Research Paper: Correlation Between Serum and Seminal Leptin and Nitric Oxide (Nitrite) in Asthenoteratozoospermic Males

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ABSTRACT

Introduction: Leptin is a critical metabolic signal linking nutrition and reproductive functions. Leptin receptors are expressed in germ cells, Sertoli and Leydig cells. Oxidative stress increases sperm DNA damages. Nitric oxide is a free radical, produced by most cells via Nitric Oxide (NO) synthase and plays stimulatory and inhibitory roles on cells. Physiological concentrations of NO are essential for the biology and physiology systems. However, a low level of NO has a detrimental effect on cells. This study aimed to determine the correlation between seminal and serum leptin and NO in asthenoteratozoospermic men.

Methods: Semen and blood samples were obtained from (n=52) males referring to the Arak Infertility Clinic, in Arak City, Iran. We examined each patient for serum hormones (LH, testosterone) by chemiluminescence. Serum and seminal leptin were measured by ELISA and seminal NO was measured by Griess reaction.

Results: There were differences in serum leptin levels, and total testosterone, LH, and NO concentrations among the 2 groups. Asthenoteratozoospermic groups showed higher testosterone and LH levels than the normozoospermic group. Serum leptin levels of asthenoteratozoospermic men significantly increased in comparison with normozoospermic men. Seminal leptin levels of asthenoteratozoospermic men significantly decreased compared to normozoospermic men. NO levels in asthenoteratozoospermic men significantly decreased in comparison with normozoospermic cases. There were no significant correlations between serum and seminal leptin and seminal plasma NO.

Conclusion: Our results do not support the hypothesis that in vivo serum and seminal leptin affect NO synthesis. Also, our results revealed that nitrite in the seminal plasma is not able to entirely indicate in vivo NO synthesis.

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1. Introduction

Infertility is among the most serious health problems worldwide. The prevalence of infertility is growing. One out of every 6 to 7 couples experience difficulties with conception, globally [1]. Infertility affects people both medically and psychosocially. Male partner contributes to about 40% of all infertility cases [2]. Spermatozoa travels through the seminiferous tubules to the ampulla of the oviduct. Therefore, sperm motility is necessary for normal fertilization. The prevalence of infertility cases with sperm motility issues was 81.84% (asthenozoospermic cases), in which 63.13% of asthenozoospermic samples were associated with oligo- and/or abnormal sperm morphology [3].

The role of metabolic substances in mechanisms controlling reproductive system has been recently highlighted. Epidemiological studies have found a link between male infertility and lifestyle patterns including dietary habits [4]. A study indicated an increased serum leptin level and decreased sperm motility in high-fat-diet rat models [5]. Leptin is a polypeptide hormone secreted by white adipose tissue [6]. Leptin regulates body weight and energy balance [7, 8]. Leptin is mainly known as a hormonal link between energy storage and energy homeostasis [9].

Numerous functions are associated with the reproductive system. Puberty and gestation are energy-dependent. Thus, long-term fluctuations in energy reserves can result in reproductive dysfunctions [10]. Therefore, it is also involved in reproductive activities. Leptin is a permissive regulator of human reproductive maturity [11]. In addition, several studies have reported that leptin is related to spermatogenesis and may directly impact the maturation of spermatocytes to spermatids [12, 13]. Leptin receptors belong to cytokine families and are responsible for the biological function of it. splicing of OB-R mRNA produce several isoforms of leptin receptors (OB-Ra to OB-Re).

A study revealed that exogenous leptin administration enhance testicular and seminal vesicle weight and increase sperm count and restore reproductive capacity in leptin-deficient mice [14]. Leptin controls reproductive functions at hypothalamus-pituitary levels. Leptin also regulates gonadal functions indirectly via the central neuroendocrine system and directly via membrane receptors in peripheral tissues [15]. A study reported the acute effects of leptin in vitro stimulation is not only GnRH release from hypothalamic explants, but also the release of LH and FSH from anterior pituitaries. Leptin essentially affects stimulation of GnRH, LH and FSH by being coupled with its receptors [16].

Leptin can directly affect the anterior pituitary to stimulate LH secretion [17, 18]. Leptin receptors are also expressed in the pituitary gland [17]. Studies reported that leptin enhances Nitric Oxide (NO) production in other cell types. Leptin enhances NO production in human spermatozoa [19]. NO is of reactive nitrogen species that may act as an antioxidant or a free radical in inter- and intra-cellular signaling [20]. NO is synthesized in all mammalian cells via oxidation of L-arginine by NO Synthase (NOS), an enzyme existing in 3 isoforms. NO is involved in physiological and pathophysiological functions in different systems, like the male reproductive tract.

