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Evaluation of the Utility of Seminal Plasma Resistin and Leptin in Predicting Successful Surgical Sperm Retrieval in Men with Non-Obstructive Azoospermia

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Abstract

Background: The purpose of the current study was evaluation of the utility of seminal plasma (SP) resistin and leptin in predicting successful surgical sperm retrieval (SSR) in men with non-obstructive azoospermia (NOA).

Methods: This prospective comparative study was conducted in the andrology clinic of a specialized fertility center. In total, 53 NOA men as candidates for either first time micro-testicular sperm extraction (micro-TESE) or repeat micro-TESE and 28 normozoospermic controls were included. ELISA was used for measurement of SP resistin and leptin levels in all participants. Significance level was defined as $p< 0.05$.

Results: The current study demonstrated a significant positive correlation between estradiol (E2) level in serum and SP resistin ($r=0.342$, $p=0.025$). Also, there was a highly significant positive correlation between SP leptin and SP resistin ($r=0.568$, $p= 0.001$). Interestingly, SP leptin was the only variable that demonstrated a significant correlation with eventful micro-TESE outcome in men who underwent micro-TESE for the first time. Finally, ROC curve showed that SP leptin level of 4.05 *ng/ml* predicted successful SSR in men who underwent micro-TESE for the first time with a sensitivity of 73.3% and a specificity of 75% as 11 out of 27 (41%) cases showed eventful micro-TESE at or above this cut-off level [AUC of 0.747, 95% CI, lower bound of 0.555, and upper bound of 0.939, $p=0.030$].

Conclusion: SP leptin can be used as a non-invasive biomarker to predict successful SSR in NOA cases undergoing first time micro-TESE, while SP resistin failed to play the same role.

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Introduction

Non-obstructive azoospermia (NOA) represents decreased sperm production within the testes. Extraction of spermatozoa from the testes in combination with intracytoplasmic sperm injection (ICSI) offers a hope for a subset of NOA patients to achieve biological parenthood (1). However, sperm retrieval was successful only in 50% of reported cases (2). Many peptides, for instance, leptin, obestatin, ghrelin, and resistin are effective in the balance between reproductive function and

energy status (3, 4). The hormones of the hypothalamic-pituitary-gonadal (HPG) axis play a pivotal role in the regulation of spermatogenesis. Thus, the serum hormonal profile and the seminal plasma (SP) contain useful information about spermatogenesis status and can potentially predict the chance of testicular sperm retrieval from NOA patients (5, 6). It has been reported that leptin, a 167-amino acid protein which is the product of leptin (LEP) gene, plays an important role in the reproduction (7). Leptin has a significant role in controlling gonadotropins (FSH, LH) (8) and gonadal hormone secretion (total and free TT) (9). In addition, leptin might accelerate the development of spermatocytes in to spermatids (10). Leptin and leptin receptors are present in the testicular tissue and their expressions are closely related to hypoxia (11). Leptin levels in the seminal fluid have been found to correlate with poor sperm motility and increased sperm DNA fragmentation, suggesting that high concentrations of leptin in the SP can adversely influence the spermatogenesis process (12). Ma et al. in 2011 stated that NOA patients with failed sperm recovery exhibited significantly higher levels of leptin in seminal plasma than men with successful testicular sperm extraction (TESE) outcomes (7). Resistin is a 12.5 *kDa* adipokine belonging to the family of cysteine-rich proteins that has been detected in Leydig and Sertoli cells of adult rats (13).

The expression of this peptide is controlled by gonadotropins, demonstrating that resistin has a hormonal impact upon the testes (4). Toll like receptor-4 is a binding site for resistin and has been found in human sperm (13). The data on the existence and the effects of resistin in human seminal plasma is scarce (14, 15). Resistin has been reported to be correlated with interleukin-6 and elastase in human semen (16). Thomas et al. studied a group of adipokines and noticed increased resistin levels in the SP compared with serum levels (17). Moretti et al. revealed an inverse correlation between the concentrations of resistin in seminal plasma and sperm motility and viability (14). Resistin in seminal plasma was significantly higher in cases of leukocytospermia or if the patients were smokers. In contrast, two other groups studied the relationships between resistin concentrations in seminal plasma and semen parameters but did not display significant correlation (16, 17). However, given the low number of studies available, it is difficult to conclude the role of resistin which seems to have a rather negative effect on spermatozoa and thus fertility (15). Therefore, the purpose of the current study was evaluation of the utility of resistin and leptin in predicting successful surgical sperm retrieval among men with NOA.

