Journal of **R**eproduction & **I**nfertility
Volume 23, Issue no. 4
https://doi.org/10.18502/jri.v23i4.10810

Original Article

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Estimation of Serum and Seminal Plasma Levels of Glactin-1 in Non-Obstructive Azoospermia Cases and Their Correlations with the Rate of Sperm Retrieval: A Comparative Prospective Study

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Received: Apr. 16, 2022
Accepted: Jul. 18, 2022

Abstract

Background: Remarkably, the current study is one of the first to deploy galectin-1 (Gal-1) in determining the degree of impairment of spermatogenesis among cases with non-obstructive azoospermia (NOA) as well as utilizing it as a biomarker to predict the rate of sperm retrieval in these patients. The purpose of the study was to evaluate the seminal plasma and serum levels of Gal-1 in NOA patients as well as their correlations with Johnsen's tubular biopsy scoring (JTBS).

Methods: The current case control study included totally 48 patients with NOA whose ages ranged from 24 to 46 years old and 50 age matched healthy controls. Gal-1 levels were measured in both seminal plasma and serum of all subjects by the enzyme-linked immunosorbent assay (ELISA).

Results: A significant negative correlation between seminal plasma levels of Gal-1 and JTBS was detected (r= -0.281, p=0.048) in the NOA cases. Interestingly, the receiver operating characteristic (ROC) curve had demonstrated that the cutoff value of seminal plasma levels of Gal-1 in determining azoospermia was >0.735 *ng/ml* and the area under the curve (AUC) was 0.858. The sensitivity, specificity, positive predictive, and negative predictive values for seminal plasma levels of Gal-1 were 76, 92, 90.5, and 79.3, respectively. In addition, sensitivity, specificity, positive predictive, and negative predictive values for serum levels of Gal-1 were 38, 66, 52.8, and 51.6, respectively.

Conclusion: Seminal plasma levels of Gal-1 are higher in NOA men versus healthy controls. Interestingly, negative correlation of seminal plasma levels of Gal-1 with JTBS was determined. Thus, it can be used as a good predictor for NOA cases.

Keywords: Non-obstructive azoospermia, Seminal plasma levels of Gal-1, Serum levels of Gal-1.

To cite this article: GamalEl Din SF, Abougabal Kh, Saad HM, Mohamed MS, Mohamed Ali AM, Abo Sief A. Estimation of Serum and Seminal Plasma Levels of Glactin-1 in Non-Obstructive Azoospermia Cases and Their Correlations with the Rate of Sperm Retrieval: A Comparative Prospective Study. J Reprod Infertil. 2022;23(4):257-263. https://doi.org/10.18502/jri.v23i4.10810.

Introduction

Approximately, half of the infertile couples suffer from male factor infertility (1, 2). The causes of male factor infertility vary from genetic alterations to environmental and lifestyle-related factors (2, 3). Semen analysis is the cornerstone in diagnosing male infertility (4). Till

now, testicular sperm extraction (TESE) in men with non-obstructive azoospermia (NOA) remains the gold standard approach for these patients (5). Azoospermia occurs in about 15% of infertile males (6, 7). Galectins are characterized by their carbohydrate recognition domains (CRDs). They share a common sequence of 130 amino acids that enables them to bind b-galactoside-rich glycol conjugates (8). Galectins play essential role in homeostasis, cell adhesion, cellular turnover, and immunity through a variety of extracellular and intracellular mechanisms (9). Galectin-1 (Gal-1) was the first member of the lectin family, reported more than 3 decades before, as a +15 *kDa* protein existing in a non-covalent homodimeric form which was named electrolectin, b-galactoside-binding lectin, galaptin or L-14 (10). The aforementioned protein became known as galectin-1 (Gal-1) encoded by the gene LGALS1 (lectin, galactoside binding, soluble 1) after nomenclature for galectins. It is expressed in different organs and tissues as shown by many researchers, including thymus (11), spleen (12), smooth muscle (13), colon (14), ovary (15), and also the nervous system (16). It is an endogenous protein that might play a key role in Leydig cell biology and it might regulate the development of normal Leydig cells in an autocrine or paracrine manner (17). On the other hand, cells such as Sertoli or peritubular cells might be responsible for Gal-1 regulation in Leydig cell functions (17).

