



Assessment of SPEM1 Expression and Inhibin B in Patients with Dysfunctional Azoospermia and Their Relation to Successful Sperm Retrieval

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Abstract

For successful sperm retrieval in azoospermic men, a reliable prediction of success rate is necessary. The aim of this study is to assess the gene expression of SPEM1 in testicular tissue and serum inhibin B levels in patients with primary azoospermia and to evaluate their predictive power for sperm retrieval.

Material and Methods: *60 non-obstructive azoospermic patients and 20 obstructive azoospermic patients undergoing testicular tissue microdissection for intracytoplasmic sperm injection were studied. SPEM1 gene expression was analyzed by real-time reverse transcription-polymerase chain reaction using the DDcT method. Chemiluminescence technique was used to measure the levels of serum inhibin B.*

Results: *The relative expression of SPEM1 and serum inhibin B levels were significantly lower in patients compared to controls. The relative expression of SPEM1 and serum inhibin B levels was significantly higher in patients with positive sperm retrieval than patients with negative sperm retrieval. A positive correlation was found between SPEM1 relative expression and serum Inhibin B levels in the studied group.*

Conclusion: *Results suggest that SPEM1 and serum inhibin B can both be utilized in predicting the success of sperm retrieval. SPEM1 could be considered a stronger predictor of sperm retrieval than serum inhibin B.*

Keywords: *Azoospermi, Inhibin B, Microdissection testicular sperm extraction, SPEM, Sperm retrieval.*

Introduction

Infertility is becoming a major public health concern with significant psychological and economic impacts^[1]. Globally, Up to 15% of

couples suffer from infertility, in which almost a quarter of the cases is due to the male factor^[2]. Azoospermia refers to the complete lack of spermatozoa in the ejaculate affecting 1% of all

males and up to 15% of the infertile male population^[3]. Azoospermia can be categorized into obstructive azoospermia (OA); that is due to blockage along the male reproductive tract and non-obstructive azoospermia (NOA): that is due to dysfunctional spermatogenesis affecting about 60% of azoospermic men^[4]. Both cases of NOA and OA can benefit from testicular sperm extraction (TESE)^[4].

In cases of NOA, micro-TESE has improved the chances of sperm retrieval by identifying foci of spermatogenesis in the testis with a success rate of 40-60%. In order to improve this outcome, it is necessary to have a reliable predictor of sperm retrieval^[5].

Several clinical parameters such as semen analysis, testicular size, follicle-stimulating hormone (FSH) levels, inhibin B, and histopathology are among predictive factors for successful sperm retrieval in men with dysfunctional azoospermia. However, no accurate predictors have been identified.^[6-8]

Serum inhibin B (a heterodimer glycoprotein synthesized by Sertoli cells) has been shown in several studies to predict spermatogenesis^[9]. On the contrary, other studies failed to show that inhibin B can predict sperm retrieval^[8].

Mounting evidence have shown that spermatogenesis failure could be predicted by analysing germ cell-specific genes involved in spermatogenesis.^[10,11] For instance, several molecular markers, including SPEM1, were analyzed by Hashemi et al. (2018) in testicular samples in patients undergoing microTESE for ICSI to evaluate their relation with sperm retrieval. SPEM1 had the highest predictive power hence the best molecular marker for predicting sperm retrieval^[12].

SPEM1 (spermatid maturation 1) is a 330 amino acid protein that is expressed in the cytoplasm of stages 14–16 elongated spermatids and is necessary in the removal of cytoplasm during spermiogenesis. SPEM1 Knockout mice show deformed sperms in addition to male infertility^[13].

In view of the scarcity of studies on SPEM1 in predicting sperm retrieval in the literature and the

lack of studies in our ethnic population, there is an unmet need to determine its value in this set-up. Thus, the present study aimed to analyze SPEM1 in testicular samples and serum inhibin B in patients undertaking micro-TESE for ICSI and their correlation with sperm retrieval.