Methods

This prospective comparative study was conducted in the andrology clinic of a specialized fertility center (Adam international hospital-fertility and sterility, Giza, Egypt). In total, 53 NOA men for either first time micro-testicular sperm extraction (micro-TESE) (group I; n=27) or repeat micro-TESE (group II; n=26) and 28 normozoospermic controls (group III) were included. Eligible participants were asked to sign an informed consent prior to enrolment into this study according to the regulations mandated by the Research Ethics Committee (REC) of Faculty of Medicine at Beni Suef University which conforms to Declaration of Helsinki 2013 (18) [Approval number: FMBSUREC/06062021, June 6th, 2021]. To protect confidentiality, participants were assigned numbers and data retrieved based on their numbers were only used for research purposes.

All patients who had undergone first time or repeat micro-TESE with testicular volume ≤15 *ml*, azoospermic ejaculates, and high or normal FSH were included. Men with diabetes, bilateral varicocele, severe systemic disease (end stage renal or liver disease), cryptozoospermia or leucocytospermia, NOA due to hypogonadotropic hypogonadism or complete AZFa and AZFb microdeletions, and the cases receiving anabolic steroids and/or exogenous testosterone therapy within at least 6 months prior to the time of surgery were excluded. Finally, patients with complete retrograde ejaculation or any sign of obstructive azoospermia (OA) were also excluded. Normozoospermic healthy fertile men were recruited as controls.

Fertility history taking and clinical examination (general and genital) were performed for all participants. Semen samples were obtained by masturbation after 2–7 days of sexual abstinence. After liquefaction at 37°*C*, seminal fluid was investigated macroscopically (evaluating the appearance, volume, pH, and viscosity) and microscopically (evaluating sperm concentration, sperm motility, and sperm morphology) according to WHO guidelines (2010), fifth edition (19).

Diagnosis of azoospermia was confirmed by at least 2 semen analyses done 2 weeks apart. Azoo-

spermia was differentiated from cryptozoospermia by observation of pellet post centrifugation. The remaining specimens were centrifuged at 3000 *rpm* for 15 *min* to obtain seminal plasma (SP). SP was dispensed into labeled and sterile tube and kept at -20°*C* to estimate the level of leptin and resistin. Following the manufacturer's instructions, the leptin level in seminal plasma was measured using a commercial ELISA kit (LEP ELISA Kit, Shanghai Sunred Biological Technology Co, China). Similarly, an ELISA technique was used for measurement of resistin level in seminal plasma (RETN ELISA Kit; Shanghai Sunred Biological Technology Co, China). The results were expressed in *ng/ml*. All patients were evaluated for serum FSH, LH, total testosterone, free testosterone, and estradiol. A morning fasting sample was used for basal hormones determination prior to micro-TESE, using an ELISA method (ElA; DiaPlus Inc., USA) according to the manufacturer's instructions. Giemsa karyotype was offered to all cases with NOA (n=53) by analysis of at least 20 G-banded metaphases from a peripheral blood lymphocyte culture. All patients with NOA underwent micro-TESE under optical magnification (X24) as described by Schlegel in 1999 and Amer et al. in 2000 (20, 21). If no morphologically dilated tubules were observed on the initial side, the contralateral testis was exposed by the same technique. If motile spermatozoa were retrieved from the first testicle, no further intervention was performed. A fragment of testicular tissue harvested from both testes during micro-TESE procedure was used for histopathological evaluation. Results were classified as follows; hypospermatogenesis (HS) was diagnosed if all stages of spermatogenesis were present but were reduced to a varying degree. Maturation arrest (MA) was diagnosed if there was total arrest of spermatogenesis at a particular stage. Sertoli cell-only syndrome (SCOS) was diagnosed if all tubules showed a total absence of germ cells (22). Other histologic parameters were evaluated including the thickness of tubular wall, intertubular cell compartment for the presence of fibrosis, and the appearance of Leydig cells (22-24).