The dual role of NO as a protective or toxic molecule is due to several factors such as the concentration of NO and type of cell. Most studies have reported the stimulatory effects of it [21]. However, the inhibitory effect of it have been also reported [22]. The low concentration of NO increases the motility of spermatozoa, the Acrosome Reaction (AR) and the zona pellucida binding ability of human spermatozoa. Whereas, higher NO concentration appears to exert harmful effects on the motility, viability and metabolism of human spermatozoa in vitro [23].

The literature is limited on the correlation between leptin and NO and their effect on the reproductive function, especially in asthenoteratozoospermic cases. This study aimed to evaluate the relationship between serum and seminal leptin and NO in asthenoteratozoospermic cases.

2. Materials and Methods

In total, 52 patients referring to a fertility and infertility center in Arak City, Iran were selected to collect semen samples. Sperm samples were obtained by masturbation following 3–5 days of sexual abstinence. Semen samples were collected from 28 individuals diagnosed with male factor infertility and 24 healthy males. Asthenoteratozoospermia cases and the controls were selected after their visit to the centers. This selection was according to the results of spermiogram analysis, once the initial infertility examination were conducted for every couple. All samples were allowed to liquefy for at least 30 minutes at 37°C. Blood samples were collected after an overnight fast, and serum was separated after centrifugation and stored at -80°C until analyzed.

Assessment of sperm concentration, motility and morphology

Sperm concentration and immotile spermatozoa percentage were evaluated by a Neubauer chamber and sperm morphology was assess by papanicolaou staining according to the WHO standard guidelines [17].

Serum hormonal measurements

Hormone assays (LH, testosterone) were examined in serum for all patients. We collected blood samples. The blood was centrifuged (at 120 G for 10 min), to separate plasma that was frozen at -80°C for subsequent measurements of LH, and testosterone levels. LH and testosterone Levels were examined by chemiluminescence as per manufacturer's instructions (Monobind Inc. Lake Forest, CA 92630, USA- product code: 3775-300) for testosterone and (Monobind Inc. Lake Forest , CA 92630, USA- product code: 675-300) for LH. Analytical sensitivity of each test was ≤0.05 ng/mL and <0.010 mIU/mL for testosterone and LH, respectively.

Measurement of nitrite in seminal plasma

After centrifuging the liquefied semen at 300 g for 10 minutes, the supernatant was withdrawn and stored in sterile tube at -80ºC until Nitrite assay. Nitrite concentration was measured by adding 100 µL of seminal plasma to 100 µL of Griess reagent and the absorbance of this added record at 540 nm with a microplate reader after 10 minutes incubation in the dark [19]. Nitrite concentration was expressed as µmol/mL [12].

Serum and seminal plasma leptin assay

We assessed serum leptin and seminal plasma with a solid-phase sandwich ELISA (RECI Diagnostics GmbH, Inc., Her-renberg, Germany). The lowest detection limit was 0.25-100 ng/L and the detection range was 1-100 ng/L with an intra- and inter-assay CV of <15%. All samples were assayed in duplicate, according to manufacturer's instructions by the mean value recorded for data analysis.

3. Results

Results are presented in Tables 1, 2 and 3. Values are described as Mean±SD and P<0.05 indicate statistically significant values. There was a statistically significant difference in the control group of seminal leptin. The asthenotratozoospermic seminal leptin concentration was lower than the normozoospermic group. There was a statistically significant difference in the serum leptin concentrations of the control groups (normozoospermic).

The scores of asthenotratozoospermic group was higher than the normozoospermic group. There were no significant differences in the testosterone and LH concentrations of the control groups (normozoospermic). The asthenotratozoospermic group scores were higher than the normozoospermic group. There were no significant differences in the NO concentrations of the control groups (normozoospermic).The scores of asthenotratozoospermic group were lower than the normozoospermic group.

Correlations analysis

Table 3 demonstrates the correlations between the 2 leptin levels; serum and seminal leptin levels, with each other, with concentrations of LH, serum testosterone, and NO in all investigated patients. According to Figures 1 and 2, there were no statistically significant correlation between the concentrations of seminal and serum leptin and NO levels in all samples (n=52).

