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Naael H Ali
College of Medicine, University of Basrah, Basrah, Iraq, naael.ali@uobasrah.edu.iq

Abdulkareem M Jewad
Department of Medical Laboratory Technology, College of Health and Medical Technology, Southern Technical University, Basrah, Iraq

Attayia K Attayia
Department of Medical Laboratory Technology, College of Health and Medical Technology, Southern Technical University, Basrah, Iraq

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ORIGINAL STUDY

Investigate the Seminal Plasma Biomarkers in Differentiation Between Obstructive- and Non-obstructive Azoospermia

Naael H. Ali [a,*], Attayia K. Attayia [b], Abdulkareem M. Jewad [b]

[a] College of Medicine, University of Basrah, Basrah, Iraq
[b] Department of Medical Laboratory Technology, College of Health and Medical Technology, Southern Technical University, Basrah, Iraq

Abstract

Background: **Azoospermia is a very severe form infertility in males. Today, clinicians depend on semen analyses for the prediction of the reproductive potential of males, and testicular biopsies are the only reliable methods for diagnosing various subtypes of azoospermia. In the recent times, advances in proteomics contributed to look for new infertility biomarkers in males whose seminal plasma proteins are rich with proteins of various genital tract origins. Therefore, the fields of proteomics helped to develop new infertility biomarkers in males. Extracellular Matrix Protein 1 and Seminal plasma proteins Testis Expressed Protein 101 assays currently existing of are under the last development for the purpose of clinical uses. Therefore, this study aimed to assesses the capability of Testis Expressed Protein 101 and Extracellular Matrix Protein 1, Seminal Plasma proteins, for differential diagnosing between Obstructive azoospermia from non-obstructive azoospermia by noninvasive methods. A case control study was conducted on 40 infertile azoospermia males who were clinically examined, having their seminal fluid analyzed and hormonally investigated and SP proteins Testis Expressed Protein 101 and Extracellular Matrix Protein 1 were assessed. Other fifty healthy fertile men were included as control group.**

Results: **The studied biomarkers were significantly lower in non-obstructive azoospermia then Obstructive azoospermia were had higher level, and the control group participants were higher than the others.**

Conclusion: **Extracellular Matrix Protein 1 and Testis-expressed 101 protein should combined and could make a differences between non-obstructive azoospermia and obstructive azoospermia thus eliminating most of the diagnostic testicular biopsies. Seminal plasma needs for pre-treatment before processing for diagnose any biomarker specially to differentiate between obstructive azoospermia and non-obstructive azoospermia. By the cutoff value of 2.3 μg/ml for Extracellular Matrix Protein 1 obstructive azoospermia and normal spermatogenesis are distinguished with 100% specificities, and obstructive azoospermia from non-obstructive azoospermia with 73% specificities, 100% sensitivities. Yet Testis Expressed Protein 101 lack could help distinguishing the non-obstructive azoospermia in specifics.**

Keywords: Azoospermia, Obstructive azoospermia, Non-obstructive azoospermia, TEX101, ECM1

1. Introduction

Men are responsible for more than fifty percent of infertility cases among couples suffering from infertility worldwide. These cases range from oligozoospermia to azoospermia, which is diagnosed in about two percent infertile men in the general population [1]. Two forms of azoospermia; non-obstructive azoospermia (NOA) and obstructive azoospermia (OA). The main cause for the first is results from pathological changes in epididymis or congenital anomalies because of the physical obstructions in reproductive tracts [2], while the NOS is failure of testicular function in production of sperms, sub classified, according to the testicular tissue histopathological examinations, into; maturation arrest (MA) hypospermatogenesis (HS); and Sertoli cell−only syndrome (SCO) [3,4]. Currently, the invasive, surgical procedure (testicular biopsy) is the only definitive diagnostic method to distinguish between OA and NOA. Also it is used to identify the NOA subtypes. However, this invasive approach for

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* Corresponding author.
E-mail addresses: naael.ali@uobasrah.edu.iq (N.H. Ali), shatalarabmedlab@yahoo.com (A.K. Attayia), drkreem.mohammed@stu.edu.iq (A.M. Jewad).

