

# Insulin-like Peptide-6 Levels in Non-obstructive Azoospermia

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## ABSTRACT

**Objective:** To investigate the serum *versus* insulin-like peptide-6 (INSL-6) levels in men with non-obstructive azoospermia (NOA) and normospermia.

**Study Design:** Descriptive study.

**Place and Duration of Study:** Department of Urology, Balıklıgöl State Hospital and Harran University, Sanliurfa, Turkey, between July and October 2020.

**Methodology:** The serum and seminal levels of INSL-6 were measured in men with NOA, and normospermia using a commercially available enzyme-linked immunosorbent assay. Age, body mass index (BMI), hormone profile, testicular volumes and seminal and serum INSL-6 levels were compared between the study groups.

**Results:** In total, 80 men were included in the study, 40 of whom have NOA and 40 have normospermia. No significant difference was found in the mean age and BMI between the groups ( $p > 0.05$ ). Seminal INSL-6 levels were higher in the normospermia group, although serum and seminal INSL-6 levels were not significantly different in this group ( $p > 0.05$ ). No significant correlation was observed between serum INSL-6 levels and age, BMI, testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and varicocele presence ( $p > 0.05$ ). No significant association was found between seminal INSL-6 levels and age, BMI, FSH, LH, and testosterone levels ( $p > 0.05$ ). However, a significant negative association observed between seminal INSL-6 levels and testicular volume ( $p < 0.05$ ).

**Conclusion:** The seminal INSL-6 levels were approximately 5.5 times higher than the serum INSL-6 levels and survival level of INSL-6 were higher in the normospermia. This suggests that INSL-6 plays an active role in the male reproductive system. However, the mechanism and extent of this effect remain to be elucidated.

**Key Words:** Infertility, Non-obstructive azoospermia, Insulin-like peptide-6.

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## INTRODUCTION

Infertility is defined as the inability to conceive even after participating in regular unprotected sexual intercourse for at least 12 months. Nearly 15% of the world's population experiences infertility and affected individuals search for the aetiologies and treatments for infertility to continue their lineage.<sup>1</sup> Such individuals suffer both psychological and material losses during these endeavours.<sup>2</sup> Infertility aetiologies range from easily correctable to irreversible, with 50% of the cases involving a male-factor. The appropriate and adequate functioning of several genetic, hormonal, psychological, and environmental factors is required for spermatogenesis and fertilization.<sup>2,3</sup>

Approximately 1% of men and 10%-15% of men with infertility have azoospermia, which is described as the absence of sperms in the ejaculate. Non-obstructive azoospermia (NOA) is defined as the absence of sperms in the semen despite an unobstructed genital tract. The most common causes of NOA are Y-chromosome microdeletion, varicocele presence, cryptorchidism, testicular torsion, radiation, toxins, and idiopathic. Although, numerous studies have been conducted to investigate the idiopathic causes of NOA, which remain unclear.<sup>4</sup>

Relaxin and relaxin-like peptides comprise relaxin-1 (H1-relaxin), H2-relaxin and H3-relaxin, as well as insulin-like peptide-3 (INSL-3), INSL-4, INSL-5, and INSL-6. These peptides are present in the heart, bone, muscle, prostate, and Leydig cells. Little has been determined regarding the physiology and pharmacology of these peptides in the male reproductive system, especially in humans.<sup>5</sup> INSL-3 is secreted from Leydig cells and is responsible for the descent of the testes from the abdomen to the scrotum during the intrauterine period and for anti-apoptotic factors in germ cells. A study involving mice reported that INSL-5 affected spermatogenesis due to altera-

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tions in glucose metabolism.<sup>6</sup> Testicles exhibit the highest INSL-6 expression.<sup>6,7</sup> A mice study showed that impaired INSL-6 gene function induced impairment in spermatogenesis. However, numerous steps remain unknown regarding the physiology or pharmacology of human spermatogenesis.<sup>6</sup> Therefore, this study aimed to investigate the serum *versus* INSL-6 levels in men with NOA compared to the use with normospermia.

## METHODOLOGY

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Harran University Clinical Studies Ethics Committee approved this study (Decision No. HRU/20.12.04). The study included men who applied to the infertility outpatient clinic at the Department of Urology, Balıkligol State Hospital and Harran University, Sanliurfa, Turkey, between July and October 2020 and were diagnosed with NOA. The control population comprised of men with normal seminal parameters who agreed to participate in the study. Patients with a history of undescended testicles who had undergone orchiopexy and testicular sperm extraction were excluded from the study. All subjects were informed about the study and written consents were obtained.

