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Evaluation of the Clinical Role of Testis Expressed Protein 101 (TEX101) and Extracellular Matrix Protein 1 (ECM1) as Novel Predictive Markers in Relevance to Testicular Sperm Retrieval and Differentiation of Obstructive from Non-Obstructive Azoospermia

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ABSTRACT

Seminal Plasma (SP) proteins are rich with many proteins of different genital tract origin so the fields of proteomics were promise for the development of novel male infertility biomarkers. Seminal plasma proteins Testis Expressed Protein 101 (TEX101) and Extracellular Matrix Protein 1 (ECM1) assay are already available or under final development for clinical use, so the aim of study, evaluation of TEX101 and ECM1 Seminal Plasma (SP) proteins for assessment the predictive of Sperm Retrieval Rate (SRR) in testicular sperm retrieval and diagnosis obstructive from non-obstructive azoospermia.

A case control study was included 65 infertile azoospermic men were subjected to clinical examination, seminal fluid analysis, hormonal investigation and SP proteins TEX101 and ECM1 assessment by Enzyme Linked Immuno-Sorbent Assay (ELISA) as well as they were subjected to the conventional Testicular Sperm Extraction (TESE) technique, mincing with searching for sperm.

The results of study included mean age of 65 men were recorded 33.37 ± 6.99 years which were divided into 10 (15.38%) Obstructive Type (OA) and 55 (84.62) Non-Obstructive (NOA) type. The SRR account 36 out of 65 patients (55.4%) were divided into OA (100%) and NOA (47.3%) and the difference was significant (P=0.014). The TEX101 and ECM1 were a significantly (P<0.001 and P=0.007, respectively), higher in NOA than OA. The receiver operating characteristic curve or ROC curve show that the SP TEX101 cut-off values above 0.9 ng/ml is candidate to sperm retrieval technique. The ECM1 protein, the cut-off values (>943.11 pg/ml for differentiation of NOA versus OA.

Keywords: Proteomics, TEX101, ECM1, Sperm retrieval technique, Reproductive hormones, Testicular histopathology

INTRODUCTION

Infertility is a common condition among men and women and it is inability to achieve pregnancy within 12 months of a regular unprotected intercourse. It is occurred in 15% of the cases of the reproductive aged couples [1,2]. Infertility may be related to a female factor, a male factor, a combination of both or it may be unexplained. Two thirds of the cases are attributed to male factors [3].

The clinical categories of male infertility range from lowered production of sperm (oligozoospermia) to severe cases of azoospermia with non-measurable levels of sperm in semen [4].

Azoospermia affects about 1% of all men and 15% of infertile men [5]. It is absent of spermatozoa in the semen sample following the standard seminal fluid analysis as recommended by the World Health Organization (WHO). When spermatozoa are absent in the wet preparation, an examination of the centrifuged sample (3000x g) for 15 min is recommended. Otherwise, no sperm are observed in the

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centrifuged sample on at least two occasions as azoospermia [6].

Azoospermia is classified to Non-Obstructive (NOA) and Obstructive Azoospermia (OA). Based on histological evaluation of testicular tissue, the NOA subtype is further classified into Hypo Spermatogenesis (HS), Maturation Arrest (MA) and Sertoli Cell-Only syndrome (SCO) [7].

Obstructive azoospermia results from physical obstruction in the male reproductive tract due to congenital or acquired defects in the epididymis or vas deferens [8].

The level of spermatogenesis and the presence of sperm in the testis are diagnosed by testicular biopsy till now it was a standard tool for differential diagnosis of azoospermia [9]. However, it is an invasive surgical procedure with potential complications. So that there is an important need for substitute, non-invasive procedures for differential diagnosis of azoospermia of male infertility and further classification of its subtypes.

The SP is derived from male reproductive organs which was rich with epididymis and testis-derived proteins, mRNA and metabolites. It has been used as a suitable clinical sample for the non-invasive diagnosis of a wide range of male reproductive system disorders [7,10].

The SP composed of 3200 proteins secreted by different genital organ origin like testes, epididymis, prostate, seminal vesicles and Cowper's glands and these are directly involved in the production and maturation of sperm or in the interaction with the zona pellucida and fusion with oocytes [11-13].

Testis-specific biomarkers are not found in other biological fluid like blood due to stringent blood-testis and blood-epididymis barriers, semen and SP remain the only available fluids for the non-invasive diagnosis of male infertility [14,15].

