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ARTICLE

Observations of high variability in DNA fragmentation of epididymal sperm in men

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Objectives: *Men with obstructive azoospermia (OA) or infertility often require surgical sperm retrieval for assisted reproductive techniques. While sperm can be successfully obtained from either the testis or epididymis in these patients, sperm DNA integrity may differ between retrieval sites, which could influence reproductive outcomes. This study aimed to determine whether bilateral epididymal and/or testicular sperm extraction is necessary in men with OA or infertility and elevated DNA fragmentation index (DFI).*

Methods: *We retrospectively analyzed men who underwent bilateral testicular biopsy and/or microscopic epididymal sperm aspiration (MESA) by a single surgeon from 2020–2022. TUNEL assays were performed to assess DFI (normal ≤15%). The primary endpoint of the study was to evaluate the variability in DFI between the right and left testes/epididymis in men undergoing sperm extraction.*

Results: *In total, 24 men met criteria to be included in this analysis who underwent sperm extraction with DFI analysis via MESA and/or testicular biopsy. Among patients with OA, testicular sperm demonstrated significantly lower DFI compared to epididymal sperm on both sides (right testis 9.52 vs. right epididymis 17.61, $p = 0.01$; left testis 9.22 vs. left epididymis 14.71, $p = 0.04$). For each individual patient with OA, the mean intra-patient difference in DFI between right and left epididymal sperm was significantly higher than the mean intra-patient difference in DFI between right and left testicular sperm (12.09% ± 6.58, compared to 2.27% ± 1.59, $p < 0.001$, respectively).*

Conclusion: *Bilateral epididymal sperm extraction may be warranted in men with OA given the observed intra-patient variability in DNA fragmentation between epididymides. Conversely, bilateral testicular extraction may be unnecessary, as no significant difference in DNA fragmentation variability was observed between sperm retrieved from either testicle.*

Key Words: DNA fragmentation, microscopic epididymal sperm aspiration (MESA), testicular biopsy, bilateral sperm extraction, male infertility, TUNEL

Introduction

Infertility affects approximately 15% of couples, with male factor infertility contributing to nearly half of those cases.[1–3] Obstructive azoospermia (OA) accounts for an estimated 40% of azoospermia cases.[4,5] In cases of OA where reconstruction is not indicated, surgical sperm retrieval can be employed for assisted reproductive technology (ART), specifically *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).[6–8] In men with OA, rates of sperm retrieval are near 100% with the use of microscopic epididymal sperm aspiration (MESA), with live birth rates nearing 40% after MESA-ICSI.[9–11] Beyond OA, infertility can also result from poor semen parameters, including elevated sperm DNA fragmentation index (DFI), which is associated with lower fertilization and live birth rates.[12,13] In such cases, testicular sperm extraction may be utilized, as

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testicular sperm often have lower DFI than ejaculated sperm, potentially improving ART outcomes.[14]

A key factor influencing fertility outcomes is the DNA integrity of the sperm utilized, as higher levels of sperm DNA fragmentation have been associated with poorer embryo quality, reduced implantation rates, lower pregnancy rates, and higher miscarriage rates.[15–18] While the effect of DNA fragmentation on fertility outcomes in the setting of IVF/ICSI specifically is still debated, numerous studies show that high sperm DNA fragmentation is correlated with poor fertility outcomes.[17,19–22] As sperm progress through the male reproductive tract, they are exposed to oxidative stress, which contributes to increased DNA fragmentation.[23–27] Studies have shown that, in infertile men with elevated ejaculate sperm DNA fragmentation, sperm retrieved directly from the testis demonstrate better DNA integrity compared to ejaculated sperm.[28–30] In men with OA, sperm DNA integrity is unchanged in the testicles as compared to their fertile counterparts, and the degree of DNA fragmentation is higher in epididymal sperm as compared to testicular sperm.[14,31,32] However, it remains unstudied whether laterality differences exist in sperm DNA fragmentation within the epididymides and testicles of men with OA.

