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Original Article

The ghrelin and orexin activity in testicular tissues of patients with idiopathic non-obstructive azoospermia

Seyma Ozkanli [a,*], Mehmet Murad Basar [b], Serkan Selimoglu [c], Bulent Erol [d], Oguz Ozkanli [e], Fuad Nurili [f], Semra Kahraman [g]

[a] *Department of Pathology, Faculty of Medicine, Istanbul Medeniyet University, Istanbul, Turkey*
[b] *Department of Urology, Memorial Sisli Hospital, Istanbul, Turkey*
[c] *Andrology Laboratory, Memorial Sisli Hospital, Istanbul, Turkey*
[d] *Department of Urology, Faculty of Medicine, Istanbul Medeniyet University, Istanbul, Turkey*
[e] *Department of Anesthesiology, Memorial Sisli Hospital, Istanbul, Turkey*
[f] *Department of Radiology, Memorial Sloan Kettering Cancer Center, USA*
[g] *Department of IVF, Memorial Sisli Hospital, Istanbul, Turkey*

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Abstract The aim of the present study is to evaluate the presence of ghrelin and orexin in the testicular tissue of patients who have undergone microscopic testicular sperm extraction (micro-TESE) due to idiopathic non-obstructive azoospermia. Seventy azoospermic cases were included in this study; serum hormone levels were measured and genetic investigations were performed. The patients were divided into two groups: micro-TESE (+) and micro-TESE (−). The number of Leydig cells and stained cells in the seminiferous tubules were counted under a light microscope, and we analyzed ghrelin and orexin activity. The relationship between serum hormone levels and ghrelin and orexin distributions in testicular tissue was evaluated according to micro-TESE results. While sperm was found in 33 cases (47.1%), micro-TESE was negative in 37 cases (52.9%). Peptide hormone activity in testicular tissue was higher in micro-TESE (+) cases. However, interstitial orexin ($p = 0.038$) and ghrelin ($p = 0.002$) activity showed statistically meaningful differences. Many different peptides, genes, and other

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* Corresponding author. Tütüncü Mehmet Efendi Cad. Karanfil Sokak.Uğur Apt. No: 16 D: 3 Göztepe, İstanbul 34730, Turkey.
E-mail address: seymaozkanli@gmail.com (S. Ozkanli).

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unknown mechanisms play important roles in testicular function. In particular, the peptides orexin and ghrelin may play regulatory roles in testicular function in humans.

Introduction

Spermatogenesis is a complex process controlled by hormonal and genetic factors. While follicle stimulating hormone (FSH) and testosterone are well-known regulators of sperm production, it has been suggested that several other paracrine and genetic factors can also affect spermatogenetic activity.

Ghrelin, a 28-amino-acid peptide, is primarily expressed in the hypothalamus and has been found to affect growth-hormone secretion [1,2]. It has also been observed to play important roles in the stimulation of food intake and the development of adipose tissue, with central neuroendocrine activity [3–5]. Recently, ghrelin expression and activity have been described in experimental and human studies [6–8]. Ghrelin expression has been shown in Leydig cells *in vitro*, and it has been reported that ghrelin activity in testicular tissue is inversely correlated with testosterone levels [9]. Although it has been suggested that ghrelin is an endocrine, paracrine, and autocrine regulatory substance in human testes, there are no studies on ghrelin activity and spermatogenesis in the literature.

Orexins, which are hypothalamic neuropeptides, were first identified in the lateral hypothalamic area and found to be related to feeding behavior [10,11]. Two different orexins are derived from the 130-amino-acid prepro-orexin: orexin A (Ox-A), with 33 amino acids, and orexin-B (Ox-B), with 28. Both affect their own specific receptors, called OX1R and OX2R, respectively. In experimental studies, OX1R has been described in the genitourinary system, especially in Leydig and Sertoli cells, seminiferous tubules, epididymis, and vesicle seminalis [12–14]. However, the relationship between orexin expression and steroidogenesis/spermatogenesis in humans is unknown.