Statistical analysis: Statistical analysis was performed using the SPSS *vs*. 26 (IBM, US) with significance set at $p<0.05$. Nonparametric statistical tests were applied for data analysis. Mann-Whitney U test was used to assess differences in leptin and resistin levels between the two NOA groups, and NOA cases with positive and negative Sperm Retrieval (SR). Moreover, ANOVA and post hoc using the LSD test were used to assess differences in leptin and resistin levels among the two NOA groups and controls. For comparing categorical data, a Chi-square test was used and the exact test was applied instead when the expected frequency was less than 5. Correlations between quantitative variables were evaluated using the Pearson correlation coefficient. ROC curve analysis was performed to assess the potential of leptin and resistin to discriminate NOA groups 1 and 2 and to discriminate NOA cases with positive and negative SR outcomes. In all cases, the area under the curve (AUC) was calculated and optimal cutoff points that maximize the sum of sensitivity and specificity were determined.

Results

Fifty-three NOA patients were included where 27 men underwent first time micro-TESE (first timers) and 26 men had undergone SSR (redo cases). The mean age was significantly higher among the 28 fertile controls compared to the NOA groups (p=0.001) (Table 1). However, there was no significant difference in the leptin and resistin levels in seminal plasma among the three groups (Table 1). Furthermore, the current study demonstrated a significant positive correlation between estradiol (E2) level in serum and resistin in seminal plasma (r=0.342, p=0.025) (Table 2). Also, there was a significant positive correlation between leptin and resistin in seminal plasma (r= 0.568, p=0.001) (Table 2). All serum hormones measured in the first timers and the redo cases did not show any significant difference between the two groups (Table 3). Moreover, there was no significant difference between the first timers and the redo cases regarding the testicular volume and the dominating testicular histopathology (Table 3). Among NOA cases, 28/57 (49.1%) opted to proceed with peripheral blood karyotype, while 29 (50.9%) refused cytogenetic testing. Klinefelter syndrome (47, XXY) was detected in 6/28 cases (21.4%). There was no significant difference between the two NOA subgroups regarding the proportion of men with abnormal karyotype (Table 3). Although the mean resistin and leptin levels were higher in the MA and HS, they did not show significant correlation with these two testicular histological types in the current study (Table 4).

Table 1. Sociodemographic characteristics and laboratory findings of all groups

		N	Mean	Std. deviation	p
Age (years)	First time	27	35.37	±6.41	0.001
	Redo	26	35.27	±5.34	
	Fertile group	28	41.17	±5.88	
BMI (*kg/m*^{2})	First time	27	26.84	±2.70	0.641
	Redo	26	27.65	±2.82	
	Fertile group	28	27.36	±3.88	
Infertility duration (years)	First time	27	5.96	±4.88	0.519
	Redo	25	5.16	±3.93	
	Fertile group	28	-	-	
SP leptin (*ng/ml*)	First time	27	4.01	±1.77	0.980
	Redo	26	4.07	±1.55	
	Fertile group	28	3.98	±1.76	
SP resistin (*ng/ml*)	First time	27	5.39	±1.41	0.766
	Redo	26	5.61	±1.29	
	Fertile group	28	5.32	±1.62	

a: ANOVA and post hoc test (LSD) were used for statistical analysis. BMI: Body mass index, SP: Seminal plasma

Table 2. Correlations between SP leptin/resistin and serum levels of reproductive hormones in NOA cases

		SP leptin (*ng/ml*)	SP resistin (*ng/ml*)
SP leptin (*ng/ml*)	r	1	0.568
	P [a]		0.001
	N	53	53
Serum FSH (*mIU/ml*)	r	-0.077	-0.211
	P[a]	0.588	0.134
	N	52	52
Serum LH (*mIU/ml*)	r	-0.054	-0.327
	P [a]	0.797	0.111
	N	25	25
Serum total testosterone (*ng/ml*)	r	-0.084	0.060
	P [a]	0.560	0.681
	N	50	50
Serum free testosterone (*pg/ml*)	r	-0.188	0.028
	P [a]	0.239	0.863
	N	41	41
Serum estradiol (*pg/ml*)	r	0.143	0.342
	P [a]	0.361	0.025
	N	43	43

a: Pearson correlation coefficient. SP: Seminal plasma, FSH: Follicle stimulating hormone, LH: Luteinizing hormone