Materials and Methods

We recruited eighty patients from the Andrology outpatient clinic of the Main University Hospital, Faculty of Medicine, University of Alexandria. 60 NOA patients with verified azoospermia as per the WHO 5th edition^[14], normal quantity of serum fructose and alpha glucosidase and no evidence of obstructive causes of azoospermia in history or physical examination were included. Patients with evidence of obstruction, anejaculation, and retrograde ejaculation were excluded from the study. The control group consisted of twenty (out of 80) patients who had obstructive azoospermia (OA). After the study protocol was approved by the Research Ethics Committee (REC) of Alexandria University, all the patients provided informed consent to participate in this research.

Sperm Extraction

A longitudinal incision of about two cm on the median raphe of the scrotum was made, extending to the tunica vaginalis, exposing the tunica albuginea. Optical magnification of X6 - 8 was employed to observe blood vessels beneath the tunica vaginalis, a single wide equatorial incision was made, which allowed extensive visualization of seminiferous tubules without damaging the vasculature of the testis. With the help of an operating microscope at a magnification of x20-25, particular seminiferous tubules larger than the rest of the testicular tubes were identified, samples of 2-10 mg micro-TESE were sharply obtained from larger and more opaque tubules.^[15] The tissue surrounding each microdissected sample was then excised, and assessed the efficacy of sperm extraction with microdissection. Further smaller pieces of testicular tissue specimen were acquired to release spermatozoa from the seminiferous tubules. The suspension

obtained was studied. Retrieved viable sperm were directly utilized for ICSI or cryopreserved at -4°C in liquid nitrogen^[16].

Upon successful retrieval of spermatozoa, the procedure came to an end. Hemostasis was achieved by microbipolar cauterization, and the tunica albuginea closed with continuous suture. A single surgeon carried out all of the surgical procedures. Two extra pieces of testicular tissue were taken and fixed in Bouin's fixative solution for quantitative assessment of histopathological pattern and RNA stabilization reagent for quantitative expression of *SPEMI*.

Histopathological Analysis

By examining hematoxylin and eosin (H and E) stained paraffin-embedded specimens, testicular histology was classified according to the most common histopathological pattern into 1) Hypospermatogenesis (HS), 2) Maturation arrest (MA), 3) Sertoli cell-only syndrome (SCOS) and 4) Tubular hyalinization (TH)^[17].

Determination of *SPEMI* Gene Expression

RNA Extraction and Reverse Transcription

The stabilized tissue samples stored at -80°C were first left to thaw; afterward, total RNA was extracted per the RN easy Mini Kit (Cat. No. 74104, QIAGEN, USA) manufacturer's instructions. The RNA concentration and purity were assessed using Nano Drop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). All genomic RNA was converted into single-stranded complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814, Applied Biosystems™, USA). Reverse transcription (RT) was carried out using Arktik thermal cycler (Thermo Fisher Scientific, USA) at 25°C for ten minutes, 37°C for 2 hours, and 85°C for five minutes, then held at 4°C .

Quantitative Real-time PCR (qPCR)

Relative quantification of *SPEMI* gene expression was performed using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Cat. No. # K0221, Thermo

Fisher Scientific, USA) using Strata gene Mx3000P PCR System (Agilent, USA) according to manufacturer instructions. Custom-made primers were supplied by Applied Biosystems, USA, including four unlabeled sequence-specific forward and reverse primers to amplify cDNA of *SPEMI* and cDNA of *GAPDH* as a housekeeping gene. The sequence of the primers was as follows: *SPEMI* forward primer 5'-TAACATTAGCATCAATATAGTG-3' and reverse 5'-GTCTGCTTTCTGAGTAAT-3'; *GAPDH* forward primer 5'-CCACTCCTCCACCTTTGACG-3' and reverse 5'-CCACCACCCTGTTGCTGTAG -3'. Relative expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method^[18]. The PCR reactions were carried out in duplicate with a final volume of 25 μL , including 12.5 μL Maxima SYBR Green/ROX qPCR Master Mix (2X), 1 μL forward primer (10 pmole), 1 μL reverse primer (10 pmole), 0.05 μL 10x diluted ROX solution, cDNA (200 ng) and nuclease-free water to a final volume of 25 μL . The reaction protocol was as follows: 10 min at 95°C (one cycle) for initial denaturation, followed by 40 cycles of 15 s at 95°C for denaturation and annealing/extension for 60 s at 45°C .