This study aims to address this gap by comparing DNA fragmentation in sperm retrieved from the right and left epididymides and testicles of men with OA or infertility characterized by poor semen parameters and elevated DFI. Specifically, we sought to determine whether laterality differences exist in sperm DNA fragmentation within these sites. Clarifying this question may help refine surgical decision-making regarding the necessity of unilateral *vs*. bilateral sperm retrieval and, consequentially, minimize unnecessary surgical interventions and improve fertility outcomes.

Materials and Methods

Study design and patient population

A retrospective analysis of all men who underwent a testicular or epididymal sperm extraction by a single, fellowship-trained surgeon (MG) from 2020–2022 at New York-Presbyterian Hospital – Weill Cornell Medical Center for OA or infertility associated with abnormal semen parameters and elevated DFI. Abnormal semen parameters were defined according to WHO criteria as having at least one parameter outside the reference range.[33,34] Preoperative evaluation included a complete history and physical examination to determine testicular volume. All patients underwent a physical exam with the use of an orchidometer as a reference standard to evaluate testicular volume, and a heating pad was used to relax the scrotal dartos muscle and to optimize examination of clinical varicoceles. Varicocele grading was recorded based on the Dubin and Amelar system.[35] Hormonal profiles, including serum testosterone and follicle-stimulating hormone (FSH), were obtained prior to surgical intervention. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays were performed on all testicular and epididymal samples to assess sperm DNA fragmentation (normal DFI: $\leq$15%). The TUNEL assay was performed as previously described.[36]

Men with OA initially underwent bilateral MESA, except in cases of prior testicular trauma, surgery, or grade II/III varicocele, where affected sites were avoided. Sides with varicoceles were avoided, if possible, given prior evidence that they can increase DFI;[37] however, if no unaffected side was available, the side with a varicocele was still pursued. If the retrieved sperm was visually assessed to be of poor quantity, a bilateral testicular biopsy was subsequently performed, adhering to the same site exclusions.

For men with infertility characterized by poor semen parameters and elevated DFI, sperm extraction was performed to optimize outcomes with ART. Bilateral microsurgical testicular biopsy was performed while avoiding sites of prior surgery, trauma, or grade II/III varicocele. Similarly, a side with a varicocele was pursued if necessary. Based on the visual assessment of sperm quantity, the decision to proceed with MESA was made, again avoiding adversely affected sites as described.

This study was reviewed and approved by the Institutional Review Board (IRB) of New York-Presbyterian Hospital–Weill Cornell Medical Center (IRB #20-07022335). The study was conducted in compliance with all applicable institutional and federal regulations for research involving human participants. Due to the retrospective study design, the requirement for informed consent was waived.

Epididymal sperm extraction method

Epididymal sperm extraction was performed by microdissection according to our previously described technique.[38] Using an operating microscope, epididymal tubule dissection and aspiration are performed using X10-15 magnification. Bipolar forceps are used to create an avascular plain overlying dilated, golden clear tubules, usually in the caput area. A 3.5 mm buttonhole is made in the tunic overlaying the tubules. A 15-degree micro

knife is used to puncture the tubules. After opening the epididymal tubule, a 5-μL micropipette with a 0.5-mm internal diameter, 0.9-mm outer diameter, and scored at 1-μL intervals (Drummond Scientific Co., Broomall, PA, USA) is placed adjacent to the effluxing epididymal tubule. Sperm are drawn into the micropipette by simple capillary action. The fluid is flushed into a sterile container of phosphate-buffered saline (PBS) solution (0.5 to 1.0 mL) obtained from the sperm-processing laboratory. The lab is instructed to cryopreserve the aspirate in multiple straws (aliquots) to allow use across several IVF cycles if needed. Epididymal aspirates are diluted in an equal volume of glycerol cryoprotectant. The aliquots are then gradually cooled to 4°C, transferred to a sequential freezer for controlled cooling to −90°C, and subsequently stored in liquid nitrogen at −196°C.