Remarkably, the current study is one of the first to deploy Gal-1 in determining the degree of impairment of spermatogenesis among NOA human patients as well as utilizing it as a biomarker to predict the rates of sperm retrieval in these patients. Thus, the current prospective study aimed to evaluate the correlation of seminal plasma and serum levels of Gal-1 with Johnsen's tubular biopsy scoring (JTBS) in NOA cases which determines the degree of impairment of spermatogenesis (18). The second objective was to assess the sensitivity and specificity of seminal plasma versus serum levels of Gal-1 in determining the rate of sperm retrieval in NOA patients.

Methods

Study population: The current case control study included totally 48 NOA patients whose ages ranged from 24 to 46 years old and 50 matched healthy controls. All participants were recruited from the Andrology Department of Kasr Al Ainy Hospital from July (2020) to January (2021). An approval of the ethics committee of the institutional review board at Beni-Suef University was obtained that conforms to Declaration of Helsinki (2013) (19). They also signed an informed consent prior to recruitment in the current study.

Inclusion criteria of the patients: Any patient diagnosed with NOA was recruited after obtaining two consecutive semen analyses.

Exclusion criteria of the patients: All patients with abnormal karyotyping, small testicular volume determined by orchidometer, varicocele, abnormal hormonal profile including follicle stimulating hormone (FSH) and luteinizing hormone (LH) and prolactin as well as total testosterone and past history of epididymorchitis were excluded from the study. All patients with major systemic illnesses, smokers, drug addicts, and alcoholics were also excluded from the study. Finally, all patient with congenital bilateral absence of vas deferens or with a previous testicular histopathology showing obstructive azoospermia were excluded from the study.

Inclusion criteria of the controls: Controls were fertile and age matched individuals. All participants were subjected to history taking and general and local examinations. A hormonal profile had been obtained from all men using chemiluminescence immunoassay (CLIA) technique which included FSH, LH, total testosterone, and prolactin (1.5–14 *mIU/ml* for FSH, 1.5–8 *mIU/ml* for LH, 2.4–8.3 *ng/ml* for total testosterone, 2.5–17 *ng/ml* for prolactin). Also, a scrotal duplex ultrasound was done to exclude individuals with varicocele. Finally, micro-testicular sperm extraction was done for the patients using a surgical microscope (5 steps magnifications (4x, 6x, 10x, 16x & 25x), 45 degree inclined binocular tubes, 12.5x wide-field eyepieces, F=200 *mm* objective lens; HB Surgitech, India) where dilated tubules were selected and preserved in one ml HEPES buffered Ham's F10 medium that was taken directly into the embryo lab to determine if there was any sperm.

A testicular sperm extraction specimen was fixed in 10% formalin for 3 days and embedded in paraffin. Hematoxylin and eosin were used to stain the cells and 5 μm thick testicular sections were evaluated semi-quantitatively by two pathologists according to the Johnson's tubular biopsy scoring (JTBS) method (18). The scoring grades were as follows: score 10, complete spermatogenesis with

normal tubules; score 9, many sperm and abnormal germinal epithelium; score 8, few sperm; score 7, no sperm and many spermatids; score 6, few spermatids; score 5, no sperm or spermatids and many spermatocytes; score 4, few spermatocytes; score 3, presence of spermatogonia; score 2, presence of Sertoli cells; and score 1, no cells.

Estimation of serum Gal-1 levels and seminal plasma: Serum separator tubes (SST) were used and samples were allowed to clot for 30 *min* at room temperature before centrifugation for 15 *min* at 1000 x *g*. Serum was removed and assayed immediately or divided into aliquot and stored at ≤-20°*C*. Repeated freeze-thaw cycles were avoided. The ejaculates were obtained after 4 days of sexual abstinence into sterile containers for immediate analysis. Semen was examined according to WHO guidelines (2010). Seminal plasma was centrifuged for 15 *min* at 1000 x *g* within 30 *min* of collection. Next, it was assayed immediately or divided into aliquot and stored at ≤-20°*C*. Repeated freeze-thaw cycles were avoided.

Principle of the test: This assay employs the quantitative sandwich enzyme linked immunosorbent assay (ELISA). A polyclonal antibody specific for human galectin-1 was pre-coated onto a microplate. Samples were pipetted into the wells and any galectin-1 present was bound by the immobilized antibody. After washing away any unbound materials, an enzyme-linked polyclonal antibody specific for human galectin-1 was added to the wells. After washing to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of galectin-1 bound in the initial step. The color development was stopped and the intensity of the color was measured.