The new research in the subject of proteomics may be promised for the advancement of novel male infertility biomarkers.

The SP protein-based assessment of Tests Express protein 1 (TEX101) and epididydmal specific protein 1 or Extracellular Matrix protein 1 (ECM1) are already discovered and under final development for clinical use. Immunoassays of ECM1 and TEX101 have the potential to roll out most of the histopathological diagnosis of testicular biopsies and TESE procedures for patients with azoospermia also to facilitate prediction of the outcome of sperm retrieval procedures used for assisted reproduction and to reduce the total cost of azoospermia diagnosis.

MATERIALS AND METHODS

A case control study included 65 infertile azoospermic males were manifested as male factor infertility (normal female partners) according to history, examination and investigation, in the period from November 2017 till January 2019 at the Male Infertility Clinic of High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, the patients were undergone a detailed history, clinical examination and laboratory investigation such as seminal fluid analysis, hormonal and seminal plasma proteins (TEX101, ECM1) assessment and then they were subjected to the testicular biopsy after written consent of patients.

Seminal fluid analysis is performed before and after centrifugation to confirmed azoospermia as well as seminal plasma collection and freezing to be thawed litter for assessment seminal plasma proteins TEX101 and ECM1 by Enzyme Linked Immuno-Sorbent Assay (ELISA).

Testicular biopsies were planned to be separated into two samples one subjected to mincing and searching for sperm then cryopreservation to be used for ICSI, the other sample sent to pathologist for histopathological diagnosis of azoospermic types.

STATISTICAL ANALYSIS

Data were collected, summarized, analyzed and presented using Statistical Package for Social Sciences (SPSS) version 23 and Microsoft Office Excel 2010. Qualitative (categorical) variables were expressed as number and percentage, whereas, quantitative (numeric) variables were first evaluated for normality distribution using Kolmogorov-Smirnov test and then accordingly normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion), while those numeric variables that are not normally distributed were expressed as median (an index of central tendency) and inter-quartile range (an index of dispersion). The following statistical tests were used: Chi-square test was use to evaluate association between any two categorical variables provided that less than 20% of cells have expected count of less than 5. However, Fischer exact test was used instead when chi-square test was not valid (in case that more than 20% of cells have expected count of less than 5). Independent samples t-test was used to evaluate the difference in mean of numeric variables between any two groups provided that these variables were normally distributed; otherwise Mann Whitney U test would be used instead if those variables were not normally distributed. One way Analysis of Variance (ANOVA) was used to evaluate difference in mean of numeric variables among more than two groups provided that these numeric variables were normally distributed; but Kruskal Wallis test was chosen in case of non-normally distributed variables. One way ANOVA was followed by post-hoc LSD test to evaluate individual differences in mean values between any two groups among groups tested primarily using one way

ANOVA; whereas, Kruskal Wallis test was followed by Mann Whitney U test for the same purpose in case of non-normally distributed numeric variables.

RESULTS

The results of study included mean age of 65 men were recorded 33.37 ± 6.99 years which were divided into 10 (15.38%) obstructive type (OA) and 55 (84.62) non-obstructive (NOA) type as in **Table 1**.

Table 1. General characteristic of the study sample.

Characteristics	Values
Sample size	65
Age (years)	
Range (min-max)	26 (22-48)
Mean ± SD	33.37 ± 6.99
Type of azoospermia	
Obstructive, *n* (%)	10 (15.38)
Non-obstructive, *n* (%)	55 (84.62)

min: minimum; max: maximum; SD: Standard Deviation; IQR: Inter-Quartile Range

The SRR account 36 out of 65 patients (55.4%) were divided into OA (100%) and NOA (47.3%) and the difference was significant (*P*=0.014) as in **Table 2**.

Table 2. Sperm retrieval rate according to type of azoospermia.

Sperm retrieval	Total *n*=65	Obstructive azoospermia *n*=10	Non-obstructive azoospermia *n*=55	*P**
Positive, *n* (%)	36 (55.4)	10 (100.0)	26 (47.3)	0.014S
Negative, *n* (%)	29 (44.6)	0 (0)	29 (52.7)	

n: number of cases; *: Fischer exact test; S: significant at P ≤ 0.05

The TEX101 and ECM1 were a significantly (*P*<0.001 and *P*=0.007, respectively), higher in NOA than OA as in **Table 3**. The SP level of TEX101 was statistically highly significant (*P*=0.005), being higher in men with positive sperm retrieval 1.48 (1.55) ng/ml than negative sperm retrieval 0.31 (1.35) ng/ml as shown in **Table 4**. The ROC show that the SP TEX101 cut-off values above 0.9 ng/ml is candidate to sperm retrieval technique with CI equal to 57.8%-81%, Sensitivity (66.7%) and Specificity (69.0%) as in **Table 5 and Figure 1**.

