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Expression Patterns of Leptin, Leptin Receptor, *Kiss1*, and *HOTAIR* Genes in Blood and Semen of Infertile Males with Oligospermia

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Abstract

Background: Infertility affects about 15% of couples during reproductive age and male factor infertility accounts for half of these cases. This study aimed to evaluate the expression of *leptin*, *KISS1*, *leptin receptor*, and *HOTAIR* genes in blood and semen samples of individuals diagnosed with oligospermia in comparison to healthy controls.

Methods: In the current investigation, we studied 36 semen and 30 blood samples from fertile oligospermic men as well as the same number of healthy controls. RNA was extracted and cDNA was synthesized. Real-time polymerase chain reaction (PCR) was conducted to assess the gene expression levels. Statistical analysis was performed using Graph Pad Prism software. The results were reported as mean±SEM and any $P< 0.05$ were considered statistically significant.

Results: A significant increase in the expression of the *leptin* gene in infertile males, particularly in semen samples ($P< 0.001$) was found when compared to the healthy controls. On the other hand, significant decrease in the expression of the *KISS1* ($P< 0.0001$) and *HOTAIR* ($P< 0.001$) genes, particularly in semen samples were found when compared to the controls. As it was shown, the expression of *leptin receptor* had no significant effects on male infertility. Additionally, there may be a potential correlation between the expression of *HOTAIR* and the *leptin* and *KISS1* genes, with a negative correlation observed between *HOTAIR* and *leptin*, and a positive correlation between *HOTAIR* and *KISS1* in both blood and semen samples. However, further investigations are necessary to establish the statistical significance of these correlations.

Conclusion: According to the results, *leptin*, *KISS1*, and *HOTAIR* genes seem to be affected in oligospermia, however, further studies with higher sample sizes are necessary.

Keywords: Ene expression, HOTAIR long non-coding RNA, Infertility, Kiss1, Leptin, Leptin receptor.

Introduction

Nowadays, infertility is a complaint in about 15% of couples of reproductive age who intend to conceive (1). This phenomenon has multiple etiologies such as chromosomal abnormalities, genetic mutations, defects in reproductive organs, and environmental issues. Among these factors, extensive studies have evaluated the role of possible genetic mutations (2). Male factor infertility refers to infertility caused by abnormalities in the male

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reproductive system. This could be caused by a variety of factors including low sperm count, poor sperm motility, abnormal sperm morphology, hormonal imbalances, and genetic disorders (3), as well as secondary infertility (4). In general, more than 4000 genes have been shown to be involved in sperm production. Thus, any defect in any of these genes could disrupt spermatogenesis and possibly lead to infertility.

Leptin plays a crucial role in regulating energy balance and body weight produced by adipose tissue. On the other hand, leptin is essential to the regulation of reproductive (5).This peptide hormone binds to its receptor, leptin receptor, present in testicular tissue. Leptin has been shown to stimulate testosterone secretion in males and female sand affect sexual development and function (6,7).

Leptin receptors have several isoforms including a full-length OB-Rb form, OB-Ra, OB-Rc, OB-Rd, OB-Rf, and OB-Re short forms that are generated through alternative splicing and lack signal transduction capabilities (6,8). Previous reports indicated that the mRNA of *leptin receptor* (ObR) in adult rats was expressed in the Sertoli cells (9), while, the immune reactivity of this gene was observed in the Leydig cells of rats and the mice germ cells (10). Other *invitro* experiments based on human cells have shown that leptin receptors are present in testicular tissue and could inhibit the human chorionic gonadotropin (HCG)-stimulated testosterone secretion from rat Leydig cells (6,11). Moreover, leptin receptor was observed in pig spermatozoa and it has been proven important for fertility in boar (12). Although in humans, this receptor is present in the somniferous tubules (13), one report has indicated the gene to be observed in human seminal plasma and spermatozoa (14). Considering the highly conflicting reports on the presence of leptin receptor in human sperm; we also evaluated the presence of leptin receptor on an mRNA level in the blood and spermatozoa of fertile and infertile males.

Kisspeptin is a neuropeptide, encoded by the *KISS1* gene, which plays a crucial role in regulating the secretion of gonadotropins. The release of kisspeptin from neurons in the hypothalamus stimulates the secretion of gonadotropin-releasing hormone (GnRH) (15). Recent studies have shown that long non-coding RNAs (lncRNAs) and PIWI-interacting RNA (piRNA) play important roles in regulating spermatogenesis (16). Several lncRNAs, such as HOTAIR, have been implicated in the regulation of gene expression and chromatin modification during spermatogenesis. Evidence shows that lncRNA irregularities in sperm could be potential diagnostic and therapeutic targets for male infertility (17).