Statistical Analysis

The IBM SPSS software package version 20.0 was used to examine the data (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test, the Chi-square, the student t-test, Pearson coefficient, the Spearman coefficient, and the Kruskal Wallis test were used accordingly. Receiver operating characteristic curve (ROC) was used to evaluate the test's diagnostic performance. A p-value of $< .05$ was considered significant.

Results

The mean age of patients with non-obstructive azoospermia was 31.07 ± 7.11 vs. 29.10 ± 3.43 in the control group and was comparable between the two groups ($p=0.105$).

Serum Inhibin B

The NOA patients had significantly lower serum inhibin B than the OA controls with a median of

37.70 pg/mL (range: 2.0 – 238.0 pg/mL) vs 155.2 pg/mL (range: 79.20 – 303.0 pg/mL), respectively, $p < 0.001$. (**Figure 1A**).

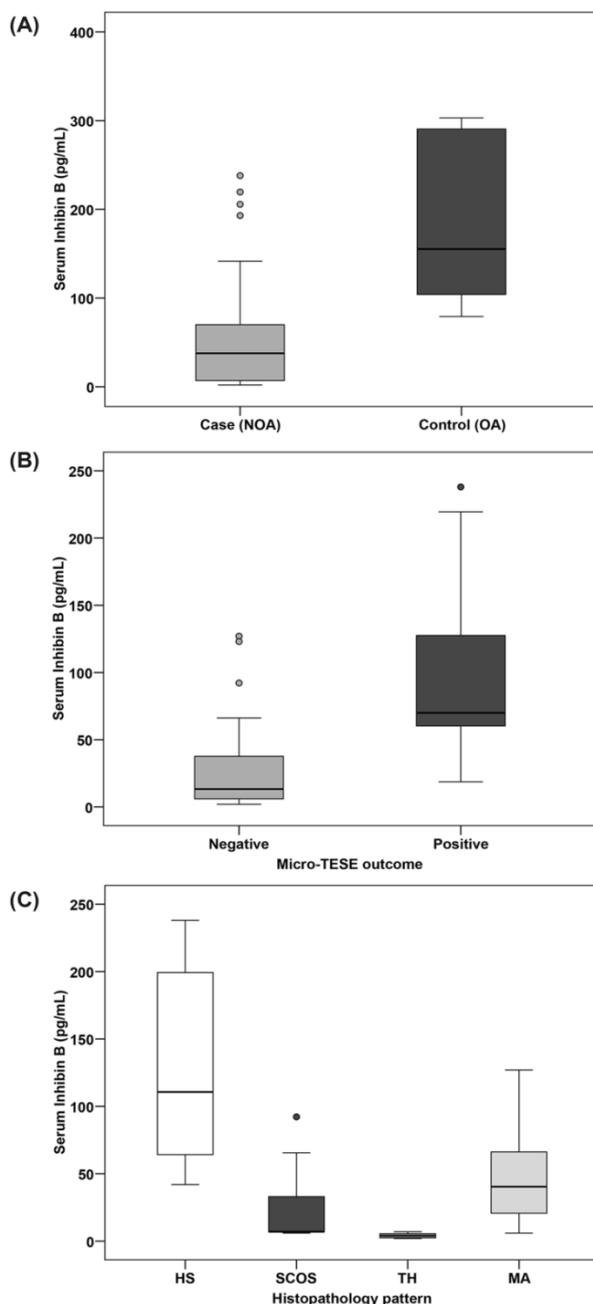


Figure. 1 (A-C)

Figure 1A. Comparison between the two studied groups (NOA vs OA) according to Inhibin B (Pg/mL) levels; **B.** Serum Inhibin B levels in relation to Micro-TESE outcome in Cases (NOA) (n = 60); **C.** Serum Inhibin B levels in different histopathology pattern (HS-Hypospermatogenesis; SCOS- Sertoli cell only syndrome; TH-Tubular Hyalinization; MA-maturation arrest) in Cases (NOA) (n = 60)