4. Discussion

This study evaluated the leptin levels in serum and seminal plasma and indicated their relationships to NO ashthenotratozoospermic cases. A previous study

Table 1. Statistical data

Parameter	No.	Min.	Max.	Mean±SD
NO µmol/mL	47	2	7	4.06±0.97
Serum leptin ng/mL	52	1.30	8.70	4.45±2.10
Seminal leptin ng/mL	51	0.02	0.24	0.09±0.06
LH mIU/mL	52	1.70	19.70	9.55±3.74
Testosterone ng/mL	52	0.90	10.40	4.97±2.24

Insignificance: P>0.05; Significance: P<0.05

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Table 2. Statistical comparisons between the asthenoteratozoospermia and control groups

Parameter	Group	No.	Mean±SD	Sig. (2-Tailed)
NO µmol/mL	1	24	4.02±0.982	0.799
	2	23	4.10±0.977	0.799
Serum leptin ng/mL	1	27	5.3593±2.03456	0.001
	2	25	3.4800±1.72820	0.001
seminal leptin ng/mL	1	27	0.0600±0.05189	0.000
	2	24	0.1433±0.05264	0.000
LH mIU/mL	1	27	9.5926±4.07827	0.933
	2	25	9.5040±3.43226	0.933
Testosterone ng/mL	1	27	4.7778±2.09731	0.512
	2	25	5.1920±2.41952	0.514

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reported that leptin is present in human semineferous tubules and seminal plasma [24, 25]. We revealed that the concentration of serum leptin are higher in asthenotratozoospermic males than normozoospermia cases. The present study demonstrated that the concentration of serum leptin in asthenozoospermic men are higher than normozoospermia cases.

The present study is consistent with Zorn et al. who reported that serum leptin increases only in males with extremely poor spermatogenic function [26]. The Findings are in line with Chen et al. and Steinman et al. who reported a correlation between increased serum leptin concentration and spermatogenesis dysfunction in azoospermia and varicocele related infertile males [27, 28]. In contrast, our results revealed that the concentration of seminal leptin are lower in asthenotratozoospermic men than normozoospermia cases. Therefore, there were no correlations between serum and seminal leptin in the present study [29].

The origin of seminal plasma leptin has not been clearly defined. However, many studies suggest that leptin passes across the blood-testis barrier and releases in the seminal plasma [30]. In contrast, some studies suggest that the molecular size of leptin prevents it from passing the testis-blood barrier and reaching the seminal plasma [31]. However, other studies suggest that seminal leptin is also secreted by ejaculated spermatozoa [32]. The lower seminal leptin and higher serum leptin in the samples might be due to the differences in the source of leptin in serum and seminal plasma.

Studies have reported that leptin enhances NO production in other cell types [19]. The presence of NOS in human testes, epididymis, vas deferens, spermatozoa

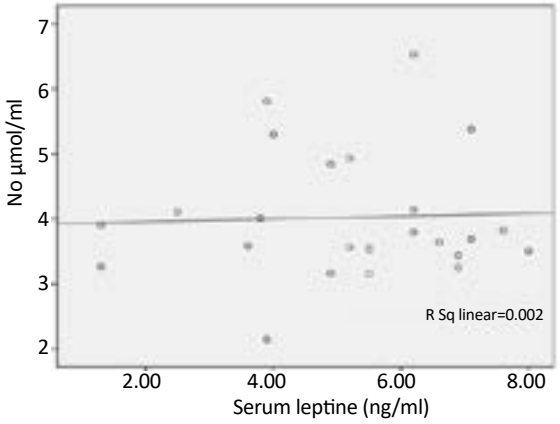

Figure 1. Correlation between seminal plasma nitrite and serum leptin

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Table 3. The relationship between serum and seminal leptin and inter- and intra-cellular signaling with LH and testosterone concentration patients (n=52)

Parameter	Correlation	NO	Serum Leptin	Leptin Seminal	LH	Testosterone
NO	Pearson correlation	1	-0.046	0.018	-0.203	0.457*
	Sig. (2-tailed)		0.757	0.907	0.172	0.001
	n	47	47	47	47	47
Serum leptin	Pearson correlation	-.046	1	-0.217	0.084	0.063
	Sig. (2-tailed)	.757		0.126	0.553	0.659
	n	47	52	51	52	52
Seminal leptin	Pearson correlation	0.018	-0.217	1	0.102	0.079
	Sig. (2-tailed)	0.907	0.126		0.478	0.581
	n	47	51	51	51	51
LH	Pearson correlation	-0.203	0.084	0.102	1	-0.352*
	Sig. (2-tailed)	0.172	0.553	0.478		0.011
	n	47	52	51	52	52
Testosterone	Pearson correlation	0.457*	0.063	0.079	-0.352*	1
	Sig. (2-tailed)	0.001	0.659	0.581	0.011	
	n	47	52	51	52	52

* P<0.05

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and blood vessels have been illustrated [33-36]. NO can strongly affect human sperm functions, such as motility, viability, metabolism, and AR. Its effects are concentration-dependent [37-40]. Our study is consistent with previous data indicating that nitrite (the stable metabolite of NO) may be measured in seminal plasma.