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differentiating OA from NOA by surgical examinations of random testicular tissues could not be an accurate reflection of the NOA histopathology as spatial distributions of spermatogenesis. Also testicular biopsy procedure may have possible complications such as; tissue damage; evoke of anti-sperm antibodies; bleeding; and chronic pain. It may need for more than one surgical procedure.

A marker test for differentiation of azoospermia patterns, that is noninvasive, is an unmet need in infertility treatment. Such test could reduce testicular biopsy, provide more accurate assessment of histopathological patterns, and facilitate better planning for assisted reproduction. Some biomarkers are already delivered [5]. Yet, there is a need to new biomarkers with better diagnostic sensitivities for the differentiation between hypospermatogenesis, maturation arrests, and Sertoli cell-only syndromes of NOA.

The SP is utilized as a non-invasive and suitable clinical samples to diagnose disorders in reproductive system of males. Seminal plasma (SP) proteomic analyses and/or spermatozoa could supply data in terms of presence and abundance of proteins (biomarkers) and its post-translational modifications. It was found that SP is made of 3200 proteins from different genital organs [6]. In the same year (2013) Rolland AD with his collogues [7] found about 2545 distinct proteins in the SP of human. Then they profiled the gene expression of the candidate protein markers, 83 in testis, 42 in epididymis, 7 in seminal vesicle and 17 in prostates. The stringent blood-testis and blood-epididymis barriers make these biomarkers not found in other parts of the body. Thus, SP from seminal fluid is still the only available fluids for the non-invasive diagnosis of male infertility [8]. TEX- 101 (Testis-expressed sequence 101 protein) is a membrane glycoprotein. Additionally, its specific expression in germ cells with no expressions in other tissues and cells may cleave from the spermatozoa surfaces and releasing into seminal plasma when the sperm maturate into epididymis, and ECM1 (Extracellular matrix protein 1), known as main infertility biomarkers in males [9].

In the present study we aimed to evaluate the diagnostic ability of TEX101 and ECM1 biomarkers to distinguish (noninvasively) between OA and NOA in view of set of seminal hormones used for azoospermia patients.

2. Materials and methods

Study population: A total of forty infertile men with azoospermia (normal female partner) were enrolled in this case control study through July, 2021 to Jan, 2022. They were diagnosed after testicular biopsy surgery and histopathology exam were done to diagnose obstructive azoospermia or non-obstructive azoospermia. Twenty of them with obstructive azoospermia aged (25–50) years and the others with non-obstructive azoospermia aged (22–51) years. Other fifty fertile men aged (19–50) years, were enrolled in the study as control group. The patients were referred to the Infertility center, Basrah Teaching Hospital, Basrah, Iraq.

Samples and processing: Routine semen analyses was performed according to the Stander method recommended by the World Health Organization [10]. Samples were left for 20–60 min for liquefaction to occur. Then semen quality was evaluated by using two parameters: macroscopically and microscopic examination.

All patients examined in details for the identification of the etiology of azoospermia, such as the history, physical investigation, semen analysis. Semen hormone profile (FSH, LH, Testosterones, Prolactin, Melatonin, ECM1, TEX 101, AMH). Audiologist with the help of an orchidometer to calculate the bilateral testicular size.

In case of Azoospermia, samples with sperm count was zero reference limit were used. Samples of related conditions, such as oligoasthenozoospermia, oligoasthenoteratozoospermia or oligoteratozoospermia were excluded.

TEX101 and ECM1 level measured by ELISA Kits (Bioassay Technology Laboratory, China, Cat. No: E4614Hu). Seminal hormones levels were by enzyme linked fluorescent assay kits (VIDAS®, BIOMÉRIEUX, France, catalog number 417011).

3. Ethical issue, approval and official permission

Prior to data collection, we obtained a signed consent from all participants on the explanation of the aims of the work ensuring data privacy.

To conduct the present study, the protocol was reviewed and approved from the Basrah Health Department, Ministry of Health. The permission was obtained from them.

3.1. Statistical analysis

The SPSS software for windows, version 26 was used to analyse the data. The data of the current work was nonparametric. So, the statistical methods match the data. The descriptive statistics were median to the quantitative data, frequencies and percentages (%) based on the kinds of the variables. We

used Chi-square and Fishers Exact Test for the comparison of the proportion and frequencies and Kruskal Wallis Test for the comparison of the median of various investigation groups, significance level (P value) of ≤0.05 is significant. We used Spearman Test was to find correlations between variables.