After detailed history and physical examination, seminal samples were obtained through masturbation without lubricant after 3 days of sexual restraint and stored in a sterile collection container. Seminal parameters were evaluated according to World Health Organization's 2010 criteria using a Macler camera. A sperm count of  $\geq 15 \times 10^6/\text{ml}$  was considered normospermia. Azoospermia was diagnosed after at least two seminal analyses. All participants showed normal karyotypes (46, XY). Semen and blood samples were centrifuged for 15 minutes at 3000 rpm and stored at  $-80^\circ\text{C}$  until INSL-6-level analysis. The serum and seminal levels of INSL-6 were measured using a commercially available enzyme-linked immunosorbent assay (Catalogue Number: 201-12-6918; Shanghai Sunred Biological Technology Co., Ltd., China). The intra- and inter-assay coefficients of variations were  $<10\%$  and  $<12\%$ , respectively, for INSL-6. The standards and samples were analysed in duplicate. The serum and seminal INSL-6 levels in the samples were subsequently determined by comparing the absorbance value of the samples with the standard curve.

The subjects were divided into two groups, the azoospermia group comprising 40 patients with NOA and the normospermia group comprising 40 patients with normospermia. Age, body mass index (BMI), hormone profile, testicular volumes, and seminal and serum INSL-6 levels were compared between the groups. Mean, standard deviation, median, minimum-maximum value, frequency and percentage were used for descriptive statistics. The Kolmogorov-Smirnov test was used to test the normal distribution of the data. Differences between the two groups were evaluated using Student's unpaired t-test or Mann-Whitney U test for parameters with a normal or non-normal distribution, respectively. The chi-square test was used

to compare categorical data between the groups. Spearman test was used for correlation analysis. SPSS 27.0 program was used for all statistical analyses. The p-value  $<0.05$  was considered significant.

## RESULTS

The mean age and BMI were similar between the azoospermia and normospermia groups ( $p > 0.05$ ) (Table I). Although the serum and seminal INSL-6 levels were higher in the normospermia group, no significant difference was found between the groups ( $p > 0.05$ , Table I). The follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were significantly higher in the azoospermia group than those in the normospermia group (FSH,  $p = 0.004$ ; LH,  $p = 0.028$ ). The testosterone levels and testicular volumes were significantly lower in the azoospermia group than in the normospermia group (testosterone levels,  $p = 0.01$ ; right testicular volume,  $p = 0.011$ ; left testicular volume,  $p = 0.028$ , Table I). No significant difference was found between the study groups in terms of varicocele presence ( $p > 0.05$ , Table I).

In the correlation analysis, no significant correlation was observed between the serum INSL-6 levels and age, BMI, FSH, LH, testosterone levels, testicular volumes, and varicocele presence ( $p > 0.05$ , Table II). No significant correlation was found between the seminal INSL-6 levels and age, BMI, FSH, LH, and testosterone levels ( $p > 0.05$ ). A significant negative correlation was found between the seminal INSL-6 levels and testicular volume (right testis,  $p = 0.003$ ; left testis,  $p = 0.014$ , Table II).

## DISCUSSION

Male infertility is an exceedingly complex condition caused by numerous factors. At least 15% of male infertility can be attributed to genetic factors. Congenital anomalies are most common in men with azoospermia (25%). Although a few of the genetic factors have been elucidated, those yet to be identified likely contribute to the azoospermia aetiology in most males with the condition.<sup>8</sup> A recent study reported that the different members of the relaxin hormone family are expressed in the male reproductive system.<sup>9</sup>

On comparing immunoreactivity levels, seminal fluid from certain species, including humans, exhibited a higher concentration of relaxin-like peptides than circulating blood.<sup>10</sup> This suggests that the relaxin hormone family plays a vital role in regulating spermatogenesis. INSL-6, a novel member of the relaxin family, plays a regulatory role in the reproductive system by influencing cell growth and differentiation.<sup>11,12</sup> INSL-6 expression is higher in mammalian testicles, unlike other relaxin family peptides. In addition to the testicle, this hormone is expressed in the kidney, small intestine, heart, brain and thymus.<sup>11,13</sup> Despite weak evidence, INSL-6 is assumed to be expressed in interstitial Leydig or Sertoli cells.<sup>9,14</sup> In the present study, it was demonstrated that the seminal INSL-6 level was nearly 5.5 times higher than the serum level. This further supports the findings that testicles exhibit the highest INSL-6 expression. Conversely, correlation analysis revealed a negative correlation between the seminal INSL-6 level and testicular volume.