The aim of the present study is to evaluate the presence of ghrelin and orexin activity in the testicular tissues of patients who have undergone microscopic testicular sperm extraction (micro-TESE) procedures due to idiopathic non-obstructive azoospermia (NOA). Ghrelin and orexin expressions were investigated in different parts of human testicular tissue, including the Leydig cells, Sertoli cells, and seminiferous tubules. Additionally, their relationships with spermatogenetic activity and their correlations with serum gonadotropins and sex steroid levels were examined.

Material and methods

The Local Ethics Committee approved all procedures, and all subjects provided written informed consent before being enrolled in the study. A total of 242 idiopathic NOA patients were evaluated with detailed anamnesis and physical examinations. Serum hormone levels, including FSH, luteinizing hormone (LH), total testosterone, and estradiol (E_2), were measured. Peripheral karyotype and chromosome Y micro-deletions were evaluated in all cases. Patients with hypogonadotropic hypogonadism, a history of cryptorchidism/orchidopexy, mumps orchids, non-specific orchids, radiotherapy/chemotherapy, or any type of genetic abnormality were excluded from enrollment. Therefore, a total of 70 cases were included in the study.

Micro-TESE and sperm retrieval

Micro-TESE was performed under general anesthesia using an operating microscope. Microdissection was performed according to the previously described technique of Schlegel [15]. Seminiferous tubules were examined under the operating microscope (25 × magnification) after longitudinal tunical incision. Enlarged tubules were dissected and removed, then minced in sperm-wash solution in a petri dish. A droplet of tissue suspension was placed on a glass slide and investigated under a light microscope at 400 × magnification. This procedure was repeated on both testicles until spermatozoa were detected. The tunica albuginea was closed with 5/0 polypropylene, and the scrotum was closed with 4/0 Vicryl. Preoperative antibiotic prophylaxis was administered in all cases.

If no spermatozoa were observed during the operation, testicular samples were prepared using a mini-gradient method and collagenase was performed for 1 h. Later, all samples were placed in ICSI dishes for spermatozoa collection. Based on micro-TESE results, the patients were divided into two groups: micro-TESE positive (+) and micro-TESE negative (−).

Histopathology and orexin/ghrelin staining

The specimens were fixed with Bouin's solution for 12 h at room temperature. The tissues were prepared in an automatic tissue processor (Shandon Excelsior ES, Thermo Scientific, Cheshire, UK) using ascending concentrations of formaldehyde, ethanol, xylene, and paraffin wax. The tissues were evaluated histochemically with hematoxylin and eosin (H&E) staining and immunohistochemically using ghrelin and OX antibodies. Sections of 3 μm were taken from paraffin blocks that were previously coated with poly-L-lysine. Specimen slides were then deparaffinized with xylene for 10 min. Hydrogen peroxide, phosphate-buffered saline (PBS), and a nonspecific blocking reagent (Ultra V block: ScyTek Laboratories, Logan, Utah, USA) were applied as antigen retrieval solutions. Rabbit anti-human ghrelin (polyclonal) (PHOENIX, Burlingame, CA, USA) and rabbit anti-human OX-A (polyclonal) (PHOENIX) antibodies were then localized at room temperature for approximately

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Table 1 Demographic data and serum hormone levels in patients according to the Micro TeSe results, *p < 0.05.

	Micro TeSe (+) (n = 33)	Micro TeSe (−) (n = 37)	P value
Age (year)	35.1 ± 2.65	34.9 ± 5.87	0.689
Infertility Time (year)	6.9 ± 1.92	7.8 ± 1.06	0.160
FSH (mIU/ml)	21.1 ± 4.23	20.5 ± 17.60	0.754
LH (mIU/ml)	14.5 ± 9.86	13.7 ± 8.42	0.761
Total testosterone (ng/ml)	4.5 ± 2.5	4.7 ± 3.30	0.387
E_2 (pg/ml)	33.6 ± 27.89	33.6 ± 26.42	0.991