4. Results

No significant differences (P > 0.05) were found between three groups of the study in age and all seminal hormones (FSH, LH, testosterone, and prolactin), while there was significant difference (P <0.05) in AMH and melatonin hormones (Table 1).

Table 2 shows the descriptive (median, min–max) and statistical differences of TEX101 and ECM1 in patients and control groups. Significant difference between groups in TEX101 and ECM1 were resulted. Its shows that healthy control group had higher level of these markers while the NOA were in the lowest level.

Table 3 shows the Spearman's correlations between age, hormones, and seminal biomarkers TEX101 and ECM1 in NOA patients group.

Resulted that FSH correlated with melatonin (R = 0.511, P = 0.021), and with TEX101 (R = − 0.501, P = 211) (Fig. 2). Testosterone with ECM1 (R = 0.503, P = 0.024) (Fig. 1).

Table 4 shows the Spearman's correlations between age, hormones, and seminal biomarkers TEX101 and ECM1 in OA patients group. Resulted that only prolactin correlated with age (R = 473, P = 0.035).

Table 5 shows the results of histopathological investigation for NOA subtypes.

Table 2. Descriptive and differences of TEX101 and ECM1 in patients and control groups.

Test		OA (20)	NOA (20)	Control (50)	p-value
ECM1 (μg/ml)	Median	21	1.75	44.5	P < 0.05
	Min-Max	4–45	1–3	31–61	
TEX101 (ng/ml)	Median	97	6.25	342.0	P < 0.05
	Min-Max	78–125	3–7	127–376	

5. Discussion

Considerable percent of infertility cases in men remain unknown, despite the evoked factors on male reproduction including environmental, genetic, and life styles. Defective in spermiogenesis when found in the routine semen analysis which reveal no spermatozoa in semen, a case known as azoospermia.

About 2–8% of males with fertility deficits show azoospermia [4] due to an obstructed or discontinuous of male genital tract (OA) or a failure of the testis to start or keep spermatogenesis because of the endogenous or exogenous abnormalities (NOA).

In view of limited diagnostic tools, always depends on a standard semen analysis. So the patients and clinicians require extra diagnostic biomarkers. Seminal plasma components originate from many sites in the male reproductive tract. It is harbor shedding antigens of sperms and provides a nutritive and protective milieu.

In the current study the azoospermia diagnosis by high-powered microscopic tests of centrifuged seminal fluids on at least two cases. Then the patients with azoospermia were evaluated for identifying the etiology of the patient's condition. For exclusion criteria, complete medical history

Table 1. Descriptive and statistical differences of age and hormonal profile in azoospermic patients and control groups.

Test		NOA (20)	OA (20)	Control (50)	p- value
Age	Median	34.0	35.5	33.0	P > 0.05
	Min-Max	22–50	25–50	19–15	
FSH	Median	3.4	2.5	2.2	P > 0.05
	Min-Max	1.3–3.8	2.13–4.8	1.8–5.7	
AMH	Median	0.2	0.15	0.64	P < 0.05[a]
	Min-Max	0.08–0.89	0.1–0.33	0.21–0.99	
LH	Median	2.3	1.7	1.3	P > 0.05
	Min-Max	0.63–2.90	1.2–4.4	0.12–1.90	
Testosterones	Median	2.5	4.25	4.2	P > 0.05
	Min-Max	1.3–3.8	3.6–4.9	3.60–4.90	
Melatonin	Median	25.5	32.5	39	P < 0.05[a]
	Min-Max	17–34	23–42	34–48	
Prolactin	Median	11.8	8.1	9.63	P > 0.05
	Min-Max	10.3–13.0	7.4–11.6	6.5–10.9	

[a] Significant by Kruskal–Wallis Test.

Table 3. Spearman's correlations between age, hormones, and seminal biomarkers in NOA patients group.