Age, reproductive hormones, and resistin in seminal plasma did not reveal any significant correlation with eventful TESE outcome (Table 5). Interestingly, seminal plasma leptin was the only variable that demonstrated a significant correlation with eventful TESE outcome (Table 5). Finally, ROC curve showed that leptin level of 4.05 *ng/ml* predicted the successful SRR with a sensitivity of 73.3% and a specificity of 75% as 11 out of 27 (41%) cases of first timers showed eventful micro-TESE at or above this cut-off level. However, seminal plasma leptin <4.05 predicted the successful SSR by 15% as only 4 out of 27 first timers showed eventful micro-TESE (Figure 1). Furthermore, the area under the curve was 0.747 and the upper and the lower bound of the 95% confidence interval were 0.939 and 0.555, p=0.03, respectively (Figure 1). On the other hand, resistin was unable to predict the successful SRR in men with first time micro-TESE, (AUC=0.678, p=0.118) (Figure 1). Similarly, both leptin and resistin were unable to predict successful SRR in men with repeat micro-TESE [AUC=0.633, p=0.362 for leptin, AUC= 0.371, p=0.380 for resistin] (Figure 2).

Table 3. Testicular volume, histopathology, cytogenetics, and hormonal profile of the NOA groups

		Group		p
		First time	Redo	
Testicular volume	Small [n=20]	11 (55.0%)	9 (45.0%)	0.80 [a]
	Moderate [n=20]	9 (45.0%)	11 (55.0%)	
	Normal [n=13]	7 (53.8%)	6 (46.2%)	
Testicular histopathology	WTH [n=9]	5 (55.6%)	4 (44.4%)	0.19 [a]
	SCO [n=22]	13 (59.1%)	9 (40.9%)	
	MA [n=18]	6 (33.3%)	12 (66.7%)	
	HS [n=2]	2 (100.0%)	0 (0.0%)	
Karyotyping	Normal [n=22]	9 (40.9%)	13 (59.1%)	0.74 [a]
	KS [n=6]	2 (33.3%)	4 (66.7%)	
Serum FSH (*mIU/ml*) [n=52]		23.22±21.27	19.70±13.94	0.484 [b]
Serum LH (*mIU/ml*) [n=25]		15.49±13.49	9.83±8.46	0.251 [b]
Serum total testosterone (*ng/ml*) [n=50]		6.23±9.61	6.45±9.79	0.935 [b]
Serum free testosterone (*pg/ml*) [n=41]		29.97±19.02	29.52±24.06	0.949 [b]
Serum estradiol (*pg/ml*) [n=43]		36.58±28.13	27.67±11.77	0.186 [b]

a: χ^2-test and the exact test were used for comparing qualitative variables. b: Mann-Whitney U test was used for quantitative variables.
SP: Seminal plasma, WTH: Wide tubular hyalinization; SCO: Sertoli cell only; MA: Maturation arrest; HS: Hypospermatogenesis; KS: Klinefelter syndrome, FSH: Follicle stimulating hormone, LH: Luteinizing hormone

Table 4. SP leptin and resistin levels among NOA cases with different testicular histopathologies

		Mean	Std. deviation	p [a]
SP leptin (*ng/ml*)	WTH	3.83	1.63	0.507
	SCO	3.71	1.64	
	MA	4.58	1.63	
	HS	4.50	2.97	
SP resistin (*ng/ml*)	WTH	5.79	0.90	0.182
	SCO	5.22	1.46	
	MA	5.80	1.14	
	HS	6.15	3.04	

a: ANOVA test was used for statistical analysis.
SP: Seminal plasma, WTH: Wide tubular hyalinization, SCO: Sertoli cell only, MA: Maturation arrest, HS: Hypospermatogenesis