Procedure: All reagents and samples were brought to room temperature for 30 *min* before starting assay procedures. All reagents, working standards, and samples were prepared after serial dilution based on instruction manuals in the kit (The Quantikine Human Galectin-1 Immunoassay, Catalog Number: DGAL10, R&D Systems, USA). Next, 100 μl of Assay Diluent RD1-9 were added to each well. Assay Diluent RD1-9 may contain a precipitate. After mixing before and during use, 100 μl of standard, control, or sample were added per well and they were covered with the adhesive strip provided.

After incubating for 2 *hr* at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500±50 *rpm*, each well was aspirated and washed and the process was repeated three times for a total of four washes. Each well was filled with wash buffer (400 μl) using a squirt bottle, manifold dispenser, or autowasher. The liquid was removed at each step which is essential for a good performance. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. Next, 200 μl of human galectin-1 conjugate were added to each well and covered with a new adhesive strip. Next, they were incubated for 2 *hr* at room temperature on the shaker. The aspiration/wash procedure was repeated similar to the measures in step 4. Then, 200 μl of substrate solution was added to each well, incubated for 30 *min* at room temperature on the benchtop, and protected from light. Afterwards, 50 μl of stop solution were added to each well. The color in the wells should change from blue to yellow. The optical density (OD) was read at 450 *nm* using an ELISA reader within 30 *min* after adding stop solution (20-21).

Calculation of results: Readings for each standard, control, and sample were obtained and the average zero standard optical density (OD) was subtracted. Using the professional curve fitting software to make a standard curve (usually most of the curves were linear), the level of this analyte was calculated. Since samples were diluted, the concentration was calculated from the standard curve.

Statistical methods: Collected data was coded, entered, and analyzed using Microsoft Excel software. Data was imported into statistical package for the social sciences (SPSS) software *vs.* 25 (IBM SPSS Inc., USA). The Kolmogorov-Smirnov test was used to verify the normality of the distribution of variables and accordingly Mann-Whitney test was used as a test of significance for the studied variables as they were not normally distributed. The level of significance was set at 5%. Spearman's coefficient was calculated to assess linear correlations.

Sample size calculation: The primary outcome of our study was evaluated by comparing the level of galectin-1 between azoospermic patients and normal healthy matched controls. As reported in previous publication (22), the mean±SD of semen fibronectin (the same galectin family) in azoospermic group was approximately 2.01±0.8 *ug/ml*, while in control group it was approximately 0.65±

0.25 *ug/ml*. Accordingly, minimum proper sample size was calculated to be 11 participants in each group for detecting a significant difference with 80% power at a significance level of 0.05 using Student's t-test.

Sample size calculation was done using PS Power and Sample Size Calculations software, *vs.* 3.0.11 for MS Windows developed by William D. Dupont and Walton D. Plummer, Vanderbilt University, USA.

Results

The current study did not show any statistically significant difference in age as the mean age of the cases and the controls was 30±4.5 and 30±4.3 years, respectively (p=0.59). Conversely, there was no statistical difference in the median serum level of Gal-1 in the cases compared to the controls (1.19 *ng/ml*, 1.34 *ng/ml*, respectively, p= 0.087) (Table 1). In contrast, there was a highly significant difference in the median seminal plasma level of Gal-1 in the cases compared to the controls (0.97 *ng/ml*, 0.53 *ng/ml*, respectively, p= <0.001) (Table 1). The frequencies of the histopathological examination of testicular tissue are detailed in table 2. The current study showed a significant negative correlation between the seminal plasma level of Gal-1 and Johnsen's score (r= -0.281, p=0.048) (Table 3). Meanwhile, the current study did not show any correlation between the serum level of Gal-1 and Johnsen's score (r= 0.13, p=0.40) (Table 3). Interestingly, the receiver operating characteristic (ROC) curve demonstrated that the cutoff value of the seminal plasma level of Gal-1 in determining the degree of spermatogenesis impairment in NOA cases according to JTBS was >0.735 *ng/ml* and the area under the curve was 0.858 while the 95% confidence interval (CI) was 0.78-0.94; therefore, seminal plasma level can be used as a good predictor for determining the rate of sperm retrieval in NOA cases (Table 4, Figure 1). The sensitivity, specificity, positive predictive, and negative predictive values of the seminal plasma of Gal-1 were 76, 92, 90.5, and 79.3, respectively (Table 4, Figure 1).