In the present study, nitrite, the stable end-product of the NOS/NO pathway, was detected in the seminal plasma. In addition, NO concentrations were lower in the asthenotratozoospermic samples than normozoospermic samples. In contrast with the results of Rosselli et al. our results does not indicate any correlation between nitrite concentration in seminal plasma and the proportion of immotile spermatozoa [12]. The current

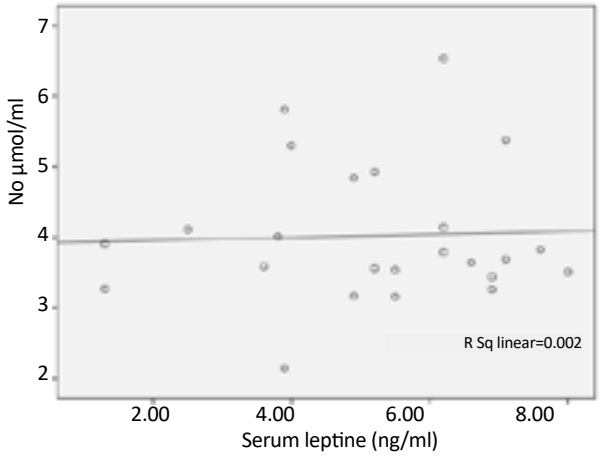

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Figure 2. Correlation between nitrite concentration in seminal plasma and concentration of seminal leptin (ng/ml)

study included both normozoospermic and asthenotratozoospermic sperm samples whereas, Rosselli et al. evaluated normozoospermic or light asthenozoospermic cases [17]. The discrepancy between our data and Rosselli can be due to different sample sizes, in which our sample size was 3 times greater than theirs [17, 39].

Rosselli et al. detected NO values in nmolar range of seminal plasma, whereas in our experience, nitrite concentrations were measured in the micro molar range [39]. Such low concentrations cannot be determined using the Griess reaction, which is not able to measure nmolar range [33]. Rosselli et al. correlated the combined amount of nitrite and nitrate in seminal plasma with the percentage of immotile spermatozoa [39]. Whilst, we preferred to measure the nitrite level alone. Thus, the differences between our results and those of Rosselli et al. can be related to the different methods used to measure NO production [39].

Although the nitrite/nitrate ratio can alter in different conditions, nitrite level is always comparable with nitrate concentration [23]. Indeed, the measurement of both nitrite and nitrate is only applied to increase the sensitivity. However, when sensitivity is not a limiting factor, the determination of nitrite alone is sufficient. In the current study nitrite was clearly detected in seminal plasma. We also did not find any correlation between nitrite levels and the leptin concentration in semen or serum. This could be explained by the presence of a metabolic pathway transforming nitrogen oxides into some other compound(s) not detectable by Griess reagent. It could also be due to the existence of an NOS inhibitor in human seminal plasma. Lewis et al. reported less NO concentrations in asthenozoospermic samples than those with an acceptable motility [33]. They suggested that motility parameters were decreased by the addition of the NO synthesis inhibitor L-NAME [20, 33, 41].

These Findings are in agreement with Alberto Revelli et al. [38] who reported no correlation between nitrite levels, sperm number, and sperm motility. They suggested it could be related to heat-insensitive NOS inhibitor that has been already found in human seminal plasma. Moreover, the preparation and maintenance of sperms in the laboratory environment may develop favorable conditions for imbalance in antioxidant capacity and NO production. Also, part of NO could react to other compounds and escape detection by Griess reagent. Alberto Revelli et al. suggested part of NO could react with thiols and form S-Nitrosothiols and escape detection by Griess reagent [23]. NO or its derivatives might be metabolized via other ways by some cellular components of the urogenital system [23].

The obtained results did not suggest that serum and seminal leptin influence NO production, at the levels generated in the male genital tract. The effect of leptin on NO synthesis is still controversial [42]. Furthermore, we suggest that seminal plasma nitrite could not clearly indicative NO presentation. Thus the use of it as a marker of local NO formation, requires further investigations.

Ethical Considerations

Compliance with ethical guidelines

The study was conducted after approval by the Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC1395.74).

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Authors contributions

The Authors contributions is as follows: Nastaran Azarbarz performed experiments and collected the data. Parvindokht Bayat analyzed and prepared figures. Farideh Jalali Mashayekhi analyzed and interpreted data, prepared figures and performed experiments. Asghar Ghafarizadeh analyzed and interpreted data. Mohamad Bayat developed the concept and designed the study, and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors certify that they have no affiliation with or involvement in any organization or entity with any financial interest, or non- financial interest in the subject matter or materials dismissed in this manuscript.

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