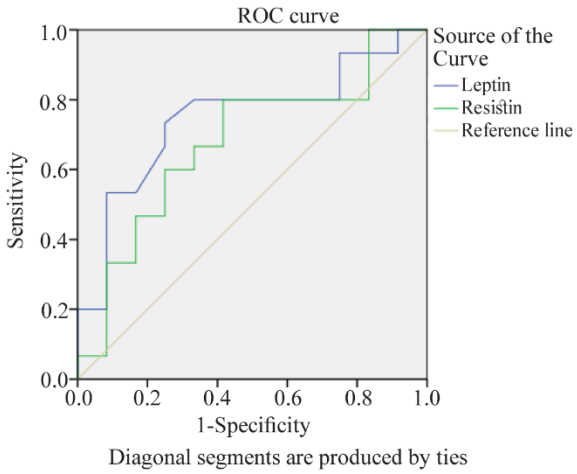

Figure 1. ROC curve: the diagnostic accuracy of the SP leptin and resistin in predicting SRR in men with first time micro-TESE

Discussion

In this study, leptin in seminal plasma was the only variable that demonstrated a significant correlation with eventful TESE outcome. Furthermore, there was a significant positive correlation between E2 level and resistin in seminal plasma. Also, there was a significant positive correlation between leptin and resistin in seminal plasma. The leptin receptor has been detected in the human seminiferous tubules (25). However, only one study proved that seminal plasma and sperm contain this receptor (26). Furthermore, leptin receptors were also detected on the tail of spermatozoa which affect sperm motility (26). Thus, two studies had demonstrated a positive correlation between seminal leptin and motility. The first one was an in vivo study conducted on 96 fertile men that showed a positive correlation between sem-

Table 5. Comparison between TESE negative and TESE positive men in relation to patients' age, BMI, reproductive hormones, and SP leptin and resistin

TESE outcome		N	Mean	Std. deviation	Std. error of the mean	p [a]
Age (years)	Negative	33	35.42	6.46	1.12	0.73
	Positive	20	35.15	4.85	1.08	
BMI (kg/m^2)	Negative	33	27.49	2.92	0.51	0.57
	Positive	20	26.82	2.49	0.56	
Serum FSH (*mIU/ml*)	Negative	32	22.41	19.25	3.40	0.75
	Positive	20	19.95	15.85	3.54	
Serum LH (*mIU/ml*)	Negative	15	14.89	14.46	3.73	0.68
	Positive	10	10.73	6.24	1.97	
Serum total testosterone (*ng/ml*)	Negative	31	7.82	11.93	2.14	0.29
	Positive	19	3.93	2.04	0.47	
Serum free testosterone (*pg/ml*)	Negative	26	34.23	25.81	5.06	0.15
	Positive	15	21.87	8.39	2.17	
Serum estradiol (*pg/ml*)	Negative	24	33.28	26.25	5.36	0.94
	Positive	19	30.90	15.51	3.56	
SP leptin (*ng/ml*)	Negative	33	3.70	1.61	0.28	0.03
	Positive	20	4.60	1.59	0.36	
SP resistin (*ng/ml*)	Negative	33	5.40	1.34	0.23	0.59
	Positive	20	5.66	1.38	0.31	

a: Mann-Whitney U test was used for statistical analysis.
BMI: Body mass index, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, SP: Seminal plasma

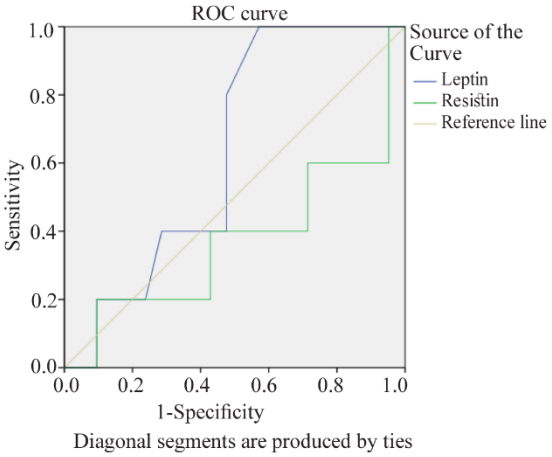

Figure 2. ROC curve: The diagnostic accuracy of the SP leptin and resistin in predicting SRR in men with repeat micro-TESE