Microsurgical testis biopsy method

Testis biopsy was performed with the assistance of an operating microscope according to our previously described technique.[39] Briefly, the tunica albuginea was inspected under X15 magnification, allowing for visualization and avoidance of subtunical vessels. An avascular region on the mid-anterior surface was identified for a tunical incision, which was carefully made using a 15-degree micro knife, measuring approximately 5 to 10 mm in length. Subtunical vessels were cauterized with bipolar cautery before excising tissue, targeting large, white or yellow tubules, which are more likely to contain sperm. Each sample (usually <1 mg tissue/sample) was removed, mechanically cut, and dispersed in 0.1–0.3 cc simulated human tubal fluid buffered by HEPES and supplemented with 5% plasmanate in a microfuge tube. Specimens were passed several times through a 24-gauge Angio catheter to confirm disruption of tubules.

Statistical analysis

Patient and disease characteristics were analyzed using descriptive statistics, including proportions, median, and mean ± standard deviations. Distribution of the outcome variables (testicular and epididymal TUNEL assays) was assessed via the Shapiro-Wilk test, confirming skewed distribution. As such, differences between right *vs*. left-sided TUNEL assays were assessed by the Wilcoxon rank-sum test.

All data were analyzed using R v4.3.2 (R Foundation for Statistical Computing, Vienna, Austria). The significance level for all statistical tests was set at 0.05, and all tests were two-sided.

Results

In total, 24 men met the criteria to be included in this analysis who underwent sperm extraction with DFI analysis via MESA and/or testicular biopsy. Cohort characteristics are detailed in Table 1. Median (IQR) age and body mass index (BMI) were 38.0 (32.75–49.25) and 26.9 (26.07–29.49), respectively. The majority of men were never smokers (87.5%), while 2 men were former smokers (8.3%) and 1 man was actively smoking (4.2%). The most common surgical indications were history of vasectomy (N = 7, 29.2%), high DFI with poor semen parameters (N = 8, 33.3%), and CF-related congenital bilateral absence of vas deferens (CBAVD) (N = 3, 12.5%). The median (IQR) of preoperative serum total testosterone was 416 ng/dL (364–576), and the median (IQR) of preoperative serum FSH was 4.60 mIU/mL (3.30–4.88). Two men had bilateral varicoceles, while ten men had a left varicocele. In both cases of bilateral varicocele, the left-sided varicocele was of greater grade. 10 men underwent bilateral testicular biopsy alone; 5 underwent both bilateral testicular biopsy and unilateral MESA; 3 underwent bilateral MESA alone; 2 underwent bilateral MESA with unilateral testicular biopsy; and 4 underwent both bilateral testicular biopsy and bilateral MESA. Among those (n = 4) who underwent simultaneous bilateral testicular biopsies and bilateral MESA, the DFI across all sites were 9.70 ± 2.23; 21.85 ± 10.91; 7.00 ± 2.49; and 9.88 ± 3.13 for right testis, right epididymis, left testis, and left epididymis, respectively. Within this small subset, there were no statistically significant differences between right testicular and right epididymal DFI, between right and left testicular DFI, or between left testicular and left epididymal DFI (all $p > 0.05$).

We stratified the cohort by surgical indication (N = 16 with OA *vs*. N = 8 with increased DFI) and then subsequently by laterality of DFI measurement. These results are detailed in Table 2. When comparing the group as a whole, there was no significant difference between the right and left DFI for testicular sperm or for epididymal sperm, regardless of surgical indication ($p > 0.05$ for all). Testicular volume differed significantly between sides, with an average of 21.75 ± 4.71 cc on the right and 18.62 ± 5.62 cc on the left among those with OA ($p = 0.001$); similarly, right testis volume was increased among those with increased DFI (20.12 ± 6.06 cc *vs*. 15.38 ± 5.53 cc, $p = 0.01$).

