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RESEARCH

Serum FSH levels can predict sperm identification in semen once diagnosed azoospermia

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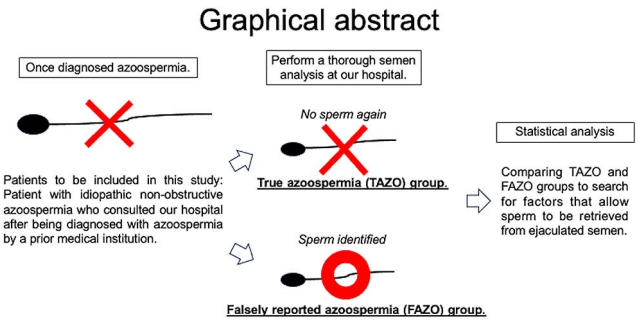
Graphical abstract



Abstract

Multiple semen analyses are important for identifying patients with severe oligozoospermia (SOS) or cryptozoospermia (CZO). Moreover, clinical predictive factors for CZO and SOS are warranted. Therefore, we aimed to identify predictors of sperm retrieval in patients with a prior diagnosis of nonobstructive azoospermia (NOA) based on repeat semen analysis. We retrospectively included 209 patients diagnosed with NOA. Data regarding age at diagnosis, body mass index, testicular volume, serum luteinizing hormone, follicle-stimulating hormone (FSH) and testosterone levels, smoking history and testicular microlithiasis were analyzed. Patients were classified into the falsely reported azoospermia (FAZO) and true azoospermia (TAZO) groups. Furthermore, FAZO-related factors were evaluated using the Mann–Whitney *U* test and univariate and multivariate analysis logistic regression models. Regarding FAZO-related factors, the cut-off level was determined using receiver operating characteristic (ROC) curve analysis. Among 209 patients with NOA, 33 (15.8%) had spermatozoa identified in subsequent semen analyses. Multivariate analysis revealed that the FAZO group had significantly lower FSH levels than the TAZO group. ROC curve analysis showed that the cut-off value for the FSH level was 15.3 mIU/mL, with 26 (78.8%) and 29 (16.5%) patients in the FAZO and TAZO groups, respectively, having FSH levels ≤15.3 mIU/mL. In conclusion, the FSH level was a predictive factor for FAZO. In patients diagnosed with azoospermia

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who have relatively low FSH levels, multiple semen analyses might facilitate identification of sperm in ejaculated semen.

Lay summary

We evaluated 209 patients diagnosed with spermless semen at prior medical institutions. After thorough semen analyses at our hospital, sperm were identified in the ejaculates of 33 (15.8%) patients. We performed comparisons between patients with and without identified sperm. The serum FSH level was identified as a significant predictive factor for sperm presence. FSH stimulates testicular growth and function and promotes sperm development. Patients who had relatively low and high FSH levels for patients with spermless semen had an increased and decreased chance, respectively, of having sperm identified in ejaculated semen through repeat thorough semen analyses. Sperm might be identified in ejaculates of patients diagnosed with spermless semen who have relatively low FSH levels.

Keywords: sperm identification; ejaculated semen; nonobstructive azoospermia; repeat semen analysis; FSH

Introduction

Semen analysis (SA) is among the most important diagnostic examinations for male infertility and can inform treatment strategies, including microdissection testicular sperm extraction (MD-TESE). Specifically, the differential diagnosis between azoospermia (AZO) and severe oligozoospermia (SOS) or cryptozoospermia (CZO) is vital for determining whether to perform MD-TESE and significantly influences the invasiveness of the procedure. The SA guidelines in the World Health Organization (WHO 2021) Laboratory Manual for the Examination and Processing of Human Semen, sixth edition (2021), recommend centrifugation and repeat SA in cases where sperm cannot be identified via routine SA. However, the quality of SA varies across laboratories and it may not be beneficial in some patients.

CZO is characterized by the lack of sperm in the initial SA without centrifugation and the detection of a very small number of sperm in the subsequent SA with centrifugation. SOS is not defined in the WHO Laboratory Manual for the Examination and Processing of Human Semen, sixth edition (World Health Organization 2021) guidelines; however, it is reportedly characterized by sperm levels $\leq 5 \times 10^6$/mL, representing severe spermatogenesis dysfunction (Stahl *et al.* 2010, Song *et al.* 2012). These low sperm levels could affect reproductive function and impede spontaneous conception.