90 min at dilutions of 1:400 and 1:200, respectively. After incubation with the primary antibodies, the slides were incubated with Ultra Tek anti-polyvalent biotinylated antibody (ScyTek Laboratories, Logan, UT, USA) as the secondary antibody for 15 min. After rehydration, Ultra Tek HRP (ScyTek Laboratories) was added. After rehydration with PBS, the DAB chromogen system (DAB substrate kit, ScyTek Laboratories) was added. The slides were counterstained with hematoxylin and the immunohistochemically stained sections were examined under a light microscope (Olympus BX-51, Tokyo, Japan). Cytoplasmic and nuclear staining was accepted as positive. The findings were evaluated via quantitative morphometric analysis. In the evaluation of all testicular biopsies, 10–15 areas were evaluated under 40 × high-power. In these areas, the number of ghrelin-positive Leydig cells for every 50 Leydig cells were evaluated. The seminiferous tubules were also examined at 40 × high-power. All ghrelin- and orexin-positive cells per 20 seminiferous tubules were counted.

Statistical analysis

Statistical analyses were performed with SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was applied to evaluate the distribution of the data, then the Mann–Whitney test for non-normally distributed data and the independent *t*-test for normally disturbed data were used to compare the groups. The Pearson correlation test was used for the evaluation of the relationships between peptide immunoactivity and serum hormone levels. All data were expressed as mean ± standard deviation (SD). P-values of <0.05 were accepted as statistically meaningful.

Results

While spermatozoa were found in 33 cases (47.1%), micro-TESE was negative in 37 cases (52.9%). The patients' ages, length of infertility, and serum hormone levels are shown in Table 1. No significant differences were observed among these parameters based on micro-TESE results.

The ratios of ghrelin- and orexin-immunostained Leydig cells and seminiferous tubules are shown in Table 2. While ghrelin immunoreactivity was predominantly observed in Leydig cells within the testicular interstitium, slight activity was also observed in the seminiferous epithelium (Fig. 1a and b). The same findings were observed for orexin immunoreactivity (Fig. 2a and b). Both types of peptide hormone activity in the testicular tissue higher in micro-TESE (+) cases; however, only the interstitial activities of these two peptides showed statistically significant differences.

Serum testosterone levels showed positive correlations with the immunoreactivities of these two peptides in testicular tissue; however, there were negative correlations with serum E_2 levels. In contrast, while gonadotropin levels showed positive correlations with testicular orexin activity in both localizations, there were negative correlations with ghrelin activity (Table 3).

Discussion

This study showed that steroidogenesis and spermatogenesis are related not only to hormonal regulation but also to certain autocrine mediators in the testes. Ghrelin expression has also been described in the Leydig and Sertoli cells of human testes [8,9,16]. The authors have concluded that this plays a role in cellular differentiation and also testosterone secretion via cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) stimulation. In previous studies, ghrelin expression was found to be inversely correlated with serum testosterone levels in patients with normozoospermia. This condition can be explained by receptor activity [7,17]. It was concluded that over-expressed ghrelin could lead to inhibition of testosterone production in the human testes. The same finding was observed by Ishikawa et al. [9], who did not find any

Table 2 The ratio of the number of ghrelin and orexin immunostained testicular structures according to the Micro TeSe groups, *p < 0.05.

	Micro TeSe (+) (n = 33)	Micro TeSe (−) (n = 37)	p value
Ghrelin Interstitial Tissue (%)	45.9 ± 3.801	33.4 ± 3.66	0.002*
Ghrelin Seminiferous Tubules (%)	25.9 ± 3.80	22.7 ± 5.50	0.634
Orexin Interstitial Tissue (%)	21.1 ± 2.84	13.3 ± 2.39	0.038*
Orexin Seminiferous Tubules (%)	2 ± 1.17	0.83 ± 0.45	0.323