Patient group			FSH	AMH (ng/ml)	LH	Testosteron (ng/ml)	Prolactin (ng/ml)	Melatonin (Pg/ml)	ECM1 (μg/ml)	TEX 101 (ng/ml)
Non-Obstructive Azoospermia	Age (year)	R	.429	.280	.036	−.142-	−.293	.190	−.197	.175
		Sig.	.059	.231	.881	.549	.210	.422	.405	.459
	FSH	R		−.031	.039	.440	.012	.511	−.069	−.501
		Sig.		.895	.869	.052	.959	.021*	.771	.0211*
	AMH	R			−.089-	−.019-	.201	.126	.196	.276
	(ng/ml)	Sig.			.708	.938	.395	.596	.408	.239
	LH	R				.305	−.218	.098	.346	−.444
		Sig.				.191	.356	.681	.135	.050
	Testosterone	R					.004	.219	.503	−.388
	(ng/ml)	Sig.					.987	.354	.024*	.071
	Prolactin	R						−.098	−.106	.155
	(ng/ml)	Sig.						.680	.656	.515
	Melatonin	R							−.325	−.396
	(Pg/ml)	Sig.							.162	.084
	ECM1	R								−.249
	(μg/ml)	Sig.								.290

includes; childhood diseases such genital trauma or prior pelvic or inguinal surgery; as viral orchitis or cryptorchidism; radiation exposure therapy/ chemotherapy and infections including epididymitis or urethritis; a very late fever or heat exposure and current medications; and family past records. Physical examination includes; testis sizes (normal testis volume greater than 19 ml) and consistency; secondary sex characteristics; and consistent vasa differentia and epididymides; a varicocele; and masses on digital rectal tests. For hormone examination, seminal plasma level of hormones was measured by enzyme linked fluorescent assay kits (VIDAS®, BIOMÉRIEUX, France) which have high sensitivity. All This initial evaluation work was done to clarify the underlying cause of the azoospermia, and whether this cause of azoospermia is amenable to therapy. The evaluation for causes results were divided into: pre-testicular, testicular and post-testicular. Endocrine abnormalities were the cause for pretesticular causes of azoospermia [11]. Disorders of spermatogenesis related to testicular etiologies, while post-testicular etiologies were due to either ejaculatory dysfunction or obstruction of sperm delivery to the urethral meatus. The last cause we found in 40% of azoospermia patients. Because it is a protein which epididymis discharges in the semen mainly, the ECM1 biomarkers is used

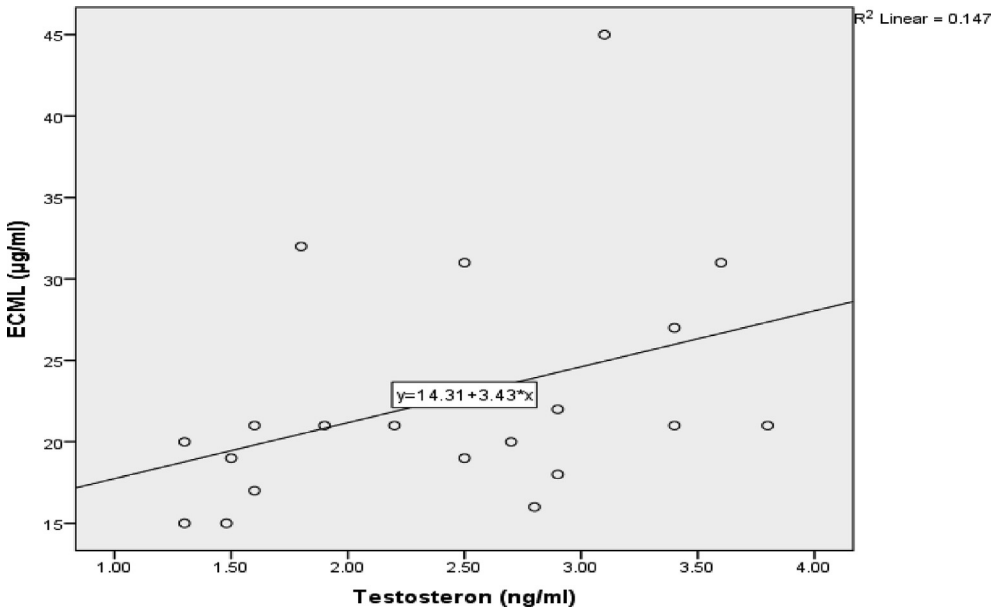

Fig. 1. Regression figure of testosterone and ECM1 in NOA patients.