The advent of assisted reproductive technologies (ART) has allowed individuals with access to a few ejaculated sperm to have a baby (Pinheiro *et al.* 1999, Mazzilli *et al.* 2023). Accordingly, multiple SAs are important for identifying patients with CZO and SOS; moreover, predictive factors for CZO and SOS should be established in the clinical field.

In this study, patients with falsely reported azoospermia (FAZO) were defined as those with a previous diagnosis of AZO, followed by a diagnosis of CZO or SOS, following sperm identification in a subsequent SA performed at our hospital. Patients with true azoospermia (TAZO) were defined as those without sperm identified in the SA at our hospital. This study aimed to investigate predictive factors for sperm identification in patients undergoing repeat SA, which could inform future medical treatment.

Materials and methods

Patients

We included patients diagnosed with AZO in a previous clinic between April 2017 and October 2020, who were referred to the Reproductive Center of Dokkyo Medical University Saitama Medical Center (DMUSMC) for further examination and treatment. There remains no standardized SA procedure across hospitals. Although most hospitals report conducting SA in accordance with the WHO Laboratory Manual for the Examination and Processing of Human Semen, fifth edition (WHO 2010), some remain uncertain of their compliance with the guidelines. In DMUSMC, the included patients underwent physical examination, scrotal ultrasonography, blood endocrinological tests, genetic testing and a thorough SA. The obtained data were used for analysis in this study. After the examinations, MD-TESE was performed at DMUSMC, as needed. This study was approved by the Ethics Review Committee of DMUSMC (approval no. 21123). Furthermore, we provided an opt-out option from this retrospective study in our institution's website.

Data collection

We retrospectively collected patient background data by reviewing the medical charts of patients who visited DMUSMC. The background data included age at

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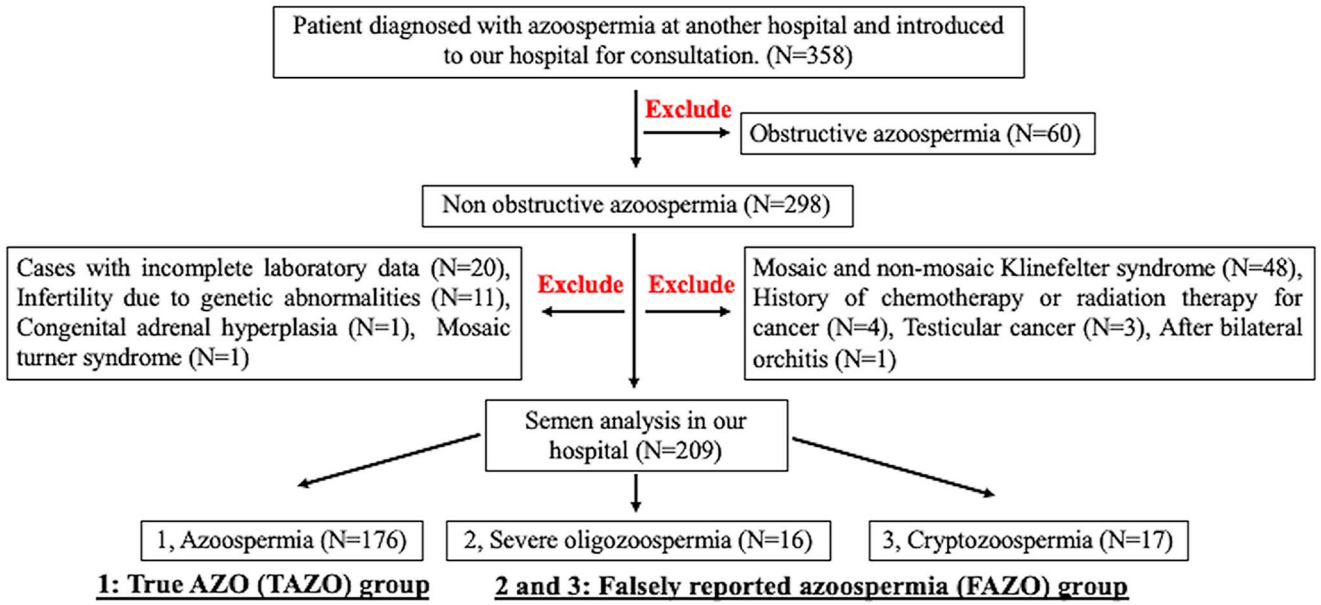

Figure 1

Study flow diagram.