Table 3. ECM1 and TEX101 in azoospermia men.

Variables	Total *n*=65	Obstructive *n*=10	Non-obstructive *n*=55	*P*
ECM1 pg/ml	1530.30 (857.99)	469.60 (737.29)	1629.10 (458.13)	<0.001 HS
TEX101 ng/ml	1.05 (1.56)	0.22 (0.52)	1.44 (1.63)	0.007 HS

Table 4. ECM1 and TX101 according to sperm retrieval outcome.

Variables	**Positive sperm retrieval** ***n*=26**	**Negative sperm retrieval** ***n*=29**	***P***
ECM1 (pg/ml)	1492.40 (1026.06)	1613.20 (431.12)	0.180 NS
Tex101 (ng/ml)	1.48 (1.55)	0.31 (1.35)	0.005 S

Table 5. Characteristics of the ROC curve.

Characteristics	**TEX101**
Cut-off value	>0.92
Area Under the Curve (AUC)	0.703
Accuracy	70.3%
95 % Confidence Interval (CI)	0.577-0.810
P	0.002 HS
Sensitivity	66.7%
Specificity	69.0%

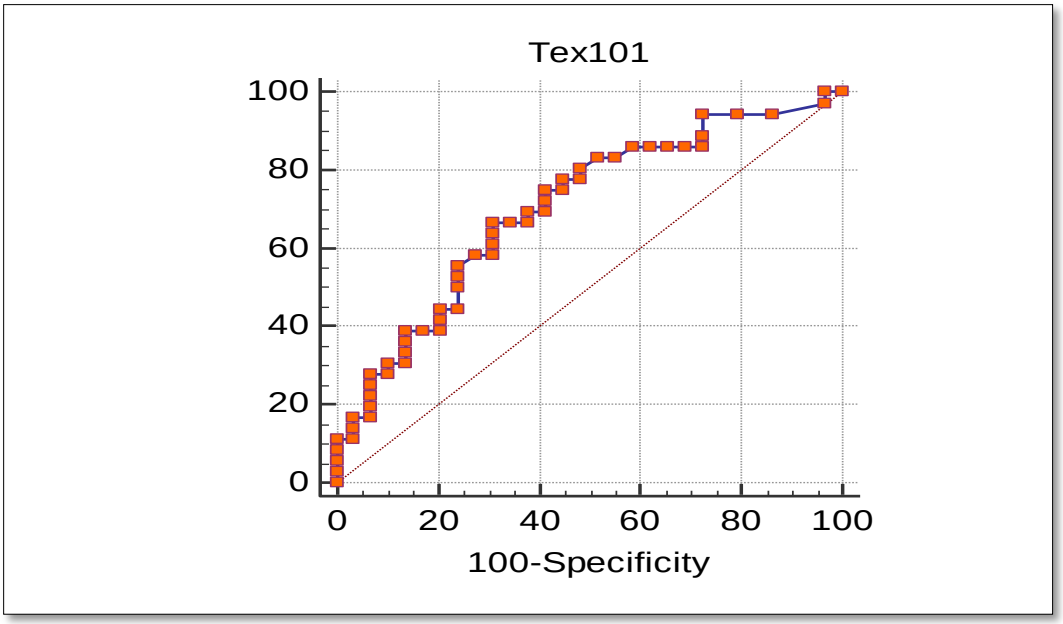

Figure 1. Receiver operator characteristic (ROC) curve to identify serum TEX101 cut-off values that predict positive sperm retrieval.

To test the validity of ECM1 and TEX101 in the differentiation between obstructive and non-obstructive azoospermia an ROC analysis was carried out and the results are shown in **Figure 2 and Table 6**. ECM1 cut-off value was >943.11 pg/ml with a sensitivity rate of 87.3% and specificity rate of 90%. In addition, the accuracy rate was 87.1%. On the other hand, TEX101 cut-off value was >0.79 ng/ml with a sensitivity rate of 61.8% and specificity rate of 90%. Moreover the accuracy rate was 76.9%. In both situations the level of significance was high (p<0.001).