**Table I: Comparison of the azoospermia and normospermia groups.**

	<b>Azoospermic</b>	<b>Normospermic</b>	<b>p-value</b>
Age (year), median (IQR)	29 (22-43)	32 (21-41)	0.058
BMI (kg/m <sup>2</sup> ), median (IQR)	27.2 (19.3-37.9)	28.5 (20.3-36.3)	0.791
Serum INSL6 (ng/mL), median (IQR)	0.4 (0.4-2.7)	0.4 (0.4-4.3)	0.337
Semen INSL6 (ng/mL), median (IQR)	2.2 (1.6-6.1)	2.6 (1.3-7.3)	0.464
FSH (iu/l), median (IQR)	15.5 (1.6-57.3)	7 (2.8-20.1)	<b>0.004</b>
LH (iu/l), median (IQR)	7.1 (1.2-37.4)	5 (1.7-10)	<b>0.028</b>
Testosterone (ng/dl), median (IQR)	359.6 (133-590.6)	378.5 (298.7-740.7)	<b>0.010</b>
Right Testis Volume (cc), median (IQR)	13.6 (7-18)	15 (11-20)	<b>0.011</b>
Left Testis Volume (cc), median (IQR)	13.5 (7-18)	14.7 (11-19)	<b>0.028</b>
Varicocele, n (%)	15 (37.5)	14 (41.2)	0.747

Statistically significant results are in bold italics ( $p < 0.05$ ); SD: Standard deviation; BMI: Body mass index; INSL6: Insulin-like peptide-6; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone.

**Table II: Correlation analysis.**

		<b>Age</b>	<b>BMI</b>	<b>FSH</b>	<b>LH</b>
Serum INSL6	r	-0.085	0.033	0.017	-0.020
	p	0.471	0.779	0.885	0.865
Semen INSL6	r	0.098	0.215	-0.067	-0.089
	p	0.407	0.066	0.569	0.449
		<b>Testosterone</b>	<b>Right Testis Volume</b>	<b>Left Testis Volume</b>	
Serum INSL6	r	0.148	-0.070	-0.053	
	p	0.207	0.555	0.652	
Semen INSL6	r	-0.105	-0.337	-0.286	
	p	0.372	0.003	0.014	

Spearman correlation; BMI: Body mass index; INSL6: Insulin-like peptide-6; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone.

Although INSL-6 levels influence spermatogenesis, no specific INSL-6 receptor has been discovered yet.<sup>15</sup> Furthermore, the physiological function of INSL-6 in the testicle and whether it acts *via* autocrine/paracrine pathways remains to be elucidated.<sup>12</sup> However, high INSL-6 levels have been demonstrated in male germ cells before and after meiosis, and INSL-6 may be a secretion peptide that aids the release of semen from the testicle.<sup>16</sup> Burnicka-Turek *et al.* reported a significant deterioration in spermatogenesis with meiotic arrest in INSL-6 knockout mice. Based on their results, the authors concluded that INSL-6 regulates male fertility by affecting spermatogenesis and sperm motility.<sup>17</sup>

Similarly, another study showed that the INSL-6 gene affects the pachytene phase of spermatogenesis and impairment in this gene function results in impaired spermatogenesis.<sup>6</sup> In a recent study evaluating 249 men with a spermatogenesis-associated disorder and 249 normospermic healthy men, the INSL-6 gene was screened and a heterozygous R171H missense mutation was detected in the men with the spermatogenesis-associated disease. The results indicated that the mutation disrupted the *in vivo* function of the INSL-6 prohormone and emphasised the notion that INSL-6 might be responsible for human spermatogenic failure.<sup>7</sup> Although not statistically significant, seminal INSL-6 levels were higher in the normospermic group in the present study. Despite weak evidence, this suggests that INSL-6 might positively affect seminal parameters. Further studies involving populations other than those with azoospermia and normospermia can be planned to evaluate whether INSL-6 affects semen volume, sperm motility, and morphology.

This study had certain limitations. One was the small sample

size. Another limitation is that the participants with male reproductive system disorders other than azoospermia were not included in the observational group. The effect of INSL-6 on seminal parameters can be evaluated more accurately in studies including these populations.

## CONCLUSION

The seminal INSL-6 level was nearly 5.5 times higher than the serum INSL-6 level. Seminal INSL-6 levels were higher in the normospermia group, although this difference was not significant. This suggests that INSL-6 plays an active role in male fertility. However, the molecular aspects of this effect remain to be elucidated. Further studies are warranted to examine the subgroups of seminal parameters in larger populations.

### ETHICAL APPROVAL:

All procedures performed in studies involving human participants were in accordance with the ethical standards of our Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Harran University Clinical Studies Ethics Committee approved this study (Decision No. HRU/20.12.04).

### PATIENTS' CONSENT:

Written informed consents were obtained from the patients prior to sample collection.

### COMPETING INTEREST:

The authors declared no competing interest.

### AUTHORS' CONTRIBUTION:

KG: Conceptualised and designed the study/article, did sample collection, lab work and acquisition of data, data

entry, analysis and interpretation of data. Drafted the primary draft of the manuscript.