The level of serum inhibin B was statistically significantly higher in cases with positive micro-TESE outcomes than cases with negative outcomes ($p < 0.001$) (**Figure 1B**). Inhibin B levels in the patients grouped according to the histopathological pattern are shown in **Figure 1C**. Serum level of inhibin B was highest in hypospermatogenesis (median=110.7 pg/mL) and lowest in tubular hyalinization (median=4.0 pg/mL), in maturation arrest and SCOS the median values were 40.40 pg/mL and 7.0 pg/mL respectively. (**Figure 1C**) Serum FSH and inhibin B had a significant negative correlation ($r = -0.595$, $p < 0.001$) whereas, *SPEMI* relative expression had a significant positive correlation with serum inhibin B. ($r = 0.621$, $p < 0.001$). (**Table 1**)

Relative *SPEMI* expression was significantly lower in NOA patients (ranged from 0.02 – 1247.7 with a median of 1.79) than in OA controls (ranged from 70.0 – 2098 with a median of 1174.6) ($p < 0.001$) (**Figure 2A**). A statistically significant higher relative expression of *SPEMI* was observed in cases with successful sperm retrieval than cases without sperm retrieval ($p = 0.001$). (**Figure 2B**).

The relative expression of *SPEMI* in patients with non-obstructive azoospermia was highest in hypospermatogenesis (median=824.4) and lowest in SCOS (median=0.13), in tubular hyalinization and maturation arrest, the medians were 0.17 and 3.11 respectively ($p < 0.001$). (**Figure 2C**). In addition, a negative correlation between *SPEMI* expression and FSH level was observed ($r = -0.582$). (**Table 1**)

Table (1): Correlation between serum Inhibin B, *SPEM1* and FSH in cases group (NOA) (n = 60)

	r_s	P
Serum Inhibin B (pg/mL) and FSH (mIU/mL)	-0.595	<0.001*
<i>SPEM1</i> relative expression and FSH (mIU/mL)	-0.582	<0.001*
Serum Inhibin B (pg/mL) and <i>SPEM1</i> relative expression	0.621	<0.001*

r_s : Spearman coefficient

*: Statistically significant at $p \leq 0.05$

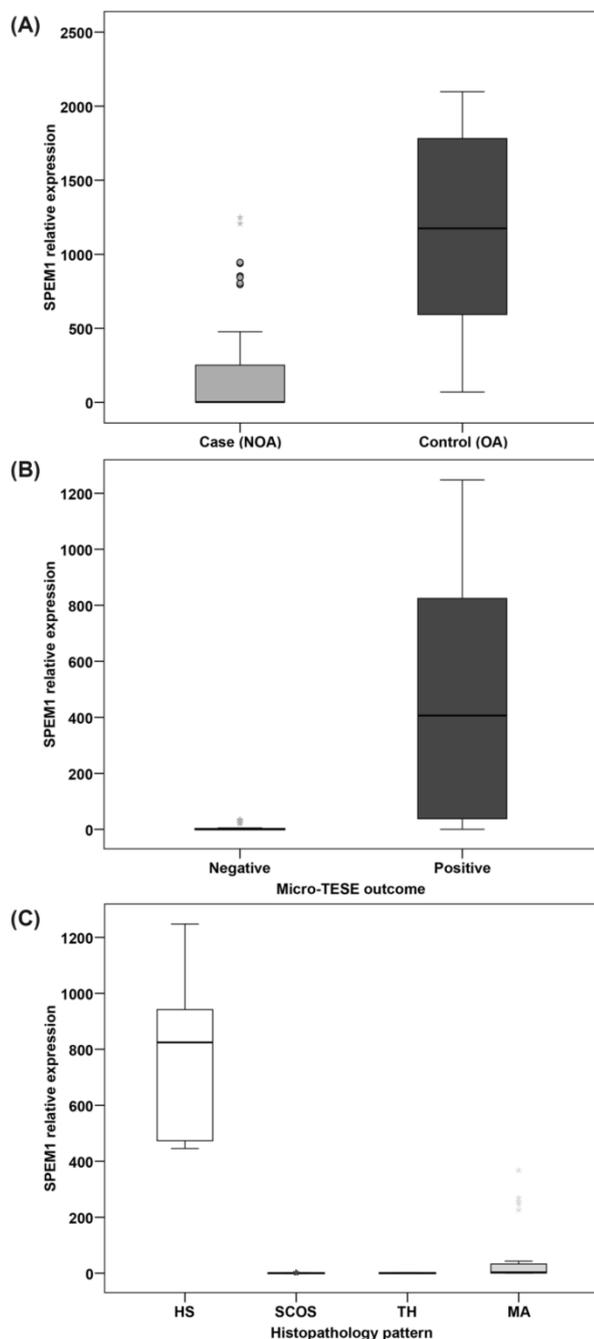


Figure 2 (A-C).