inal leptin and progressive and total motility (17). The mean leptin concentrations in normal weight men and overweight or obese groups were 0.91 *ng/ml* and 0.83 *ng/ml*, respectively (17). The second one was an in vitro work conducted by Lampiao and du Plessis in 2008 who studied the effect of leptin on sperm motility and the results revealed a positive impact, since leptin significantly increased the total and progressive motility after 1, 2, and 3 hours of incubation. The aforementioned study was also conducted on spermatozoa of fertile donors (27). In contrast, Guo et al. showed an inverse correlation between sperm motility and seminal leptin in patients with idiopathic asthenozoospermia (28). Remarkably, it must be noted that our study was conducted on NOA cases although seminal leptin did not show any correlation between first timers and redo NOA cases. Yet, there was a significant correlation between positive and negative TESE outcomes. Furthermore, the concentration was higher in MA and HS compared to severe impairment of spermatogenesis (including tubular hyalinization and sertoli cell only syndrome).

Ellithy et al. measured the serum, testicular, and seminal leptin levels at the same time in NOA, OA, and fertile men, and they found that seminal leptin levels were lower in TESE-positive NOA

patients than NOA patients with negative TESE and both levels in the above mentioned groups were much higher than fertile men; also, testicular leptin levels were significantly lower in TESE-positive and TESE-negative NOA patients, while no differences in serum leptin levels were found among the study groups (29). They concluded that seminal leptin as an assistant marker plus FSH can be used in increasing the production accuracy of sperm retrieval in NOA patients (29). The current study failed to demonstrate any correlation between seminal resistin of men who underwent TESE for the first time and redo NOA cases. Additionally, it failed to show any correlation between seminal resistin and positive and negative TESE outcomes. To the best of our knowledge, SP resistin was measured only in three studies. Moretti et al. demonstrated a negative correlation between the concentrations of seminal resistin and sperm motility and vitality (14). In contrast, the other two studies (16, 17) failed to show any correlation between seminal resistin and sperm parameters. Once again, it should be noted that the aforementioned studies in evaluation of the role of seminal resistin were conducted among patients having sperm in their ejaculates not NOA cases like the explored cases of the current study. Remarkably, the current study showed that seminal leptin was more sensitive compared to seminal resistin in predicting successful sperm retrieval. It is worth mentioning that seminal leptin was slightly higher in NOA cases compared to the fertile group that can be explained by the fact that seminal leptin has a detrimental effect on spermatogenesis and that the average body mass index in the 3 groups indicated the high frequency of overweight obese men. However, seminal leptin was also slightly higher in eventful TESE cases compared to uneventful cases. The current finding can be attributed to the significant role of leptin at physiological levels in men with normal BMI.

In contrast, high concentrations of leptin may have a detrimental impact on spermatozoa in overweight or obese men. Notably, the direct effects of seminal leptin is attributable to the presence of human receptor of leptin on the spermatozoa itself. Furthermore, its effect may also be associated to decreased testosterone production by Leydig cells as a result of higher circulating levels of leptin in obese or overweight men which finally interferes with the normal cycle of spermatogenesis (30). Furthermore, leptin can also modify the nutritional support of spermatogenesis by human Sertoli cells (30). In fact, when exposed to leptin, human Sertoli cells dramatically suppress the production of acetate, which is a crucial metabolite for spermatogenesis (31). In view of the aforementioned facts, our finding can be contradictory as seminal leptin was slightly higher in eventful TESE cases compared to uneventful cases despite the normal BMI in all cases. This can be explained by the small sample size of the current study. Similarly, it is obvious that seminal resistin has a rather negative effect on spermatozoa and thus fertility (15). However, the exact role of seminal resistin cannot be determined due to the low number of studies available (15). The current study has demonstrated that resistin levels are increased in infertile patients with leukocytospermia and varicocele and are correlated with impaired sperm quality, lipid peroxidation, and sperm redox imbalance (32). Admittedly, the small number of the participants can be considered as a major limitation. Additionally, the average body mass index in the 3 groups indicated the high frequency of overweight and obese males which is another limitation of the current research.

Conclusion

Seminal leptin can be used as a non-invasive biomarker to predict successful sperm retrieval in NOA cases undergoing first time micro-TESE while seminal resistin failed to play the same role. However, further studies are needed to confirm these findings.

Conflict of Interest

All authors declare no conflict of interest.

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