Research Article

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Ghrelin and orexin levels in infertile male: evaluation of effects on varicocele pathophysiology, relationship seminal and hormonal parameter

[İNFERTİL ERKEKTE GHRELİN VE OREKSİN DÜZEYLERİ: Varikosel patofizyolojisi, seminal ve hormonal parametre ilişkisi üzerine etkilerinin değerlendirilmesi]

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Abstract

Objective: The aim of the present study was to investigate serum ghrelin and orexin levels in patients with varicocele and compare these levels with idiopathic infertile male and healthy control cases.

Methods: This study enrolled 24 men with varicocele, 24 males having idiopathic infertility, and 21 fertile men as the control group. Hormonal analyses, ghrelin and orexin levels were measured samples. Semen was analyzed after 3 and 5 days of sexual abstinence.

Results: Serum ghrelin levels were statistically different among the three groups ($p=0.015$), and it was due to a statistically lower level in group-1 than the level in the control cases ($p=0.012$). On the other hand, serum orexin

levels were lower than healthy subjects in infertile groups with/without varicocele, but there was no difference ($p=0.685$) among three groups. Serum ghrelin level showed a negative and significant correlation only with sperm motility ($r=-0.646$, $p=0.022$), there was no correlation with other parameters. On the other hand, serum orexin levels did not show a significant correlation with seminal parameters.

Conclusion: Both new investigated peptides ghrelin and orexin have regulatory effects on testicular function. However, ghrelin has a more obvious and complex effect on spermatogenesis. Impaired seminal parameters, especially motility was associated with increased serum ghrelin levels in infertile patients, especially with varicocele.

Keywords: ghrelin; infertility; orexin; semen analysis; varicocele.

ÖZ

Amaç: Bu çalışmanın amacı varikoseli olan hastalarda serum ghrelin ve oreksin düzeylerini incelemek ve bu düzeyleri idiyopatik infertil erkek ve sağlıklı kontrol vakaları ile karşılaştırmaktır.

Yöntemler: Bu çalışmaya 24 varikoselli erkek, idiyopatik infertiliteye sahip 24 erkek ve kontrol grubu olarak 21 fertil erkek alındı. Hormonal analizler, ghrelin ve oreksin seviyeleri ölçüldü. Üç ile beş günlük cinsel perhiz sonrası ardışık iki semen analizi ile sperm parametreleri değerlendirildi.

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Bulgular: Serum ghrelin düzeyleri üç grup arasında istatistiksel olarak farklıydı ($p = 0.015$) ve bu durum grup-1’de kontrol olgularından daha düşük istatistiksel değerde olmasına bağlıydı ($p = 0.012$). Diğer taraftan, varikoseli olan/olmayan infertil gruplarda serum oreksin düzeyleri sağlıklı bireylere göre daha düşüktü, ancak üç grup arasında fark yoktu ($p = 0.685$). Serum ghrelin düzeyi sadece sperm motilitesi ile negatif ve anlamlı korelasyon gösterdi ($r = -0.646$, $p = 0.022$), diğer parametrelerle korelasyon izlenmedi. Bununla birlikte, serum oreksin seviyeleri seminal parametrelerle anlamlı bir korelasyon göstermedi.

Sonuç: Yeni araştırılan peptidler olan ghrelin ve oreksin, testis fonksiyonu üzerinde düzenleyici etkilere sahiptir. Bununla birlikte ghrelinin spermatogenez üzerinde daha belirgin ve karmaşık bir etkisi vardır. Bozulmuş seminal parametreler, özellikle motilite, infertil hastalarda, özellikle varikoselde artmış serum ghrelin seviyeleri ile ilişkilidir.

Introduction

Varicocele is the most common reason for surgically treated problems in infertile males. While it has been reported around 35–40% of infertile men, it is observed around 15% in healthy population and is also reported around 14–20% in adolescent young boys [1]. Varicocele affect testicular spermatogenesis in testis with different pathophysiologic mechanisms. The exact mechanism is not still clear in varicocele physiopathology and there is some unexplained mechanism such as increased scrotal hotness, toxic effects of renal and adrenal metabolites on testis, diminished arterial inflow, testicular hypoxia and toxic effects of increased reactive oxidation substances [2–3]. However, there are still some undefined pathophysiological mechanisms of varicocele. Also, because, 40–50% of patients that underwent surgical treatment, their sperm parameters didn’t improve after the operation. This likely suggests that autocrine and paracrine mediators except knowing mechanisms mentioned before might also play an important role in the pathophysiology of varicocele [1, 4].