- A. *SPEM1* relative expression in cases and control;
- B. *SPEM1* relative expression in relation to Micro-TESE outcome in Cases (NOA) (n = 60);
- C. *SPEM1* relative expression in different histopathology pattern (HS-Hypospermatogenesis;

SCOS- Sertoli cell only syndrome; TH-Tubular Hyalinization; MA-maturation arrest) in Cases (NOA) (n = 60)

ROC Curve Analysis

The diagnostic value of *SPEM1* and serum inhibin B for sperm retrieval was assessed using ROC curves. As shown in **Figure 3**, the best cut-off value of serum inhibin B was >50.8 pg/mL. Under the cut-off value, AUC was 0.875, specificity 83.33%, sensitivity 79.17%, and accuracy 81.67%, whereas the best cut-off value of *SPEM1* was >2.8. Under the cut-off value, AUC was 0.909, specificity of 86.11, the sensitivity of 91.67, and accuracy of 88.33. This data suggest that *SPEM1* has better predictive power than serum inhibin B.

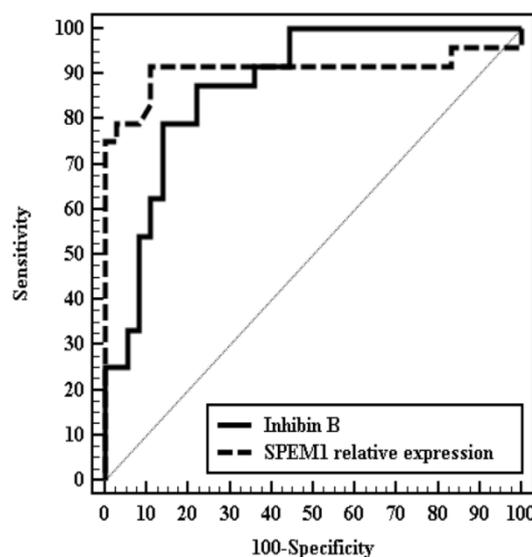


Figure 3 ROC curve for Inhibin B and *SPEM1* relative expression to discriminate Retrieval patients (n = 24 vs. 36) in cases (NOA) groups

Discussion

Micro-TESE has been considered a more efficient method of sperm retrieval in dysfunctional azoospermia with minimal invasion than multiple biopsies. Conventional TESE has been associated

with testicular atrophy, intratesticular bleeding, devascularisation, and scar formation, disrupting the hormone synthesis and spermatogenic pathway^[19].

Several authors reported that testicular histology is the best single predictor of micro-TESE, but its utility is restricted because the diagnosis requires an extra surgical operation.^[20,21]

The histopathological patterns in the present study included; maturation arrest 30/60 (50%), hypospermatogenesis 12/60 (20%), SCOS 10/60 (16.7%), and tubular hyalinization 8/60 (13.3%). There was positive sperm retrieval in 100% of the cases with hypospermatogenesis. On the contrary, SCOS and tubular hyalinization had a 0% chance of sperm retrieval, and there was a statistically significant association between testicular histopathology and micro-TESE outcome ($p < 0.001$). These findings resonate with a study done by Seo et al. (2001), who demonstrated that cases with SCOS had a lower rate of sperm retrieval compared to cases with hypospermatogenesis and maturation arrest^[22]. On the contrary, Tsujimura et al. (2002) reported a higher retrieval rate of 22.6% in cases of SCOS. This finding could be attributed to incomplete SCOS^[23].