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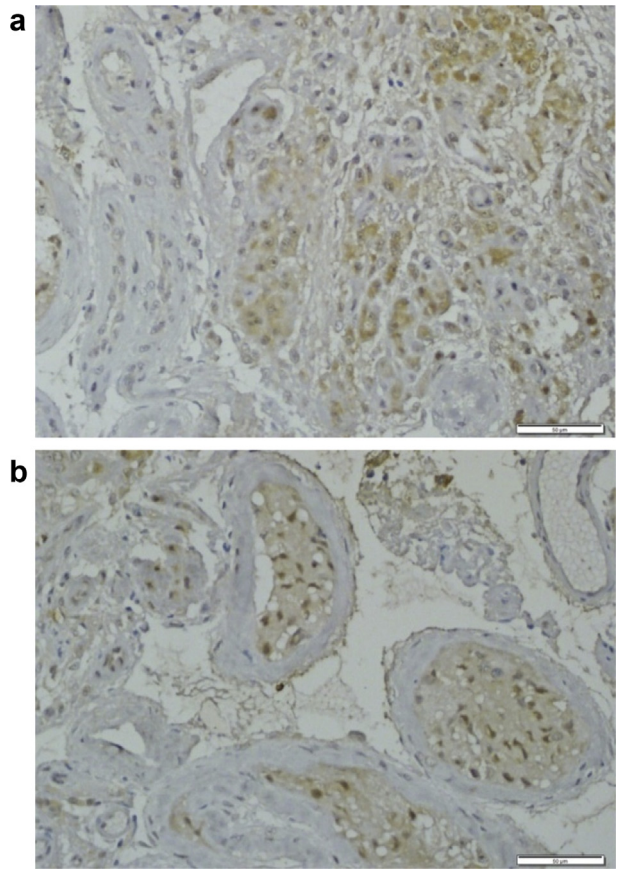

Figure 1. Positive Ghrelin immunreactivity: (a) Leydig cells (Ghrelin x 40); (b) germ cells in seminiferous tubulus (Ghrelin X40).

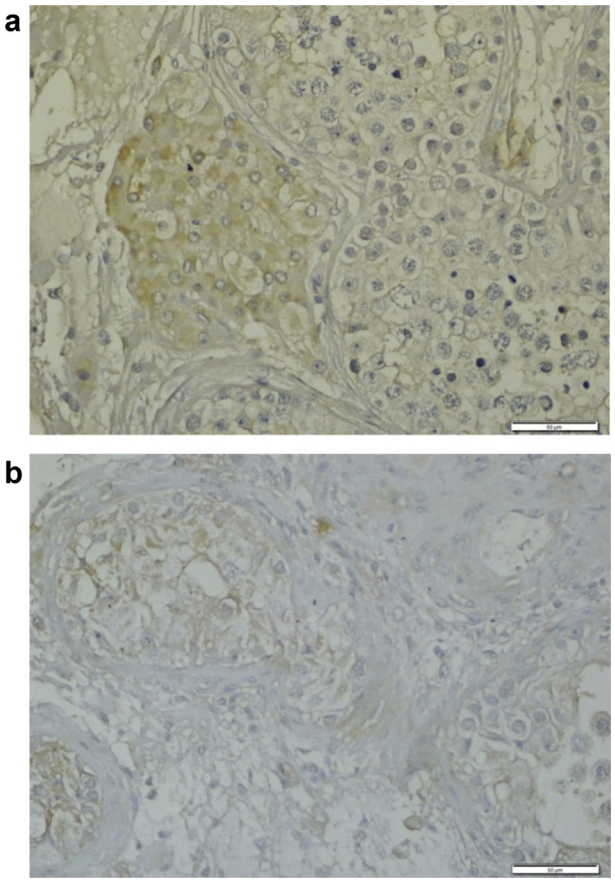

Figure 2. Positive Orexin-A cytoplasmic immunreactivity: (a) Leydig cells (Orexin-A x 40); (b)the germ cells in seminiferous tubulus (Orexin-A x 40).