DFI assay demonstrated significantly lower fragmentation of testicular sperm than epididymal sperm on both sides among patients with OA (right testicular DFI 9.52 ± 5.18 *vs*. right epididymal DFI

TABLE 1. **Cohort characteristics**

Characteristic	Overall
Number, N	24
Age, median [IQR]	38.00 [32.75, 49.25]
BMI, median [IQR]	26.90 [26.07, 29.49]
Smoking status, n (%)	
Never	21 (87.5)
Former	2 (8.3)
Current	1 (4.2)
Varicocele on exam, n (%)	
No	12 (50.0)
Unilateral (left-sided)	10 (41.7)
Bilateral	2 (8.3)
Surgery, n (%)	
Bilateral testicular biopsy	10 (41.7)
Bilateral MESA	3 (12.5)
Bilateral testicular biopsy with unilateral MESA	5 (20.8)
Bilateral MESA with unilateral testicular biopsy	2 (8.3)
Bilateral testicular biopsy with bilateral MESA	4 (16.7)
Surgical indication, n (%)	
Vasectomy	7 (29.2)
High DFI	8 (33.3)
CBAVD due to CF	3 (12.5)
CBAVD not due to CF	1 (4.2)
Iatrogenic OA following bilateral inguinal hernia repair	1 (4.2)
Idiopathic OA	4 (16.7)
Duration trying to conceive (years), median [IQR]	3.00 [1.81, 4.00]
Obstructive interval[a] (years), median [IQR]	8.00 [7.00, 12.50]
Preoperative serum total testosterone (ng/dL), median [IQR]	416 [364–576]
Preoperative serum FSH (mIU/mL), median [IQR]	4.60 [3.30–4.88]

Note. [a]Calculated among those n = 8 patients who underwent vasectomy (n = 7) or had iatrogenic OA (n = 1). Abbreviations: IQR, interquartile range; BMI, body mass index; DFI, DNA fragmentation index; CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; OA, obstructive azoospermia; MESA, microsurgical epididymal sperm aspiration; FSH, follicle-stimulating hormone.

17.61 ± 9.19, $p = 0.01$; left testicular DFI 9.22 ± 6.09 *vs*. left epididymal DFI 14.71 ± 10.04, $p = 0.04$) (Table 2). Among those patients with elevated DFI, there was only n = 1 patient who underwent MESA, and therefore, no comparison of testicular *vs*. epididymal DFI could be made.

The mean intra-patient difference in DFI between the right and left epididymis among patients with OA was 12.09% ± 6.58, compared to 2.27% ± 1.59 ($p < 0.001$) between the right and left testis (Table 3). Among men who underwent bilateral epididymal sperm extraction and had a concurrent varicocele, 100% (3/3) demonstrated a higher DFI on the right side. Of these, two had a unilateral varicocele and one had a bilateral varicocele.

Discussion

This study compares DNA fragmentation in sperm retrieved from both the right and left testicles and epididymides of men with OA or infertility with poor semen parameters associated with elevated DFI. Given that elevated sperm DNA fragmentation is associated with poorer IVF outcomes,[20] we aimed to determine whether testicular or epididymal sperm

TABLE 2. **Comparison of DFI by surgical indication and laterality**

Characteristic	Cohort stratified by surgical indication					
	Patients with OA (n = 16)			Patients with increased DFI (n = 8)		
	Right	Left	*p*-value	Right	Left	*p*-value
Testicular volume (cc), mean (SD)	21.75 (4.71)	18.62 (5.62)	0.001	20.12 (6.06)	15.38 (5.53)	0.01
Bilateral samples only						
Testicular DFI, mean (SD)	9.52 (5.18) (N = 11)	9.61 (6.59) (N = 11)	0.83	10.32 (4.49) (N = 8)	9.56 (4.69) (N = 8)	0.20
Epididymal DFI, mean (SD)	19.22 (9.77) (N = 9)	15.51 (10.3) (N = 9)	0.43	NA (N = 0)	NA (N = 0)	
	Testicular	**Epididymal**	***p*-value**	**Testicular**	**Epididymal**	***p*-value**
All cases						
Right DFI, mean (SD)	9.52 (5.18) (N = 11)	17.61 (9.19) (N = 12)	0.01	10.32 (4.49) (N = 8)	6.70 (NA) (N = 1)	NA
Left DFI, mean (SD)	9.22 (6.09) (N = 13)	14.71 (10.04) (N = 10)	0.04	9.56 (4.69) (N = 8)	NA (N = 0)	NA

Note. Abbreviations: SD, standard deviation; DFI, DNA fragmentation index; OA, obstructive azoospermia; NA, not applicable.