The aim of this study was to investigate the expression of *leptin*, *leptin receptor*, *KISS1*, and *HOTAIR* genes in oligospermia-infertile individuals and healthy controls.

Materials and Methods

Study design and participants

The sample collection was performed in the Motazedi Hospital, affiliated with Kermanshah University of Medical Sciences, Kermanshah, Iran. Patients and controls aged and weighed between 18-40 years and 65-90 kg, respectively, were enrolled in this study. Also, for the infertile group, oligospermia was defined as a sperm count of less than 15 million/mL. The exclusion criteria were male infertility which had only a child and normal sperm count. The current study was performed on 60 blood (30 oligospermia and 30 healthy controls) and 72 seminal fluid samples (36 oligospermia and 36 healthy controls). Some, but not all, of the blood and semen samples belonged to the same individuals. After the sample collection, sperm characteristics including motility, morphology, and count were evaluated. The aims and methods of the study were clearly explained to the participants according to their level of knowledge and they were asked to sign a consent form freely.

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RNA extraction and cDNA synthesis

Semen samples were prepared for RNA extraction by mixing with Hams F10 medium and centrifuging at 500×G for 14 minutes at 5 ºC. Additionally, peripheral vein blood was collected from the study subjects and stored in tubes containing EDTA in the refrigerator until further use. Total RNA was extracted from whole blood samples using the RNX PLUS RNA extraction kit (SinaClon Bioscience, Iran) according to the manufacturer's instructions. The Trizol kit was employed to extract RNA (TRIzol™ Reagent, Invitrogen, USA) following the manufacturer's guidelines. The concentration and purity of the extracted RNA were evaluated using the Nanodrop 2000 spectrophotometer (NanoDrop™ 2000/2000c Thermo Scientific, USA). The cDNA was then synthesized using the Parstous commercial kit (Parstous Biotechnology, Iran) following the manufacturer's instructions.

Primer design

The desired gene sequence and its related transcripts were obtained using NCBI and Ensemble databases. Then, a region of the gene related to the exon-exon connection was selected. It was also checked that the selected region is present in all gene transcripts. Primers for *leptin* (ACCESSION NM_ NM_000230.3), *leptin receptor (*ACCESSION NM_002303.6), *KISS1 (*NM_002256.4), *HOTAIR (*ACCESSION NR_186241.1), and *beta-actin* (housekeeping gene) were designed using an online software, oligoanalyzer (Table 1). The accuracy and specificity of primers were confirmed using the Basic Local Alignment Search Tool on the US National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Synthetizing of designed primers was performed by Sina Clone Company (Tehran, Iran).

Table 1. The sequences of used primers.

Gene	Primer sequence (5′ to 3′)	Length (bp)
Leptin	F- TTTCACACACGCAGTCAGTC R- CGTGAAGAAGATCCCGGAGG	195
Leptin receptor	F- ACACCAGAGTGATGCAGGTT R- CTCAAACGTTTCTGGCTTCTG	184
Kiss1	F- GCACTTCTAGGACCTGCCTC R- TGGGTGGCACAGAGGAAAAG	81
HOTAIR	F- AAGTGAAACCAGCCCTAGCC R- TAGAGTTGCTCTGTGCTGCC	131
Beta-actin	F- CACCATTGGCAATGAGCGGTTC R- AGGTCTTTGCGGATGTCCACGT	135

Real-time PCR

The expression levels of *leptin*, *leptin receptor*, *KISS1*, *HOTAIR*, and *beta-actin* genes were investigated using specific primers presented in Table 1. Real-time polymerase chain reaction (RT-PCR) analysis was performed in a total volume of 25 µL including 1 µL of cDNA, 0.5 mM of each forward and reverse primer, 12.5µL of qPCR Master (SYBR) (RealQ Plus 2x Master Mix Green, Amplicon, Denmark), and 9.5 µL of H2O. The PCR reactions were performed on the Light cycler 96 (*Roche*, Switzerland) using the thermal cycling parameters (95 °C 15 min, 40 cycles of 30 s at 95 °C, 30 s at 62 °C, 63 °C, 61.5 °C, and 62 °C for *Leptin*, *KISS1*, *HOTAIR*, and *Beta-actin*, respectively, 35 s at 72 °C). The relative gene expression was evaluated using the *Pfaffle* formula. The relative amount of target mRNA in samples was calculated and normalized to the corresponding Beta-actin mRNA transcript level as a housekeeping gene. The RT-PCR products were analyzed by 2% agarose gel electrophoresis. All experiments were performed in duplicates.