the time of hospital attendance, right and left testicular volumes measured with a Prader orchidometer, testicular microlithiasis, grade and site of varicocele, smoking history, endocrinological findings (luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL) and testosterone levels), genetic examination findings, such as G-banding and AZO factor deletion, and TESE history. We excluded patients with genetically confirmed infertility, AZO caused by genital obstruction, mosaic and non-mosaic Klinefelter's syndrome, history of chemotherapy and radiotherapy for cancer, incomplete laboratory data, testicular cancer, bilateral orchitis and congenital adrenal hyperplasia. Patients who had undergone varicocelectomy without recurrence were considered as lacking varicocele. Patients determined to have CZO/SOS and AZO following SA in our hospital were included in the FAZO and TAZO groups, respectively. CZO was indicated by the lack of sperm in the initial routine SA and the presence of sperm in the repeat SA following centrifugation. Contrastingly, SOS was indicated by a sperm concentration $<5 \times 10^6$/mL in the initial routine SA.

Statistical analysis

The Mann–Whitney U test was performed for between-group comparisons of the aforementioned variables. In addition, we performed univariate and multivariate analyses using the logistic regression model. The cut-off values of identified predictive values were determined using receiver operating characteristic (ROC) curve analysis. Subsequently, we assessed the number and proportion in each group according to the determined cut-off values. All statistical analyses were performed using an R-based user interface, EZR version 3.6.1 (https://www.xquartz.org/; Saitama Medical Center, Jichi Medical University, Japan) (Kanda 2013). Statistical significance was set at $P < 0.05$.

Results

Patient characteristics

Between April 2017 and October 2020, 358 patients with a previous diagnosis of AZO visited our hospital. Among the patients who visited the hospital, we included 209 eligible patients based on the exclusion and inclusion criteria (Fig. 1). Table 1 presents the characteristics of the patients. The median (range) values of the serum hormone levels were as follows: LH, 8.7 (2.2–24.5) mIU/mL; FSH, 21.6 (2.7–79.2) mIU/mL; testosterone, 4.2 (1.1–8.4) ng/mL; and PRL, 9.1 (3.1–158.9) ng/mL. The median (range) left and right testicular volumes were 12 (2–24) mL and 12 (2–24) mL, respectively. The median (range) age at hospital attendance was 34 (22–54) years. The left varicocele was classified as grade 3, 2 and 1 in 13, 25 and 16 patients, respectively, whereas the right varicocele was classified as grade 3, 2 and 1 in zero, six and six patients, respectively. Testicular microlithiasis was identified in ten patients. Moreover, 74 patients were smokers, while 150 patients had undergone TESE.

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Table 1 Characteristics of patients (*n* = 209) diagnosed with azoospermia by prior clinic. Data are presented as the median (minimum–maximum value) or as *n* (%).

Characteristics	Values
Age	34 (22–54)
Left testicular volume (mL)	12 (2–24)
Right testicular volume (mL)	12 (2–24)
Testosterone (ng/mL)	4.2 (1.1–8.4)
LH	8.7 (2.2–24.5)
FSH	21.6 (2.7–79.2)
PRL	9.1 (3.1–158.9)
Left varicocele (G3/G2/G1)	13/25/16
Right varicocele (G3/G2/G1)	0/6/6
Microlithasis	10 (4.8%)
Smoking	74 (35.4%)
Performed with TESE	150 (71.8%)

LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; TESE, testicular sperm extraction.

Based on the SA at our clinic, 33 (15.8%) and 176 (84.2%) patients were diagnosed with CZO/SOS and AZO, respectively (Table 2); accordingly, they were included in the FAZO and TAZO groups, respectively. The characteristics of patients with AZO, SOS and CZO are listed in Table 3.

Comparison between the FAZO and TAZO groups

The Mann–Whitney U test revealed significant between-group differences in left testicular volume ($P < 0.001$), right testicular volume ($P < 0.001$), LH level ($P < 0.001$), FSH level ($P < 0.001$), PRL level ($P = 0.00792$) and TESE history ($P < 0.001$). There were no significant between-group differences in age ($P = 0.196$), testosterone levels ($P = 0.0882$), testicular microlithiasis ($P = 0.209$) or smoking history ($P = 0.352$) (Table 4). The asterisk indicates the results of the Mann-Whitney U test at a significance level of $p<0.05$.