TABLE 3. **Intra-patient difference in DFI between the right and left testes and epididymis**

Characteristic	Cohort stratified by surgical indication					
	Patients with OA (n = 16)			Patients with increased DFI (n = 8)		
	Epididymis	Testis	*p*-value	Epididymis	Testis	*p*-value
Intra-patient DFI laterality difference[a], mean (SD)	12.09 (6.58)	2.27 (1.59)	<0.001	NA	1.41 (1.12)	NA

Note. [a]For those patients with bilateral DFI data, the absolute intra-patient difference between the right and left epididymal DFI values (mean: 12.09) was computed and compared to the absolute difference between the right and left testicular DFI values (mean: 2.27). Abbreviations: SD, standard deviation; DFI, DNA fragmentation index; OA, obstructive azoospermia; NA, not applicable.

extraction should be performed bilaterally or unilaterally in this population. For each individual patient with OA, the mean intra-patient difference in DFI between right and left epididymal sperm was significantly higher compared to right and left testicular sperm (12.09% ± 6.58, compared to 2.27% ± 1.59, $p < 0.001$). Taken together, these results indicate that bilateral sampling of the testicles is unlikely to provide additional benefit (assuming equal volume and consistency), as DFI showed minimal variation between sides. In contrast, the marked side-to-side variability observed in epididymal sperm suggests that bilateral epididymal sampling may be warranted to optimize the likelihood of retrieving sperm with lower DFI.

To the best of our knowledge, this study is the first to document significant variability in sperm DFI levels between the epididymides of individuals with OA undergoing sperm extraction. It's important to note that sperm DNA damage is a significant factor in ART outcomes. Previous evidence has shown that increased sperm DNA damage, as indicated by a higher DFI, adversely affects pregnancy outcomes in ART by delaying cleavage and affecting embryo quality.[20,21,40,41] Because of this, efforts have been made to measure and optimize DFI levels for men who pursue ART.

With continuous advancements, outcomes in ART have steadily improved over time. Despite these advancements, there remains no definitive guidance on whether to prioritize testicular or epididymal sperm for use in ART. Data has consistently shown that extracted testicular sperm has less DNA damage than the epididymis.[14,42] However, the inconsistent correlation between sperm origin and clinical outcomes has contributed to the ongoing lack of consensus. For example, van Wely et al. compared live birth rates in couples with OA due to either congenital

bilateral absence of the vas deferens or prior vasectomy, who underwent ICSI using sperm obtained via testicular *vs*. epididymal extraction. Their results showed that the live birth rate was 39% for couples utilizing epididymal sperm *vs*. 24% for couples using testicular sperm.[10] Their study suggests sperm maturation that occurs during transit to the epididymis is required for more optimal ICSI results. Furthermore, in a systematic review by the Cochrane Library, which evaluated clinical trials comparing the effectiveness of sperm retrieval techniques (testicular vs. epididymal) in men with azoospermia prior to ICSI. Their analysis concluded that there is insufficient evidence to recommend any specific sperm retrieval technique, and the surgeon should utilize the least invasive technique available.[43] These findings highlight the need for further research to optimize sperm.

We also considered potential confounding factors, such as varicocele, which is associated with increased sperm DNA fragmentation.[44,45] However, varicocele alone does not appear to explain the findings in our cohort. Among the men with varicocele who underwent bilateral epididymal sperm extraction, 100% (3/3) demonstrated higher DFI on the right side, contrary to the expected left-sided predominance if varicocele were the primary driver. Furthermore, the absence of significant DFI variability between bilateral testicular samples further supports that varicocele was not the main contributor to the differences observed. While there is no specific data-driven hypothesis for why higher DFI was observed on the right side irrespective of varicocele, it is likely attributable to the high intrinsic variability in sperm DNA fragmentation that can occur within the epididymis as sperm depart from the testis.