On the other hand, the sensitivity, specificity, positive predictive, and negative predictive values of serum Gal-1 were 38, 66, 52.8, and 51.6, respectively (Table 4, Figure 2). Furthermore, the cutoff value of the serum Gal-1 in determining the degree of spermatogenesis impairment in NOA cases according to JTBS was >1.485 *ng/ml* and the area under the curve was 0.401 with 95% confidence interval (CI) of 0.276-0.526; therefore, serum Gal-1 is a poor predictor for determining the rate of sperm retrieval in NOA cases (Table 4, Figure 2).

Table 2. The frequency of different histopathological examinations in cases

Histopathology	Number	Percentage
Sertoli cell-only syndrome	37	77.08%
Arrest at spermatocyte stage	6	12.50%
Arrest at spermatid stage	5	10.41%

Table 3. Correlations between seminal plasma and serum levels of Gal-1, age, and Johnson's score

	Serum levels of Gal-1		Seminal plasma levels of Gal-1	
	r	p-value	r	p-value
Age (years)	-0.046	0.654	-0.28	0.78
Johnson's score	0.127	0.390	-0.281	0.048

r: spearman coefficient

Discussion

Our study demonstrated that seminal plasma level of Gal-1 was statistically higher in the cases compared to the controls. Also, seminal plasma level of Gal-1 was more sensitive and specific in determining the degree of spermatogenesis impairment in NOA cases. Moreover, seminal plasma level of Gal-1 had revealed significant correla-

Table 1. Descriptive data and Gal-1 levels in serum and seminal plasma of the participants

	Cases (48)			Healthy controls (50)			p-value
	Minimum	Maximum	Median	Minimum	Maximum	Median	
Gal-1 in serum (*ng/ml*)	0.9	1.77	1.19	1.26	1.6	1.34	0.087
Gal-1 in seminal plasma (*ng/ml*)	0.75	1.25	0.97	0.41	0.66	0.53	<0.001

p-values were calculated using Mann Whitney test

Table 4. Sensitivity and specificity of serum and seminal plasma levels of Gal-1 in determining sperm retrieval in NOA cases

	AUC	p-value	95% CI		Cutoff value	Sensitivity	Specificity	PPV	NPV
			Lower bound	Upper bound					
Gal-1 in serum (*ng/ml*)	0.401	0.087	0.276	0.526	>1.485	38	66	52.8	51.6
Gal-1 in seminal plasma (*ng/ml*)	0.858	<0.001	0.780	0.936	>0.735	76	92	90.5	79.3

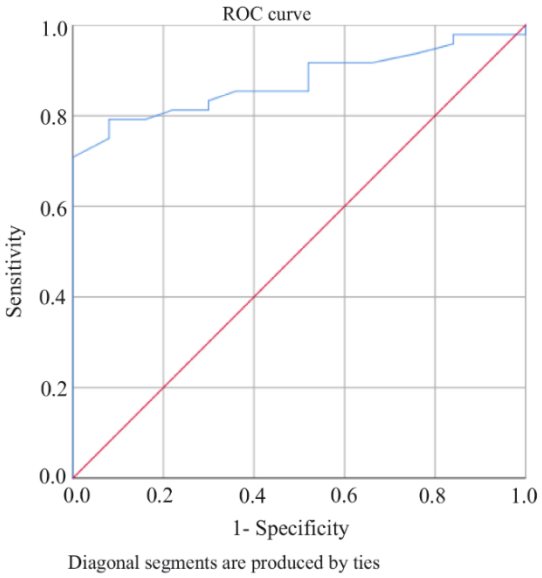

Figure 1. Receiver operating characteristic (ROC) curve. Sensitivity and specificity of seminal plasma levels of Gal-1 for predicting the severity of azoospermia