Ghrelin is a 28-amino acid peptide that had recently been identified. It is mainly secreted from the hypothalamus and stomach [5, 6]. However, Ghrelin has some central neuroendocrine effects and affects GH release [7–9]. These effects were observed in anterior pituitary cells in culture [5, 7, 8]. In addition to these central biological effects, ghrelin expression has been reported in peripheric tissues such as ovary, kidney and testis [10–12]. While testicular function is mainly regulated by the hypothalamic-pituitary

axis, paracrine and autocrine regulatory signals are also playing an important role in testicular function, ghrelin expression was observed in Leydig and Sertoli cells in recent studies [13–15].

Orexin described firstly in rat brain extract is another hypothalamic peptide and there are two subtypes: Hyporectin-1 (Orexin-B) including 33-amino acids and Hyporectin-2 including 28-1 amino acids (Orexin A). Later, receptors for these two peptides were referred to as OX1R and OX2R in CNS and peripheric tissues including also human testis [16–18].

In the literature, while there have been limited studies about the testicular activity of these two peptides, no studies exist about their role and activity in varicocele. Additionally, there are no studies about the serum levels of the two neuropeptides in varicocele. The present study aimed to evaluate serum ghrelin and orexin levels in patients with varicocele and compare these levels with the ones observed in idiopathic infertile male and healthy control cases. Moreover, the relationships among serum ghrelin, orexin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, total testosterone (TT), estradiol (E_2) levels and semen analysis were measured. Ultimately, the role of ghrelin and orexin in varicocele pathophysiology was discussed.

Material and methods

Each patient who suffered from infertility was evaluated in the Andrology outpatient with a detailed history and thorough physical examination by the same physician. The blood is taken from hormonal analysis including gonadotropins (FSH and LH), prolactin, TT and E_2 from the patients between 8 and 10 a.m. Hormonal analyses were performed on Roche Modular Analytics E 170 immunoassay analyzer. After centrifugation, half of the serum was separated and kept at -80°C for ghrelin and orexin analysis at the end of the study.

Two consequent semen analyses within 3 weeks had been performed with 3 to 5-days of sexual abstinence period and evaluated according to WHO 2010 guidelines criteria [19]. Sperm morphology had been evaluated by using SpermMac stain (Ferti Net NV, Belgium) according to the Kruger’s strict criteria. If there were no spermatozoa in basal semen analysis, gradient method was applied for further evaluation.

The patients having any scrotal pathology, previous history of any kind of scrotal surgery such as orchidopexy or previous varicocelectomy, taking any kind of hormonal treatment in the last 6 months, having abnormal serum hormone levels, abnormal liver and renal function tests, obstructive azoospermic male, having any systemic disease and overweight participants ($\text{BMI} \geq 29.9 \text{ kg/m}^2$) were excluded from the study.

Finally, a total of 24 adult men with varicocele, aged between 22 and 32 years (mean 27.8 ± 2.5 years) joined up the study (Group-1). Additionally, 24 infertile males in the same age groups without

varicocele (20 and 38 years old; mean age 29.1 ± 4.5 years) applied to Andrology outpatient were selected for the idiopathic male infertility group according to same exclusion criteria (without having any diseases related to infertility or scrotal pathology) (Group-2). A total of 21 fertile cases (fathered after spontaneous pregnancy) admitted to the Urology outpatient clinic with different urological problems were enrolled in the study as the control group (Group-3). Additionally, the same exclusion criteria were also considered for these patients. The mean age of this group was 30.9 ± 6.9 years (20–47 years). The same laboratory analyses were performed in these cases.

Varicocele grade was first evaluated on an outpatient basis by physical examination; then it was confirmed by Doppler ultrasonography. Varicocele scale was made according to the Dubin classification. According to this classification, there are 4 grade: *Grade-0*: Moderate, transient reflux during the Valsalva maneuver (physiologic findings); *Grade-1*: Persistent venous reflux that ends before the Valsalva maneuver is completed. Venous diameter is less than 3 mm; *Grade-2*: Persistent venous reflux throughout the entire Valsalva maneuver. Venous diameter is higher than 3 mm; *Grade-3*: Venous reflux is present under basal conditions and does not change during the Valsalva maneuver. The venous diameter is higher than 4 mm.

We used enzyme-linked immunosorbent assay (ELISA) for measuring serum orexin and ghrelin levels (Phoenix Pharmaceuticals, Inc. Germany). The calibration curves were prepared with orexin and ghrelin standards at between 0.01 and 100 ng/mL and the results were given as ng/mL.

Statistical analysis was performed by using SPSS 8.0 (Statistical Package for Social Sciences for Windows; Chicago, IL, USA). To evaluate the difference and compare age, seminal parameters, hormone levels, one-way ANOVA Post hoc Bonferroni tests were used. P-value < 0.016 was considered statistically meaningful. Later, the relationship between compared parameters were evaluated in all infertile males, in addition to infertility due to varicocele by using Pearson correlation and Spearman correlation for varicocele grade and peptide levels, separately. If p value was less than 0.05, it was accepted as statistically significant.