Statistical analysis

Statistical analysis and graph drawing were carried out using GraphPad Prisms® 6.0 (GraphPad Software, La Jolla, California,

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USA). The data of this study were analyzed and reported as mean±standard error of the mean (SEM). For this purpose, t-test, Spearman correlation coefficient, and simple linear regression were used. The data normality was assessed using the 1-sample Kolmogorov-Smirnov (K-S) test. Then, the Wilcoxon matched-pairs test was used to compare patients before and after treatment. Besides, the differences with *P* values<0.05 were considered to be statistically significant (*P< 0.05; **P< 0.01 ***P< 0.001; ****P< 0.0001).

Results

Relative mRNA expression level of Leptin, Leptin receptor, KISS1, and HOTAIR genes

The expression levels of the *leptin*, *leptin receptor*, *KISS1*, and *HOTAIR* genes in the semen and peripheral blood samples of fertile individuals (controls) and oligospermia cases were examined. According to the analyses, there was an increase in the expression level of the *leptin* gene in the peripheral blood of oligospermia groups when compared to the controls (P< 0.001, Fig. 1a). Also, the statistical examination revealed an increase in the expression of the *leptin* gene in the seminal fluid of oligospermia groups compared to the control group (P< 0.05, Fig. 2b). We also examined *KISS1* expression level in oligospermia groups in comparison to controls which revealed a reduction in the expression of the *KISS1* gene in the peripheral blood of the oligospermia, with no significant level (Fig. 1c). However, the expression of *KISS1* gene was significantly increased in the seminal fluid samples of individuals with oligospermia when compared to the control group (P< 0.0001) (Fig. 2c).

Furthermore, the expression level of the *HOTAIR* gene in oligospermia in comparison to the control group performed in this study. The results showed a significant reduction (P< 0.001) in the expression level of this gene in the peripheral blood of the oligospermia group in comparison to the controls (Fig. 1d). Additionally, there was a non-significant minor reduction in the expression level of the *HOTAIR* gene in the seminal fluid of the oligospermia group when compared to the control group (Fig. 2d).

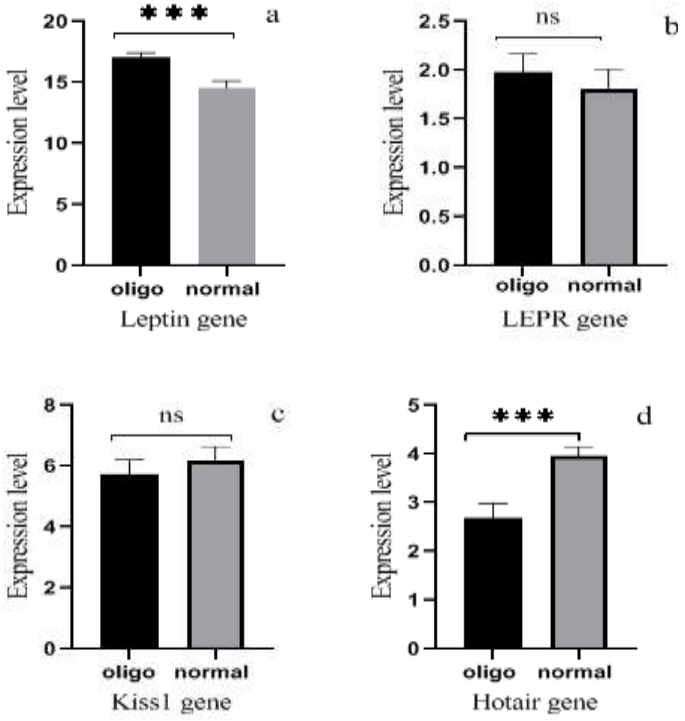

Fig. 1. Comparison of the expression levels of *Leptin*, *LEPR*, *KISS1*, and *HOTAIR* genes in the peripheral blood of the control (fertile) and oligospermia groups. The results are presented as mean ± standard deviation and a significance level of P< 0.05 is considered significant, while a P value greater than or equal to 0.05 is considered not significant (ns), *P< 0.05, ***P< 0.001.