Univariate analysis revealed significant between-group differences in left testicular volume ($P < 0.001$), right testicular volume ($P < 0.001$), LH level ($P < 0.001$) and FSH level ($P < 0.001$) (Table 5). In the subsequent multivariate analysis using the logistic regression model, we included seven factors (age, left and right testicular volume, testosterone, LH, FSH and PRL levels). Among them, the FSH level was determined to be a significant predictive factor ($P < 0.001$) for sperm identification in patients undergoing repeat SA (Table 5). The asterisk indicates the results of the logistic regression model at a significance level of $p<0.05$.

Table 2 Results of semen analysis at our hospital.

Patients	Patients, *n*	%
AZO diagnosed by prior medical clinic	209	100
TAZO group	176	84.2
FAZO group	33	15.8
SOS patients	16	7.7
CZO patients	17	8.1

AZO, azoospermia; TAZO, true azoospermia; FAZO, falsely reported azoospermia; SOS; severe oligozoospermia; CZO, cryptozoospermia.

Table 3 Patient characteristics of severe oligozoospermia (SOS), cryptozoospermia (CZO) and azoospermia (TAZO group). Data are presented as the median (IQR) or as *n* (%).

	TAZO group	SOS patients	CZO patients
n	176	16	17
Age	34 (22–54)	34 (27–47)	36 (28–46)
Left testicular volume (mL)	12 (2–24)	15 (6–22)	14 (10–24)
Right testicular volume (mL)	12 (2–24)	15 (6–22)	15 (10–24)
Testosterone (ng/mL)	4.1 (1.1–8.4)	4.9 (2.6–7.1)	4.3 (2.2–8.1)
LH	9.3 (2.2–24.5)	5.2 (3.8–15.1)	6.8 (2.2–12.0)
FSH	23.0 (2.7–64.2)	7.4 (3.2–21.5)	12.8 (3.7–21.3)
PRL	9.4 (3.1–158.9)	7.5 (4.6–16.8)	8.0 (3.7–25.1)
Left varicocele			
Grade 1	13 (7.4%)	1 (6.3%)	2 (11.8%)
Grade 2	19 (10.8%)	4 (25%)	2 (11.8%)
Grade 3	12 (6.8%)	1 (3.0%)	0
Right varicocele			
Grade 1	6 (3.4%)	0	0
Grade 2	3 (1.7%)	2 (12.6%)	1 (5.9%)
Grade 3	0	0	0
Microlithasis	7 (4.0%)	1 (6.3%)	2 (11.8%)
Smoking	60 (34.1%)	6 (37.5%)	8 (47.1%)
TESE	144 (81.8%)	0	6 (35.3%)

LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; TESE, testicular sperm extraction.

ROC curve analysis of the FSH level

ROC analysis of the FSH level revealed that the area under the curve was 0.861, with a specificity and sensitivity of 0.830 and 0.788, respectively. The cut-off value of the FSH level was 15.3 mIU/mL (Fig. 2).

Classification of patients in each group according to the cut-off FSH level

There were 26 (78.8%) and 29 (16.5%) patients in the FAZO and TAZO groups, respectively, with FSH levels ≤15.3 mIU/mL. Contrastingly, seven (21.2%) and 147 (83.5%) patients in the FAZO and TAZO groups, respectively, had FSH levels >15.3 mIU/mL (Table 6). The false-positive rate (probability of not identifying sperm in patients with FSH levels ≤15.3 mIU/mL) was 52.7%, while the false-negative rate (probability of identifying sperm in patients with FSH levels >15.3 mIU/mL) was 4.5% (Table 7). This outcome indicates that the probability of not identifying sperm is □50% if the FSH level ≤15.3 mIU/mL; contrastingly, the probability of

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Table 4 Patient characteristics of FAZO and TAZO groups.

	FAZO group	TAZO group	*P* value
n	33	176	
Age	36 (27–47)	34 (22–54)	0.196
Left testicular volume (mL)	14 (6–24)	12 (2–24)	**<0.001***
Right testicular volume (mL)	15 (6–24)	12 (2–24)	**<0.001***
Testosterone (ng/mL)	4.3 (2.2–8.1)	4.1 (1.1–8.4)	0.0882
LH	6.8 (2.2–15.1)	9.3 (2.2–24.5)	**<0.001***
FSH	12.8 (3.2–21.5)	23.0 (2.7–64.2)	**<0.001***
PRL	8.0 (3.7–25.1)	9.4 (3.1–158.9)	**0.00792***
Left varicocele			-
Grade 1	3 (9.1%)	13 (7.4%)	
Grade 2	6 (18.2%)	19 (10.8%)	-
Grade 3	1 (3.0%)	12 (6.8%)	-
Right varicocele	0	6 (3.4%)	-
Grade 1			
Grade 2	3 (9.1%)	3 (1.7%)	-
Grade 3	0	0	-
Microlithasis	3 (9.1%)	7 (4.0%)	0.209
Smoking	14 (42.4%)	60 (34.1%)	0.352
TESE	6 (18.2%)	144 (81.8%)	**<0.001***

FAZO, falsely reported azoospermia; TAZO, true azoospermia; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; TESE, testicular sperm extraction.
*values are statistically significant.