Results

The patient's data is given in Table 1. The mean age was not statistically different among the three groups

($p=0.108$). There were statistically significant differences in all seminal parameters, except ejaculate volume among the three groups. The differences in seminal parameters were due to higher values in control cases than others. In one-way ANOVA Post hoc Bonferroni test, the difference between the sperm parameters in fertile group were higher than other infertile patients regarding the sperm counts since the parameters, such as sperm counts (Post hoc Bonferroni $p_{\text{fertile}\&\text{infertile}(+)}=0.001$ and $p_{\text{fertile}\&\text{infertile}(-)}=0.023$), motility (Post hoc Bonferroni $p_{\text{fertile}\&\text{infertile}(+)}<0.001$ and $p_{\text{fertile}\&\text{infertile}(-)}<0.001$) and morphology (Post hoc Bonferroni $p_{\text{fertile}\&\text{infertile}(+)}<0.001$ and $p_{\text{fertile}\&\text{infertile}(-)}<0.001$), in the fertile group with or without varicocele were different. However, serum hormone levels were not different.

Although the lowest serum ghrelin level was observed in varicocele cases, there was not any significant difference among the three groups ($p=0.119$). Additionally, despite the higher orexin levels in infertile patients, there was no statistical difference in serum orexin levels among the three groups ($p=0.243$) (Figure 1).

Figure 1: Serum ghrelin and orexin levels in groups.

Table 1: Patients' age, seminal parameters, serum hormone, ghreline and orexine levels in groups.

	Group-1 (n=24)	Group-2 (n=24)	Group-3 (n=21)	p-Value
Age, Year	27.8 ± 2.5 (22–32)	29.1 ± 4.5 (20–38)	32.7 ± 7.3 (20–47)	0.108
Ejaculate volume, mL	3.7 ± 1.6 (1.5–7)	3.8 ± 1.8 (1.5–9)	3.8 ± 1.3 (2–6.2)	0.950
Spermatozoa count, $\times 10^6$ /mL	20.3 ± 16.4 (0–55)	29.2 ± 21.1 (0–93)	69.9 ± 29.6 (24–120)	0.001*
Motility, %	22.6 ± 16.4 (0–50)	32.3 ± 19.5 (0–58)	76.1 ± 10.2 (60–95)	0.001*
Morphology, %	2.2 ± 1.8 (0–6)	2.6 ± 2.5 (0–8)	10.3 ± 3.7 (2–18)	0.001*
FSH, mIU/mL	6.3 ± 1.2 (2.9–21.9)	4.7 ± 3.4 (1.5–16.5)	4.1 ± 1.13 (1.9–16.6)	0.509
LH, mIU/mL	5.4 ± 3.2 (1.1–15.1)	4.9 ± 2.1 (1.9–10.6)	5.2 ± 1.9 (1.9–8.9)	0.705
Prolactin, ng/mL	15.1 ± 3.0 (3.2–60.0)	12.1 ± 4.5 (5.9–24.1)	10.2 ± 8.4 (4.8–24.1)	0.262
Total testosterone, ng/mL	4.7 ± 1.74 (3.0–6.7)	5.4 ± 0.6 (2.1–5.3)	5.8 ± 1.3 (3.8–7.1)	0.643
Estradiol, pg/mL	28.1 ± 10.1 (11.9–46.2)	22.8 ± 6.8 (5.2–34.1)	21.8 ± 7.5 (11.7–43.8)	0.034
Ghrelin, ng/mL	2.8 ± 1.9 (0.3–8.6)	3.8 ± 1.7 (0.4–14.4)	4.3 ± 3.9 (0.3–15.6)	0.119
Orexin, ng/mL	0.5 ± 0.2 (0.2–0.8)	0.6 ± 0.3 (0.1–1.4)	0.5 ± 0.3 (0.1–1.2)	0.243

Mean $\pm$ SD; min–max values, *p Oneway ANOVA test, $p < 0.016$.

In Pearson correlation analysis, both peptides showed positive insignificant correlation with age ($r_{\text{ghrelin\&age}}=0.136$, $p=0.279$; $r_{\text{orexin\&age}}=0.004$, $p=0.976$). Additionally, while ghrelin showed a positive correlation with whole seminal parameters, orexin showed a negative correlation. However, none of them were statistically meaningful ($p>0.05$). Based on serum hormone levels, both peptides showed a positive correlation with serum hormone levels, but none of them were statistically meaningful ($p>0.05$).