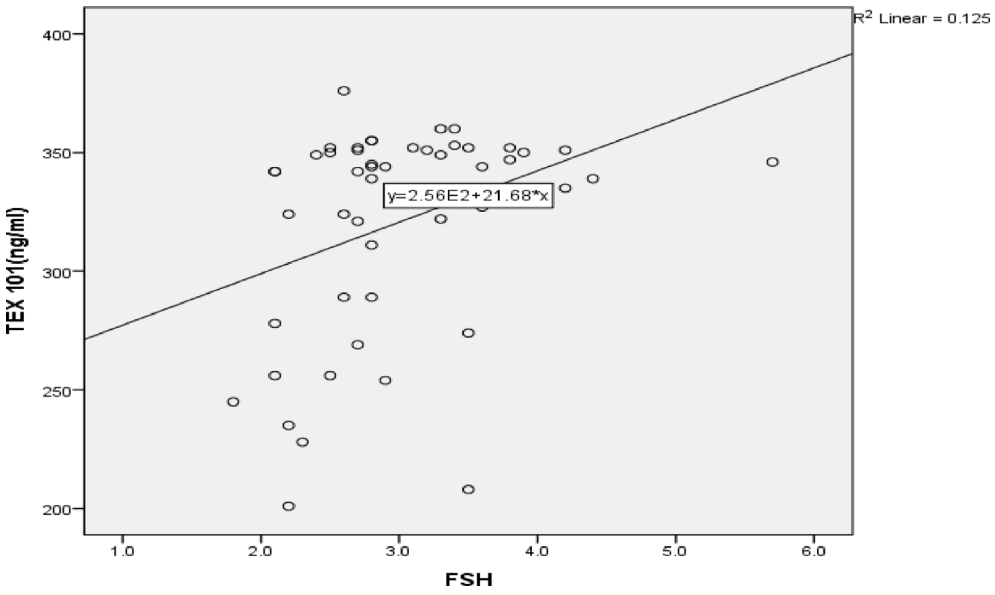

Fig. 2. Regression figure of FSH and TEX101 in NOA.

to different NOA from OA. Similarly, proteins which have specific expressions in testis include TEX101 appearing as biomarkers for NOA [11].

In the result of the present study, the biomarkers ECM1 and TEX101 were differ significantly ($P < 0.05$) between the three studded groups, the median of each biomarker was higher in healthy control group than OA and NOA group level [12]. Many researchers resulted by a seminal plasma proteomic analyses show no specific proteins to operate the sperms and proteins in azoospermia patients like both ECM1 and TEX101 seminal plasma levels of with high significance in males with NOA than males having OA [13]. According to other researchers [14], TEX101 alone was no strong markers for the non-invasive differentiation of NOA from OA (32% sensitivity and 99% specificity). Thus, combining epididymis-specific protein ECM1 and TEX101 may help differentiating NOA from OA and thus eliminating most diagnostic testicular biopsies. Furthermore, identifying the specific proteins from testis or from germ cell which spermatocytes, spermatids, or spermatozoa discharge into semen exclusively could be a provider of markers to accurately show the stages of spermatogenesis. These findings agree with other scholars [9,15,16], reporting that testis-expressed protein 101 as the biomarker for azoospermia and extracellular matrix

Table 4. Spearman's correlations between age, hormones, and seminal biomarkers in OA patients group.