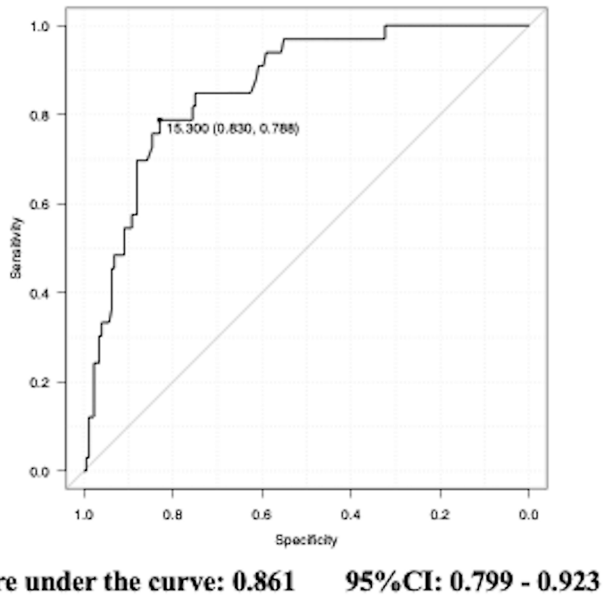

Figure 2

Receiver operating characteristic curves performed in this study.

identifying sperm is significantly lower when the FSH level was >15.3 mIU/mL.

Discussion

Our findings indicated that the FSH level was the most significant predictive factor for CZO or SOS (FAZO group), with a cut-off value of 15.3 mIU/mL. Specifically, patients with FSH levels ≤15.3 mIU/mL who undergo repeat SA may still have sperm in the ejaculated semen. Accordingly, these patients might benefit from intracytoplasmic sperm injection (ICSI), and therefore avoid invasive procedures such as TESE. Our findings may inform the development of new clinically meaningful guidelines for the treatment of male infertility and future expansion of the framework.

In our study, there were 33 (15.8%) patients in the FAZO group. Perouse *et al.* (2022) performed a second SA in 172 patients, who had been diagnosed with AZO based on the initial SA, and identified sperm in three (1.7%) patients. Among these three patients, one was a transient case due to a high fever before the first SA. Compared with this previous study, we observed a higher proportion of patients with detectable sperm in the second SA. This could be attributed to the fact that the initial SA of our patients was performed at other institutions. The WHO Laboratory Manual for the Examination and Processing of Human Semen (2021) indicates the importance of centrifugation of the semen sample (3,000 g for 15 min) and a thorough examination of the sediment for diagnosis of AZO. However, this guideline is not met in

Table 5 Logistic regression model between FAZO and TAZO groups.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age	1.040 (0.980–1.110)	0.180	1.050 (0.970–1.150)	0.214
Left testicular volume (mL)	1.200 (1.090–1.320)	**<0.001***	1.010 (0.780–1.300)	0.986
Right testicular volume (mL)	1.230 (1.120–1.360)	**<0.001***	1.050 (0.811–1.350)	0.719
Testosterone (ng/mL)	1.220 (0.968–1.530)	0.098	1.120 (0.834–1.490)	0.460
LH	0.727 (0.625–0.844)	**<0.001***	1.060 (0.856–1.300)	0.612
FSH	0.835 (0.783–0.889)	**<0.001***	0.840 (0.765–0.922)	**<0.001***
PRL	0.917 (0.828–1.020)	0.097	0.945 (0.849–1.050)	0.293
Microlithasis	2.410 (0.591–9.860)	0.220		
Smoking	1.540 (0.720–3.290)	0.266		

OR, odds ratio; FAZO, falsely reported azoospermia; TAZO, true azoospermia; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin.
*indicates statistical significance.

Table 6 Number and proportion of patients in each group according to the cut-off value of the FSH level.