When the correlation test was applied only in the varicocele group, unlike previous analyzes, both peptides showed negative, but insignificant correlations with sperm motility and morphology in these cases. Additionally, there was no difference between correlations in terms of serum hormone levels in varicocele cases.

Discussion

The present study shows that several autocrine factors have different effects on spermatogenesis and hormonal mechanisms in varicocele physiopathology. As well as the known effects of varicocele, it is thought that varicocele plays an important role in spermatogenesis through autocrine factors.

While varicocele is one of the most frequent reasons of male infertility, its' effects on spermatogenesis and testicular function have not been clear. There are many different physiopathological mechanisms have been accused mentioned before [2, 3, 20]. However, it has been suggested that there have been some indefinite factors that affect spermatogenesis in patients with varicocele. The effect of varicocele on hormonal mechanism factors was demonstrated in animal studies. In an experimental study, it was shown that intratesticular testosterone levels decreased, whereas serum testosterone levels did not change [20–22]. Nevertheless, there are also different thoughts on this issue [23–25]. In our study, serum testosterone levels were detected to be lower. Conversely, there was not statistically significant difference. However, we thought that experimental and human studies results may be show different results, and there may be different factors affecting extra and intratesticular testosterone levels.

In animal studies, ghrelin expression was observed in Leydig cells, and it encouraged the inhibition of human-CG and cAMP stimulated testosterone secretion. Therefore, it was suggested that ghrelin might also join in the regulation of testicular function [12, 13]. Then, Barreiro et al. showed ghrelin receptors were not observed in the testicle before puberty but increased significantly post-pubertal period [26]. On the contrary, ghrelin receptors were identified in an

isolated seminiferous tubule in adult testis. These receptors' immunoreactivity increased after FSH stimulation. Therefore, these findings suggest that ghrelin regulates spermatogenesis in testis with different pathways. In the present study, we found a positive correlation between seminal parameters and serum ghrelin levels in all subjects. However, there were an inverse correlation among sperm motility, morphology and ghrelin levels in varicocele subjects. This finding suggested that ghrelin has a dual effect on spermatogenesis.

The relationship between serum ghrelin and sex steroid levels were described in women having polycystic ovary syndrome by Pagotto firstly [27]. Then, they reported lower serum ghrelin levels increased with androgen replacement therapy in hypogonadal men [28]. These findings suggested that ghrelin might play a regulatory role in the reproductive system. Recently, Ishikawa et al. had investigated the relationship between ghrelin expression in testis and serum testosterone levels, and they found that ghrelin expression was contrariwise correlated with serum testosterone levels [29]. Thus, they concluded that hormonal dysfunction was associated with increased ghrelin expression. Also, in our study, serum ghrelin levels showed a positive correlation with serum testosterone levels in all cases. Additionally, the same findings were observed in patients with varicocele. So, we thought that ghrelin plays a role in testosterone secretion.

In the literature, there are several studies concerning the effects of orexin on the hypothalamic-pituitary axis [18, 30–32]. In experimental studies, it was shown that orexin inhibits the pulsatile LH secretion [20, 30]. Orexin receptors were in the male genital tract including epididymis, penis, seminal vesicle and testis [26, 33, 34]. The authors concluded that all of them had different effects based on their localization. In testis, these receptors might play a guiding in Sertoli cells and sperm DNA structure. However, there is no clear data about the relationship between spermatogenesis and serum steroid hormone levels and orexin in the literature. Therefore, this study is the first study on this issue. We found a higher orexin level in infertile cases than controls, but it did not show statistical significance. Additionally, serum orexin levels showed a negative correlation between seminal parameters and positive correlation with serum hormone levels. Moreover, its' correlation was the same in varicocele cases.

In conclusion, while varicocele has been accepted as one of the most common and treatable causes of male infertility, it has a lot of undefined pathophysiological mechanisms. Its effect on testicular function, not only due to increased scrotal temperature and reflux of toxic metabolites but also some autocrine and paracrine factors,

might be involved. Both newly investigated peptides ghrelin and orexin have regulatory effects on testicular function. While both increase spermatozoa number and motility in male testis, serum ghrelin levels decreased in infertile males and this condition was between decreased seminal parameters, especially in having varicocele. Thus, these findings suggest that ghrelin has some unexplained effects. Further studies will help to reveal the exact mechanisms of interaction between ghrelin activity and the pathophysiology of varicocele and idiopathic infertility.

Ethical considerations

In this study, all procedures involving human participants were arranged in accordance with the ethical standards of the Local Ethics Committee and the Helsinki Declaration of 1964 and subsequent amendments or comparable ethical standards.

This study was approved by the University of Kırıkkale, Faculty of Medicine Local Ethics Committee (2006/046). All patients included in the study were given detailed information about the study and the “Informed Consent Form” was signed by all participants.

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