MD: Helped in the conception of the study and drafting and editing of the manuscript.

ID: Revised the data and design of the study. Revised and critically analysed the draft of the entire manuscript and added intellectual content to it. Checked the grammar and paraphrasing of the manuscript. Helped in lab work and sample collection.

All the authors have approved the final version of the manuscript to be published.

## REFERENCES

1. Achermann APP, Esteves SC. Diagnosis and management of infertility due to ejaculatory duct obstruction: Summary evidence. *Int Braz J Urol* 2021; **47(4)**:868-81. doi: 10.1590/S1677-5538.IBJU.2020.0536.
2. Choy JT, Eisenberg ML. Male infertility as a window to health. *Fertil Steril* 2018; **110(5)**:810-4. doi: 10.1016/j.fertnstert.2018.08.015.
3. Cocuzza M, Alvarenga C, Pagani R. The epidemiology and etiology of azoospermia. *Clinics (Sao Paulo)* 2013; **68(Suppl 1)**:15-26. doi: 10.6061/clinics/2013(sup01)03.
4. Winters BR, Walsh TJ. The epidemiology of male infertility. *Urol Clin North Am* 2014; **41(1)**:195-204. doi: 10.1016/j.ucl.2013.08.006.
5. Ferlin A, Menegazzo M, Gianesello L, Selice R, Foresta C. Effect of relaxin on human sperm functions. *J Androl* 2012; **33(3)**:474-82. doi: 10.2164/jandrol.110.012625.
6. Anand-Ivell R, Dai Y, Ivell R. Neohormones as biomarkers of reproductive health. *Fertil Steril* 2013; **99(4)**:1153-60. doi: 10.1016/j.fertnstert.2012.12.023.
7. Chen GW, Luo X, Liu L, Jiang Q, Qian XM, Guo ZY. R171H missense mutation of INSL6 in a patient with spermatogenic failure. *Eur J Med Genet* 2011; **54(4)**: e455-7. doi: 10.1016/j.ejmg.2011.04.008.
8. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nat Rev Urol* 2018; **15(6)**:369-84. doi: 10.1038/s41585-018-0003-3.
9. Ivell R, Kotula-Balak M, Glynn D, Heng K, Anand-Ivell R. Relaxin family peptides in the male reproductive system- a critical appraisal. *Mol Hum Reprod* 2011; **17(2)**:71-84. doi: 10.1093/molehr/gaq086.
10. Kohsaka T, Hamano K, Sasada H, Watanabe S, Ogine T, Suzuki E, et al. Seminal immunoreactive relaxin in domestic animals and its relationship to sperm motility as a possible index for predicting the fertilising ability of sires. *Int J Androl* 2003; **26(2)**:115-20. doi: 10.1046/j.1365-2605.2003.00409.x.
11. Kasik J, Muglia L, Stephan D.A, Menon R K. Identification, chromosomal mapping, and partial characterisation of mouse InsI6: A new member of the insulin family *Endocrinology* 2000; **141(1)**:458-61. doi: 10.1210/endo.141.1.7376.
12. Brailoiu GC, Dun SL, Yin D, Yang J, Chang JK, Dun NJ. Insulin-like 6 immuno reactivity in the mouse brain and testis. *Brain Res* 2005; **1040(1-2)**:187-90. doi: 10.1016/j.brainres.2005.01.077.
13. Hsu SY. Cloning of two novel mammalian paralogs of relaxin/insulin family proteins and their expression in testis and kidney. *Mol Endocrinol* 1999; **13(12)**:2163-74. doi: 10.1210/mend.13.12.0388.
14. Balvers M, Spiess AN, Domagalski R, Hunt N, Kilic E, Mukhopadhyay AK, et al. Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. *Endocrinology* 1998; **139(6)**:2960-70. doi: 10.1210/endo.139.6.6046.
15. Ivell R, AgoulNIK AI, Anand-Ivell R. Relaxin-like peptides in male reproduction - a human perspective. *Br J Pharmacol* 2017; **174(10)**:990-1001. doi: 10.1111/bph.13689.
16. Lok S, Johnston DS, Conklin D, Lofton-Day CE, Adams RL, Jelmsberg AC, et al. Identification of INSL6, a new member of the insulin family that is expressed in the testis of human and rat. *Biol Reprod* 2000; **62(6)**:1593-9. doi: 10.1095/biol-reprod62.6.1593.
17. Burnicka-Turek O, Shirmeshan K, Paprotta I, Grzmil P, Meinhardt A, Engel W, et al. Inactivation of insulin-like factor 6 disrupts the progression of spermatogenesis at late meiotic prophase. *Endocrinology* 2009; **150(9)**:4348-57. doi: 10.1210/en.2009-0201.