FSH, mIU/mL	FAZO group (*n* = 33)	TAZO group (*n* = 176)
≤15.3	26/33 (78.8%)	29/176 (16.5%)
>15.3	7/33 (21.2%)	147/176 (83.5%)

FSH, follicle-stimulating hormone; FAZO, falsely reported azoospermia; TAZO, true azoospermia.

all reproduction clinics; accordingly, the methods and accuracy of SA vary across laboratories (Vasconcelos *et al.* 2022). Another reason for the differences in the quality of SA across facilities could be skill differences among embryologists. In Japan, there are very few specialists in the field of male infertility treatment, which may contribute to diagnostic errors.

TESE is typically used for AZO treatment and is a significantly invasive procedure. Accordingly, multiple and thorough SAs, with the inclusion of centrifugation, are crucial for avoiding unnecessary TESE and establishing a diagnosis of AZO.

The WHO Laboratory Manual for the Examination and Processing of Human Semen published its first edition in 1980 and has been regularly updated, with the sixth edition being published in 2021. In each revision, the lower limit values for SA are updated based on data reported worldwide in order to better reflect real-world clinical settings. In the 2021 edition, a new section on sperm DNA fragmentation index and semen oxidative stress assay was included. In addition, sperm quality and the surrounding environment were discussed, which coincides with our original hypothesis, but had no bearing on the current findings. Future studies are warranted to verify FSH values as a major indicator, regardless of the referenced edition of the WHO guidelines.

Low sperm levels in patients with CZO and SOS affect reproductive function and impede spontaneous pregnancy (Almagor *et al.* 2001). Recent advances in ART have allowed patients with CZO and SOS, who have even minimally detectable sperm levels in their semen, to have babies (Plouvier *et al.* 2017, Mazzilli *et al.* 2023). In cases wherein sperm can be detected in the semen, even in very small amounts, ICSI should be attempted; however, TESE may still be necessary. Sperm in patients with CZO or SOS has low quality and high rates of DNA fragmentation (Campos *et al.* 2021, Caliskan *et al.* 2022). Recent small-scale studies have indicated that the use of testicular sperm in ICSI among patients with a high rate of sperm DNA fragmentation can predominantly increase live birth and clinical pregnancy rates and decrease miscarriage rates (Esteves *et al.* 2017, Awaga *et al.* 2018, Esteves *et al.* 2023). In our study, six (18.2%) patients in the FAZO group underwent TESE, with all of them having a diagnosis of CZO (Table 3). This demonstrated that TESE is more likely to be performed in the presence of worse semen findings. It is important to consider treatment choices on a case-by-case basis in order to achieve conception.

Table 7 Presence or absence of sperm in ejaculated semen determined by the cutoff value of FSH.

FSH, mIU/mL	Sperm present in ejaculate	Sperm absent in ejaculate
≤15.3	26/55 (47.3%)	29/55 (52.7%)
>15.3	7/154 (4.5%)	147/154 (95.5%)

FSH, follicle-stimulating hormone.

A limitation of this study is the measurement of testicular volume using a punched-out Prader orchidometer. Testicular volume measured with a Prader orchidometer is larger than that measured through ultrasonography due to the thickness of the scrotal skin (Sakamoto *et al.* 2007). Accordingly, the testicular volume of our patients was slightly larger than previously reported values in patients with spermatogenesis dysfunction. Another limitation is the small number of patients in the FAZO group (*n* = 33), and further accumulation and analysis of these patients will be necessary in the future.

To our knowledge, this is the first study on the predictors of CZO or SOS in patients with a prior diagnosis of nonobstructive azoospermia (NOA) using a subsequent and thorough SA. Our findings indicated that the FSH level was a predictor of NOA diagnosed as CZO or SOS, with a cut-off value of 15.3 mIU/mL. Accordingly, patients with suspected NOA, who have FSH levels <15.3 mIU/mL, might benefit from repeat and thorough SA. However, our findings indicated that the false-positive rate is relatively high (52.7%) even in patients with FSH levels <15.3 mIU/mL. Therefore, it is important to explain to the patient that sperm detection is not guaranteed and obtain consent before repeat SA. In addition, even if a very small amount of sperm can be detected after multiple SAs, such sperm may be of poor quality and ineligible for ICSI; moreover, ICSI may yield unsatisfactory outcomes. In such cases, ICSI with testicular sperm may be required, which may necessitate TESE. Accordingly, this should also be communicated to the patient in advance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work.

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Author contribution statement

KU and TI designed and performed the study. KU, TI, AO and IH collected and analyzed the data. KU, HO, K Sugimoto and K Saito wrote the paper. KU, K Sugimoto, HO and K Saito supervised the research.

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