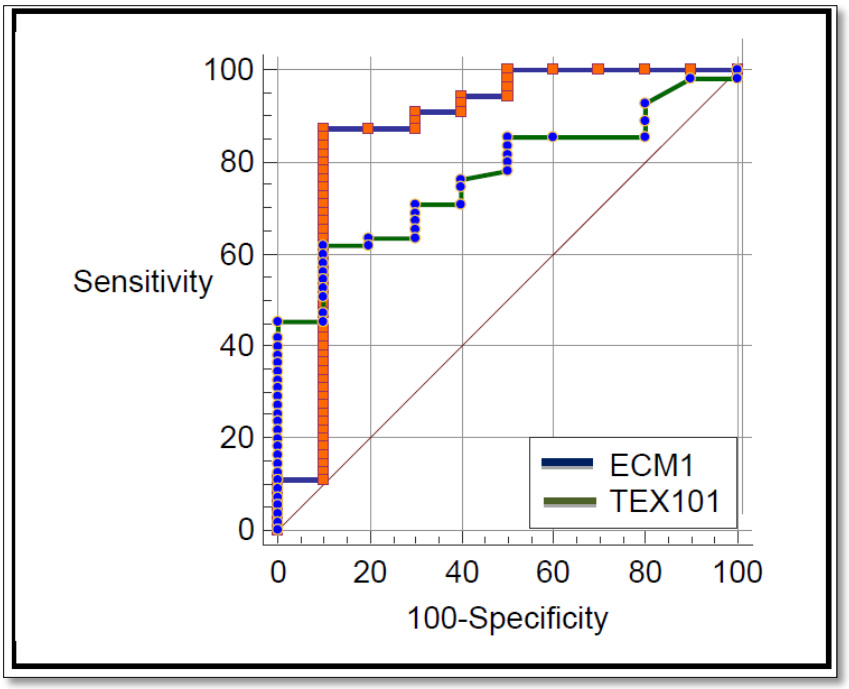

Figure 2. ROC analysis to find ECM1 and TEX 101 cut-off values that predict non obstructive azoospermia versus obstructive azoospermia.

Table 6. Characteristics of the ROC curve.

Characteristics	ECM1 pg/ml	TEX101 ng/ml
Cut-off value	>943.11	>0.79
Area Under the Curve (AUC)	0.871	0.769
Accuracy	87.1	76.9%
95 % Confidence Interval (CI)	0.765-0.941	0.648 to 0.865
P	<0.001	<0.001
Sensitivity	87.3%	61.8%
Specificity	90.0%	90.0%

DISCUSSION

The age of the patients enrolling in the study was ranging from 22 to 48 years with a mean age of 33.37 ± 6.99 years which is nearly similar to that reported by other literature's [16,17] who conducted a study on 60 and 76 azoospermic patients, they reported a mean age of patients are 33.32 ± 7.55 years and 35.1 ± 60 years, respectively.

In addition to the other literatures, it was approximately as same the mean age of azoospermic patients, 35.5 ± 8.30 years and 33.38 ± 7.44 years [18,19] they were studied 451 and 20 azoospermic patients, respectively.

About the type of azoospermia the presented study was included 15.38% obstructive type and 84.62% non-obstructive type, which is nearly the same the result of previous literatures [20-22] the OA is less common than NOA and occurs in 15-20% of men with azoospermia, whereas other study reported that NOA is diagnosed in 49% to 93% and post-testicular obstruction or retrograde ejaculation are estimated to affect from 7% to 51% of azoospermic men of azoospermic patients [23,24].

With respect to OA (normaspermatogensis) the results of Rashed et al. [25] were approximately similar to current observation, in which, the cases of normal spermatogenesis were 15% and 24%, however other study there was a higher incidence (28%) of normal spermatogenesis [26].

Regardless of the underlying etiology, management of patients with azoospermia usually relies upon the recovery of spermatozoa with a testicular biopsy/sperm extraction

procedure and a successful *in vitro* fertilization with intra-cytoplasmic sperm injection, so that one of the effective parameters that should be considered in the management of azoospermic patients is the ability to predict the rate of spermatozoa recovery in these patients. Understanding these parameters is also important for counseling the patient and his wife [27].

Regarding the SRRs, the other study was reported SRRs of 16.7-45% by conventional TESE (cTESE) [27].