Type of disease			FSH	AMH (ng/ml)	LH	Testosteron (ng/ml)	Prolactin (ng/ml)	Melatonin (Pg/ml)	ECM1 (μg/ml)	TEX 101 (ng/ml)
Obstructive Azoospermia	Age (year)	R	.108	.103	−.061-	−.230-	.473	−.280	−.348	.062
		Sig.	.652	.666	.797	.329	.035	.232	.132	.796
	FSH	R		.255	−.047-	−.275-	.080	.253	.088	.136
		Sig.	.	.278	.844	.241	.737	.282	.712	.566
	AMH	R			.280	.004	−.139	−.112	−.052	−.022
	(ng/ml)	Sig.			.233	.988	.560	.639	.829	.926
	LH	R				.147	−.190	−.087	−.165	−.183
		Sig.				.536	.423	.715	.487	.439
	Testosteron	R					−.113	−.048	−.047	.081
	(ng/ml)	Sig.					.635	.840	.843	.734
	Prolactin	R						.317	−.069	−.006
	(ng/ml)	Sig.						.173	.771	.981
	Melatonin	R							.103	.081
	(Pg/ml)	Sig.							.667	.733
	ECM1	R								.068
	(μg/ml)	Sig.								.776

Table 5. Histopathological investigation for NOA patients.

Histopathological Exam	NOA	%
Sertoli cell–only syndrome	3	15
maturation arrest	5	25
hypospermatogenesis	6	30
Mixed	6	30
TOTAL	20	100

protein 1 differentiating NOA and post-vasectomy males with threshold values of 2.3 ng/mL.

Angiotensin-converting enzyme (ACE) TEX 101 regulation on spermatozoa and removing GPI-anchored protein played a vital role in the production of fertile spermatozoa, and ACE operation did no rely done its well-known peptidase activities. This means TEX101 has unique specific substrate providing a potential targets to produce an awaited contraceptive medicines for males [17].

It is worthy to mention her that SP was not traditional to clinically diagnose as it may hamper the immunoassay performances because it has molecules features including fast protease-mediated liquefactions, high viscosity, and big quantity of seminal macrovesicles [3]. So, SP could require more treatment procedures for facilitating the quantification of the protein with high sensitivities. For enhancing ELISA sensitivities, multiple combinations of detergents, temperatures, and pH values are required for the selection of two SP pretreatment protocols: treatment of seminal plasma with guanidinium at pH 12 or deoxycholate at 63 °C may lead to the release of TEX101 from its vesicles [18].

Other authorities noticed in the present study that ejaculatory dysfunction or ejaculatory duct obstruction may be found in those with azoospermic with normal sized testes and low semen volume. Therefore, the FSH is useful and critical factors to determine of diagnostic testicular biopsies are required for establishing the existence or lack of normal spermatogenesis [13]. If FSH elevated two times than normal limit, abnormal spermatogenesis is the diagnosis. Thus, diagnostic testicular biopsies are not required these patients. Yet, if sperm retrievals with ICSI are taken into account, a testicular biopsies could be conducted for prognostic aims, for determining if spermatozoa which could possibly retrieved by later testicular sperm aspirations or extractions [19].

Significant difference in AMH in three studied groups, The NOA have very low seminal plasma concentration. It is well known that AMH produced by Sertoli cells and it is responsible for the regression of Mullerian ducts in male embryo. Yet, AMH mutations or its specific receptors in humans, or disruptions of these genes in rodents showed no key function(s) for postnatal AMH actions. Persistent Mullerian duct syndrome is very uncommon in male pseudo-hermaphroditism with continues occurrence in Mullerian derivations in otherwise usually virilized men [20]. Therefore, it is suggested that there are relationships between AMH secretion relation with, Sertoli cell function, a seminal marker for spermatogenesis [21]. In addition, there was a positive correlation between the total AMH contents in seminal plasma with sperm concentrations and number. However, the reports in healthy males on the relationship between spermatogenic parameters and circulations of AMH levels are not consistent, and post pubertal AMH secretion is still no clear requiring other examinations.

It is recommended here that assays for ECM1 and TEX101 could replace the majority of the diagnostic testicular biopsies facilitating the predicting sperm retrieval procedure results. So, the increase in the reliabilities and successes of helped reproduction approaches.

6. Conclusion

Combining extracellular matrix protein 1 (ECM1) and testis-expressed sequence 101 protein (TEX101) may help to make a difference between NOA and OA. It this help in eliminating most diagnostic testicular biopsies. Seminal plasma required pre-treatments pre-processing for diagnosing any biomarker special for the differentiation between OA and NOA.

Ethics approvals and consents for participation

We obtained written informed consents from the patients and control subjects before sampling and processing.

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Conflict of interest

All authors declared no conflict of interest.

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