frequencies between the groups. Logistic regression model was applied to estimate the odds ratio (OR) and its 95% confidence interval of

LEP and *LEPR* genotypes. For comparison of hormone levels and semen parameters among genotypes Kruskal-Wallis and Mann-Whitney U tests were used. The power of this study was 80% to detect genotype association of leptin polymorphism at α=0.05 level. The level of statistical significance was considered as $p<0.05$.

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Declaration of interests

The authors are academic members or postgraduate students of Hamadan University of Medical Sciences or Payam-e Noor University. The authors are not directly funded by the Government of Iran.

Notes on contributors

Designed the study, wrote the article and contributed in the critical revision: HT, IK, MF; Contributed in sample collection and performing experiments: SK, MH, AF; Statistical analysis: AVR,MK.

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