Salehi et al. [28] showed that the overall mean rate of SRR was 48.8%, which was approximately same as current observation.

Abdel Rahem et al. [29] were studied, 112 patients had obstructive azoospermia and 276 patients had NOA, it reported all patients in the obstructed group had a positive sperm while the sperm retrieval rate for the NOA group was 50%.which is nearly same as observation of under current study. The study of Cissen et al. [30] included 599 (43.7%) with successful sperm retrievals after a first TESE procedure of NOA.

Obstructive azoospermia is less common than non-obstructive azoospermia and occurs in 15 to 20% of men with azoospermia [31].

Although NOA indicates impaired sperm production of the entire testis by definition, it has been observed that focal normal spermatogenesis can be observed in 50 to 60% of men with NOA [32].

The laboratory technique, embryologists experience, pathologist, single or multiple, site, unilateral or bilateral testicular biopsy and type of SR technique are possible causes of difference in the SRR.

During the last decades, seminal plasma protein has gained an important role in male infertility assay and proteomics has been serving as a tool for biological research of spermatogenesis and the clinical research of male infertility.

One of research biological tool for SP proteins assay based on the previous measurement by ELISA [33]. The current study represents the first study of proteomics in IRAQ.

The ELISA technology is used for quantitative detection of TEX101 which is range 0.313-20 ng/ml and sensitivity <0.188 ng/ml, whereas ECM1 is range 31-2000 and P=0.007, respectively), higher in men with non-obstructive azoospermia than men with obstructive azoospermia, which is similar to other studies that reported, a proteomic analysis of seminal plasma has shown the absence of certain proteins responsible for sperm function and proteins were absent in azoospermic patients such as both Seminal plasma level of ECM1 and TEX101 were significantly higher in men with NOA than men with OA [34,35].

Drabovich et al. [36] have identified ECM1 and TEX101proteins in seminal plasma that could be help facilitate the differential diagnosis of azoospermia. Testing such SP, may be able to distinguish patients with OA and NOA [36].

Proteomic analysis of seminal plasma has shown the absence of certain proteins in the seminal plasma, however many proteomic analysis were perform to determine the differential expression of proteins in azoospermia [37,38].

The result of presented study is similar to Drabovich et al. [36] was reported that testis-expressed protein 101 is characterized as the biomarker for azoospermia and extracellular matrix protein 1 was able to differentiate NOA and post-vasectomy men with a threshold value of 2.3 ng/mL.

In humans, several seminal plasma proteins were found which serve as diagnostic markers of spermatogenesis, seminiferous epithelium state and azoospermia [39].

So that from these previous and current observation, high SP level of two protein in NOA versus low level in cases of OA, this fact due to a focal spermatogenesis of deferent score in between NOA as mention above [33].

According to OA and NOA subtype in the presented observation, Seminal plasma level of ECM1 was significantly lowest in men with OA (P<0.05), on the other hand, Seminal plasma level of TEX101 was significantly lowest in men with OA (P<0.05) so both proteins were characterized as biomarker for diagnosis OA from NOA. A positive significant correlation of Seminal plasma level of ECM1 to serum level of FSH, these result on the same line of other literatures which reported an emerging SP proteins assay as biomarkers for the noninvasive diagnosis of male infertility and differentiation of azoospermia forms, OA versus NOA and histopathological subtypes of the NOA azoospermia [40,41].

Sperm retrieval rat in the current study show no statistical significant between positive SR versus negative SR with respect to ECM1, whereas SP level of TEX101 was statistically highly significant (P=0.005), being higher in men with positive sperm retrieval 1.48 (1.55) ng/ml than negative SR 0.31 (1.35) ng/ml. However one of researcher reported TEX101 could differentiate between hypospermatogenesis and sertoli cell-only syndrome (but not between MA and SCO) with prediction of spermatozoa success rates for the corresponding subtypes were HS (100%), MA (55%) and SCOS (0%) [33].

Identification of both testis-specific and germ cell type-specific proteins secreted into semen exclusively by spermatocytes, spermatids or spermatozoa should provide markers to accurately pinpoint the stage of spermatogenesis failure and thus predict TESE outcome with a better diagnostic performance [42].

The levels of ECM1 protein were high in NS (~40 µg/ml) and NOA (~20 µg/ml) samples, but notably decreased in OA/PV samples (~1 mg/ml).