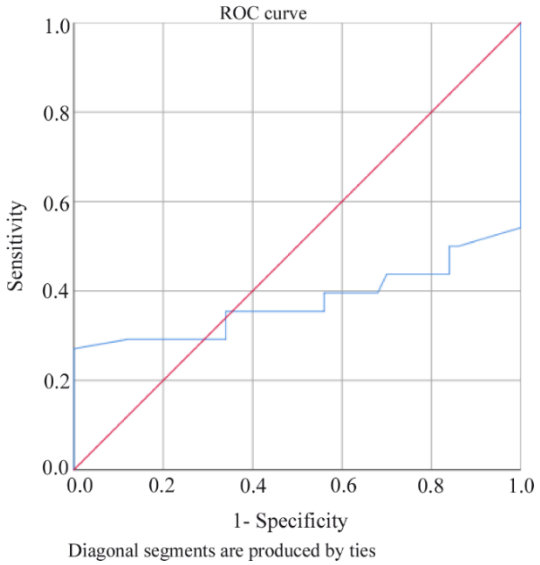

Figure 2. Receiver operating characteristic (ROC) curve. Sensitivity and specificity of serum levels of Gal-1 for predicting the severity of azoospermia

tion with JTBS score where the majority of the individuals in the current study were cases with Sertoli cell-only syndrome. In addition, there was a significant difference in the seminal plasma level of Gal-1 in the cases compared to the controls. These findings can be explained by the following facts. Gal-1 in rats is expressed mainly in Sertoli cells in stages 10-12 of the spermatogenic cycle (23). It can also be detected on the apical stalks of Sertoli cells and heads of mature spermatids (23). Following spermiation (stage 8), it is found at the basal segment of Sertoli cells, then it is expressed throughout the whole Sertoli cells along the maturation process of germ cells (23).

Thus, Dettin et al. concluded that Gal-1 is secreted mainly by Sertoli cells as they observed an intense immunological reaction throughout Sertoli cells in addition to the luminal pole of the seminiferous epithelium and the head of mature spermatids during the spermiation and it is superficially localized to spermatozoa in the caput epididymis (23). However, Gal-1 immunoreactivity was not detected in spermatocytes and round spermatids in the same study (23). Recently, Özbek et al. stated that Gal-1 and 3 might play a role in reproductive system maturation and main-tenance of the immune-privileged environment in the testis, due to their pro-apoptotic and anti-apoptotic functions (9). Interestingly, a human study conducted by Chui et al. indicated a high Gal-1 expression in the peritubular myoid cells in the human testis that surrounded the seminiferous tubules and affected the leucocytes in the interstitium (24). On the other hand, peritubular cells and macrophages were negative for Gal-1 while Sertoli, Leydig, and smooth muscle cells in the testis of the rats were positive for Gal-1 in the study conducted by Özbek et al. (9). Moreover, few studies demonstrated the developmental, cellular, and subcellular localizations of galectins in the reproductive

organs (22, 25-27). Additionally, a further explanation for the higher level of seminal plasma of Gal-1 in the cases compared to the healthy controls is that Gal-1 is secreted by the prostatic tissue (28). Another finding of the current study was the higher levels of the serum Gal-1 in the patients compared to their seminal plasma levels. This finding can be explained by that fact that Gal-1 was detected in Sertoli, Leydig and smooth muscle cells in the testis of the rats (9). Moreover, Dettin et al. observed that Gal-1 was secreted mainly by Sertoli cells as they showed an intense immunological reaction (23).

In contrast, serum Gal-1 is produced by organs other than the testis including thymus (11), spleen (12), smooth muscle (13), colon (14), ovary (15), and also the nervous system (16). Nevertheless, it should be noted that the seminal plasma of Gal-1 was more specific for determining the degree of spermatogenesis impairment in the current study. Admittedly, the major limitation of the current study can be the small target population. Also, the level of Gal-3 was not measured due to financial limitation so that the sensitivity and the specificity of Gal-1 and Gal-3 could not be compared for determining azoospermia. Finally, lack of immunohistochemistry evaluation of the patients' testes due to financial constraints was another limitation; therefore, sites for expression of Gal-1 in the human testes could not be exactly localized.

Conclusion

Seminal plasma levels of Gal-1 are higher in NOA men versus healthy controls. Interestingly, negative correlation of seminal plasma levels of Gal-1 with JTBS was determined. Thus, seminal plasma of Gal-1 can be used as a predictor for the rate of sperm retrieval in NOA cases.

Conflict of Interest

All authors declare no conflict of interest.

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