correlation between ghrelin expression in Leydig cells and spermatogenetic activity in patients with normal spermatogenesis; however, there was an inverse correlation between ghrelin expression and testosterone levels. Therefore, they concluded that ghrelin had an indirect effect on spermatogenesis. In a recent study, Zhu et al. investigated ghrelin expression in ob/ob mice and its effects on sperm production [18]; ghrelin and ghrelin surface receptor antibody 1 (GHS-R1) expression were found to be higher in ob/ob mouse testes. Additionally, both were localized in steroidogenic Leydig cells, especially inside the seminiferous tubules and elongated spermatid. The inhibition of ghrelin signaling resulted in increased steroidogenic activity, reduced germ cell apoptosis, and improved sperm output in the ob/ob testis. In the present study, ghrelin expression was found to be higher in the interstitial tissue and Leydig cells than in the seminiferous tubules. Additionally, its activity was higher in NOA cases for which spermatozoa had been retrieved with micro-TESE.

These findings suggest that ghrelin plays an important role in spermatogenesis via a hormonal mechanism. In our study, ghrelin showed a positive correlation with serum testosterone levels despite a negative correlation with E_2 levels. These findings do not coincide with a previous study that reported an inverse correlation between ghrelin and testosterone in normozoospermia. This might be related to differences in patient populations in the two studies. Moreover, the interaction between gonadotropin hormone (GnRH) levels and ghrelin activity in the present study was similar to that in the literature, and an inverse correlation was observed between GnRH levels and ghrelin immunoreactivity.

The relationship between orexin and testosterone was first described by Johren et al. [19], who concluded that adrenal orexin receptor activity was regulated by testosterone messenger ribonucleic acid (mRNA) levels. Receptor

Table 3 Correlations between the serum hormone levels and ghrelin and orexin activities in Leydig cells and seminiferous tubules (Correlation coefficient test, *p < 0.05).

	Leydig Cells		Seminiferous Tubules	
	r	p	r	p
Testosterone & Ghrelin	.221	**0.087**	.039	**0.761**
Testosterone&Orexin	.241	**0.067**	.071	**0.581**
E2 & Ghrelin	−230	**0.075**	−146	**0.254**
E2 & Orexin	−164	**0.198**	−131	**0.311**
FSH & Ghrelin	−144	**0.253**	−063	**0.625**
FSH & Orexin	.142	**0.254**	.156	**0.271**
LH & Ghrelin	−291	**0.085**	−252	**0.061**
LH & Orexin	.170	**0.192**	.167	**0.190**

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downregulation was observed in orchiectomized rats, but upregulation was observed after testosterone replacement. Later, the different effects of orexin receptors were shown in Leydig cells, peritubular myoid cells, and Sertoli cells in testes by Karteris et al. [13]. While OX-R in Leydig cells regulate testosterone secretion, they control the contraction of seminiferous tubules in peritubular myoid cells via the phospholipase C (PLC) pathway. Therefore, they play an important role in the regulation of spermatogenesis and sperm output. Orexin expression was found to be lower in Sertoli cells than in other tissues. In an experimental study, Barreio et al. reported that Ox-R expression increased with age and had a significant effect on postnatal testicular development [14,20]. Additionally, its activity was regulated by gonadotropins, especially FSH and other autocrine/paracrine mediators, and it also regulated steroidogenesis in testes.

In the present study, orexin activity was observed in human testes; however, this was more evident in Leydig cells and interstitial tissues than in seminiferous tubules. For that reason, we thought that orexin was more effective in steroidogenesis than spermatogenesis. Furthermore, we observed that orexin showed positive correlations with gonadotropins and testosterone. These findings suggest that orexin activity is regulated by a hormonal mechanism.

There were some major limitations in this study, including the following: *i*) there was a small patient population, *ii*) we did not evaluate changes in peptide activity according to testosterone levels in eugonadotropic and hypogonadotropic cases, and *iii*) we did not evaluate the effect of the medical treatment on peptide activity.

Spermatogenesis and steroidogenesis in the testes are controlled by various mechanisms, many of which are unknown, and several peptides and genes play different and important roles in testicular function. Orexin and ghrelin are two peptides that play regulatory roles in testicular function in humans. Further studies are needed to fully characterize these roles, including studies with larger sample sizes. Moreover, the evaluation of immunoreactivity before and after medical treatment might be very important for pharmacogenetic studies.

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