The level of serum inhibin B in NOA was substantially lower than in OA controls ($p < 0.001$). Meanwhile, when comparing the inhibin B level in the NOA cases, it was significantly higher in those patients with the positive micro-TESE outcome as opposed to patients with the negative micro-TESE outcome ($p < 0.001$). These results agree with the previous study done by Nagata et al. (2005). They found that the positive TESE group had statistically higher levels of serum inhibin B compared to the negative group^[24].

The present research revealed a significant negative correlation between FSH levels and serum inhibin B in NOA. Many studies have found a significant negative correlation between serum inhibin B levels and FSH levels. Illingworth et al. (2009) also showed a negative correlation between serum inhibin B and FSH^[25]. The role of inhibin B as negative feedback for FSH could explain this^[25].

In this study, inhibin B levels were statistically higher in cases that had positive sperm retrieval. The histopathological pattern and serum inhibin B had a significant correlation. Inhibin B levels were higher in cases of hypospermatogenesis with positive sperm retrieval. On the other hand, serum inhibin B was lower in cases of SCOS and tubular hyalinization with negative sperm retrieval. These findings agree with Anawalt et al. (1996) and De Krester et al. (1989), who reported that serum inhibin B levels were lower in patients with SCOC and tubular hyalinization and higher in hypospermatogenesis.^[25,26]

The ROC curve was utilized to examine the predictive potential of serum inhibin B in sperm retrieval in order to obtain a more reliable prediction. We found that serum inhibin B had an area under the curve of 0.903, with a cut-off value of > 50.8 pg/ml and an accuracy of 80%.

There are significant differences in the computed cut-off value in other research. Balleca et al. (2002) obtained a cut-off value of 40 pg/mL that was close to our study, with a 90% sensitivity and 100% specificity^[9]. Some studies had a cut-off value lower than our cut-off value. Ziaee et al. (2006) and Alhalabi et al. (2016) reported a cut-off value of 27.5 pg/mL and 35 pg/mL, respectively^[27,28]. In contrast, Bonarriba et al. (2013) and Wang et al. (2020) reported a higher cutoff value of 67 pg/mL and 77.72 pg/mL respectively [29,30]. This could be attributed to variation in sample size and different ethnicity.

SPEM1 gene expression was analyzed in testicular biopsies of 30 NOA cases and 10 OA controls. There was a significantly decreased expression of SPEM1 in the NOA cases compared to the normal spermatogenesis in the OA group.

SPEM1 relative expression was considerably lower in patients who had negative sperm retrieval compared to those with positive sperm retrieval in the NOA group. SPEM1 relative expression was highest in hypospermatogenesis (median=824.45). In all the 6 cases, there was a positive micro-TESE outcome, whereas SPEM1 relative expression was lowest in SCOS and tubular hyalinization patients

(median=0.11 and 0.18 respectively), of whom both groups had a negative micro-TESE outcome.

In line with our findings, Hashemi et al. (2018) assessed seven molecular markers, including SPEM1 to predict the success of sperm retrieval^[12]. They concluded that SPEM1 expression reduced significantly in patients with NOA compared with normal spermatogenesis (OA). In addition, SPEM1 expression also reduced significantly in patients with negative sperm retrieval compared to positive sperm retrieval^[12].

Using ROC curve analysis of SPEM1, a cut-off value of >1.88 and AUC of 0.824 were obtained. Hashemi et al. (2018) also concluded that the most predictive marker of micro-TESE outcome was SPEM1 with a cut-off value and AUC of 0.086 and 0.91, respectively^[12]. This is the only study in the literature to assess the cut-off value of SPEM1 in predicting sperm retrieval.

The present study showed that SPEM1 was a more sensitive predictor of sperm retrieval than serum inhibin B in NOA patients. The area under the ROC curve for SPEM1 was significantly larger than that of serum inhibin B.

The current work has opened up new avenues for future research into SPEM1's predictive power in sperm retrieval. Further research should be conducted on a large population of cases and controls to analyze the expression of SPEM1 and inhibin B semen samples and evaluate their association with sperm retrieval.

Conclusions

The present study revealed that SPEM1 and serum inhibin B could be utilized in predicting the success of sperm retrieval. However, SPEM1 could be considered a stronger predictor of sperm retrieval than serum inhibin B.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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