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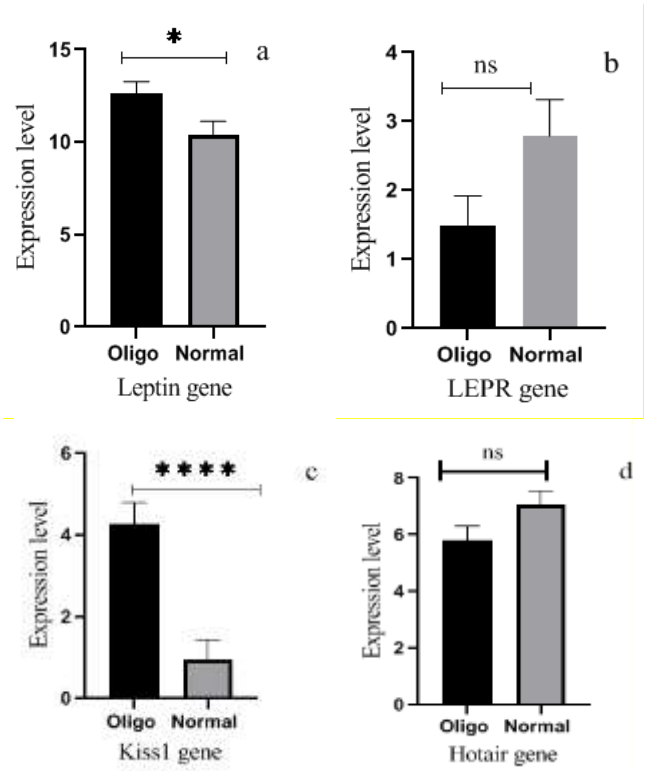

Fig. 2. Comparison of expression levels of the *Leptin*, *LEPR*, *KISS1*, and *HOTAIR* genes in the seminal fluid of a control (fertile) and an oligospermia groups. The results are presented as mean ± standard deviation and a significance level of P< 0.05 is considered significant, while a P value greater than or equal to 0.05 is considered not significant (ns), *P< 0.05, ****P< 0.0001.

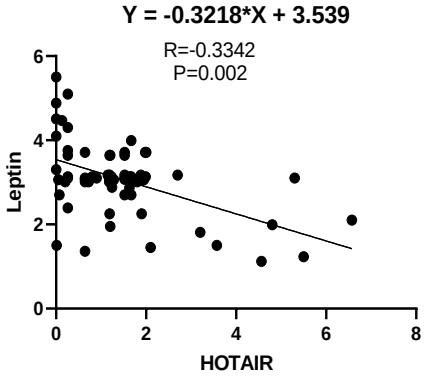

Fig. 3. Pearson's regression analysis reveals a significant negative correlation between the expression of Leptin and HOTAIR genes in seminal fluid. Significant differences are defined as *P*< 0.05. *P* stands for *P*-value, while R stands for Pearson's correlation coefficient.

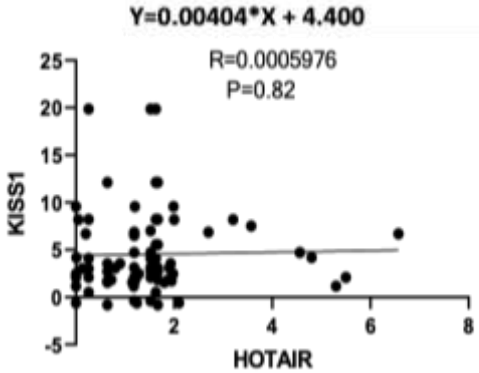

Fig 4. Pearson's regression analysis reveals a positive correlation between the expression of *KISS1* and *HOTAIR* genes in seminal fluid. Significant differences are defined as *P*< 0.05. *P* stands for *P*-value, while R stands for Pearson's correlation coefficient.