Post-vasectomy seminal plasma samples are void of proteins originating from the testis and the epididymis due to ligation of the vas deferens [43].

When azoospermia is diagnosed by semen analysis, low SP levels of ECM1 and TEX101 proteins suggest obstructive azoospermia, while high SP level of ECM1 suggests non-obstructive azoospermia.

These observations confirmed that two proteins can be used as diagnostic of choice to differentiate between OA and NOA [44].

TEX101 is a membrane protein with specific expression in germ cells only, it is GPI-anchored, mouse TEX101 is expressed in testis but released from the surface of spermatozoa by highly specific enzymatic mechanisms during sperm maturation in the epididymis [45].

These report explain why the physical obstruction to seminal out flow and the absence of germ cells lead to very low (theoretically zero) levels of TEX101 in SP of patients with OA, PV and SCO whereas in in other subtypes of NOA, TEX101 is expressed, but the male gamete that failed to mature (sperm cells) never pass through the epididymis to allow for the cleavage of TEX101 from the surface of spermatozoa. This fact suggested that TEX101 can be released from the spermatocytes membrane inside the testis by non-specific mechanisms, TEX101 expression per germ cell may vary in different individuals and TEX101 was released into SP not only by epididymal spermatozoa, but also by testicular germ cells. So it is detected in SP in low concentration (<120 ng/ml) this lead to fact, SP concentration of TEX101 alone allows for the differentiation of histopathological NOA subtypes which is more specific for differentiated sertoli cell-only syndrome from the other categories of NOA [46].

These results give an explanation of current study which reported a high SP TEX101and ECM1 level incases NOA than OA which are 1.44 (1.63) ng/ml and 1629.10 pg/ml versus 0.22 ng/ml and 469.60 pg/ml, respectively.

Furthermore, ECM1 levels was higher in fertile men and in men with non-obstructive azoospermia, but nearly absent in vasectomized men, differentiating these conditions with high specificity and sensitivity [43], on the other side, TEX101 levels were higher in fertile men and undetectable in SCOS and post-vasectomy samples [43], which was similar to current observation. These data may be strengthening the confidence in non-obstructive azoospermia and obstructive azoospermia diagnosis using these two SP and gives predictive value of Testicular Sperm Extraction (TESE) outcome [47].

Receiver Operator Characteristic (ROC) curve to identify serum TEX101 cut-off values, Area Under Curve (AUC), Accuracy, 95% Confidence Interval (CI), Sensitivity and Specificity that predict positive sperm retrieval, so that any patient with seminal plasma TEX101concentration above 0.9 ng/ml is candidate to sperm retrieval technique .The prediction of sperm retrieval by TEX101 was comparable to other study which revealed TEX101 AUC=0.69 (95% CI 0.48-0.89). With the cut-off of ≥ 0.6 ng/mL, TEX101 had 73% sensitivity, 64% specificity,70% positive and 68% negative predictive values [46].

Regarding ECM1, the ROC curve, the presented observation were nearly same as finding of other observation were reported that sensitivity, specificity and threshold value were equal to 100, 73 and >2.3 µg/ml [48]. Whereas, other study reported that AUC (0.99) with sensitivity equal to 94% and the ECM1 (<2.3 µg/ml) suggest an OA, but high seminal plasma level of ECM1 (>2.3 µg/ml) suggest NOA [36] which is approximately same the sensitivity in the current study.

CONCLUSION

Although late; but the first an Iraqi study from which it can conclude and focus light on the followings:

It should be noted that seminal plasma TEX101 and ECM1 proteins are promising to be differentiated between OA/NOA and predict the success of sperm retrieval especially when complemented with testing reproductive hormones like a follicular stimulating and luteinizing hormone while TEX101 SP alone was moderate predictive value for diagnosis of NOA subtypes and SRR but unconventional alone for clinical diagnostics. From presented observation that including testicular histopathology patterns, method of TESE surgery and seminal plasma proteins, may be able to predict the chances of obtaining spermatozoa in patients with azoospermia. Although, in despite of the efficiency of some predictive procedures, no one of them are superior to other.

AUTHORS CONTRIBUTION STATEMENT

This research was done by Dr. Hussain Khaleefa Kadhem as a part of his Ph.D. thesis under the supervision of Prof.Dr.Ula Alkawaz and Assist. Prof. Dr. Hayder A. L. Mossa (corresponding author).

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CONFLICT OF INTEREST

Conflict of interest declared none.

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