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Discussion

Herein, we examined the expression of *HOTAIR* gene as a lncRNA and its correlation with *leptin* and *KISS1* genes. The results showed that *leptin* gene expression was significantly higher in peripheral blood and semen samples of the oligospermic group than in those of the control group, consistent with previous studies on leptin levels in infertile individuals. In 2019, El Taiebet al. (18) conducted a study to examine the impact of varicocelectomy on leptin levels in the serum and semen of men with asthenozoospermia. This study involved 36 male subjects with varicocele and isolated asthenozoospermia, as well as 30 healthy fertile controls. The results showed that both serum and seminal *leptin* levels were higher in patients than in controls, and seminal leptin levels were positively associated with varicocele grade. Additionally, both serum and seminal leptin levels were negatively correlated with sperm motility. Our findings are consistent with previous research indicating increased *leptin* expression in the blood and semen of individuals experiencing infertility. In 2018, a study from Pakistan conducted on 178 infertile and 135 fertile individuals to examine some genetic affecting factors (19). Although their results are in line with ours; they observed no significant difference in serum *leptin* levels between infertile and control groups. Previous studies and current research suggest that *leptin* expression is increased in infertile individuals, particularly in their peripheral blood.

We also found a significant decrease in the expression of *KISS1* gene, particularly in semen samples, compared to the control group ($P< 0.0001$). This finding is consistent with previous studies that have also reported a reduced KISS1 expression in infertility disorders. For instance, Al-Kalabi et al. (20) found lower levels of kisspeptin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin in the serum of infertile individuals compared to the control group. Also, Rehman et al. (21) reported higher levels of kisspeptin in fertile men compared to infertile men. Another study by Minabe et al. in 2019 (22) investigated the effect of *KISS1* on infertility using an animal model. They compared the expression levels of *KISS1* in a group of infertile mice with a healthy group using Real-Time PCR. The results showed a lower expression level of *KISS1* in the infertile group. Our study, along with others, indicates that *KISS1* expression is reduced in individuals with oligospermia-infertility. Additionally, research suggests that when kisspeptin binds to KISS1R on the membrane of sperm cells, it can trigger signals for maturation and enhances sperm motility, ultimately transforming immature sperm into mature sperm (23). Consequently, it is plausible to link a decreased *KISS1* expression to infertility disorders given such suggested functions.

This study examined the expression of *HOTAIR* gene, along with *leptin* and *KISS1* genes, in oligospermic infertile individuals. The results showed a decreased expression of the *HOTAIR* gene in both blood and seminal fluid samples of infertile individuals compared to controls, with a significant decrease in peripheral blood samples ($P< 0.001$). This is consistent with previous studies that have also shown a decrease in *HOTAIR* expression in infertile individuals (17,24).

Additionally, our study found a possible correlation between the expression of *HOTAIR* and *leptin* and *KISS1* genes. A significant negative correlation was observed between *HOTAIR* and *leptin* ($P< 0.05$), and a positive correlation was observed between *HOTAIR* and *KISS1* in seminal fluid samples; however, it did not reach statistical significance. Also, there was a statistically significant negative correlation between the expression of *leptin* and *leptin receptor*, in seminal fluids of oligospermic patients ($P< 0.05$). It is noteworthy to mention that this study is the first to investigate these correlations, and further studies are necessary to determine the exact mechanism of the relationship found between the expression of *HOTAIR* and the two other genes. Overall, our study provides

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new insights into the potential role of *HOTAIR* in male infertility and emphasizes the need for additional research to fully comprehend its mechanisms.

Similar to any other study, this one is not without limitation. The main limitation of this work was the low sample size which could be addressed by future studies. Also, no measurement of the gene products in sera and semen seems and their comparison with the expression pattern is another limitation. Of the strength points of the current research is the evaluation of various related genes such as *leptin* and its receptor.

Herein, we found a reduction in the expression of the *leptin* gene in infertile male semen samples of Kermanshah province. We also found a significant decrease in the expression of the *KISS1* gene, particularly in semen samples. Moreover, the study discovered a significant reduction in the expression of *HOTAIR* gene in infertile males, particularly in blood samples. Additionally, there may be a potential correlation between the expression of *HOTAIR* and the *leptin* and *KISS1* genes. Further research is required to fully comprehend the mechanisms underlying the connection between *HOTAIR* and the other two genes. These findings support previous research on decreased *HOTAIR* expression in infertile individuals and emphasize the potential role of lncRNAs in male infertility.

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Conflict of interest

The authors declare that they have no conflict of interest. All authors have read and agreed to the published version of the manuscript.

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Approval for the study was obtained from the Medical Ethics Committee of the Kermanshah University of Medical Sciences with the IRB number of IR.KUMS.REC.1398.252.

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