The findings from this study provide critical insights for optimizing clinical outcomes in men with OA or infertility with poor semen parameters and elevated DFI. Testicular biopsy is a surgical procedure with inherent risks, including testicular pain, hypogonadism, erectile dysfunction, testicular atrophy, infection, and hematoma.[46] When counseling men and planning treatments, it is important to consider minimizing surgical interventions when possible to minimize risks. Given our data showing minimal variation in DNA fragmentation between testicles, performing testicular sperm extraction unilaterally rather than bilaterally may be a preferred approach for men with OA or infertility and elevated DFI. This strategy could help reduce the risk of associated complications while still achieving effective outcomes. Conversely, there was a significantly greater variance in DFI between the right and left epididymides in these men. Given the evidence linking DFI to outcomes in ART,[20] bilateral epididymal sperm extraction may provide an opportunity to select sperm with lower DNA fragmentation, which could be advantageous for achieving optimal outcomes. Taken together, while unilateral testicular sperm extraction may be reasonable and reduce procedural risks, bilateral epididymal sampling could offer advantages in light of the observed variability in DNA fragmentation between the right and left epididymides.

Importantly, this study highlights the distinction between group-level comparisons and within-patient variability, which addresses the true clinical question of whether bilateral sampling offers value for individual patients. While no systematic left-right difference was observed across the cohort, the magnitude of intra-patient variability in epididymal sperm DFI was substantial and unpredictable, underscoring that the side yielding sperm with lower DFI cannot be reliably anticipated. Thus, our findings support the consideration of bilateral epididymal sampling in select cases where optimizing sperm quality is critical. Ultimately, further studies are required to support the need for bilateral MESA and to identify the patients who may benefit from a bilateral approach.

This study is not without limitations. The retrospective design leaves room for selection bias and limits the ability to control for confounding variables. Although the analysis includes 24 men over several years in a busy practice, a larger sample size would only help to sturdy the findings of this study. Additionally, all procedures were performed by a single surgeon at one institution, which may not reflect variations in techniques or outcomes at other sites. While the assay is widely used and accepted, it was the sole method employed for assessing DNA fragmentation and may not capture all aspects of sperm DNA integrity. Furthermore, the study relied on a single TUNEL assay per patient, which should be considered when interpreting the results. Finally, the follow-up does not provide any insight into clinical outcomes for these men, which is necessary to fully understand the relevance of the observed differences in DNA fragmentation. Future prospective randomized clinical trials that also assess pregnancy outcomes will be needed to validate these findings.

Conclusions

Our results reveal a high variability in DNA fragmentation between the epididymides in men who

underwent epididymal sperm extraction. These findings suggest the potential importance of performing bilateral epididymal sperm extraction to select sperm with lower DNA damage and potentially improve outcomes for men pursuing ART. On the other hand, no significant difference in DNA fragmentation variability was seen between testicular sperm retrieved from either side. However, further studies are needed to determine the necessity of bilateral MESA and to identify which patients may benefit from a bilateral approach.

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Author Contributions

Conceptualization: Manish Kuchakulla, Marc Goldstein; methodology: Marc Goldstein; data collection: Manish Kuchakulla, Hriday P. Bhambhvani, Runzhuo Ma; statistical analysis: Hriday P. Bhambhvani, Runzhuo Ma; writing—original draft preparation: Manish Kuchakulla, Hriday P. Bhambhvani, Robert Fisch; writing—review and editing: Manish Kuchakulla, Marc Goldstein, Hriday P. Bhambhvani, Jonathan Gal, Robert Fisch; supervision: Marc Goldstein. All authors have contributed substantially to this manuscript. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials

All data is available upon request.

Ethics Approval

This study involved human subjects and was reviewed and approved by the Institutional Review Board (IRB) of New York-Presbyterian Hospital–Weill Cornell Medical Center (IRB #20-07022335). The study was conducted in compliance with all applicable institutional and federal regulations for research involving human participants.

Informed Consent:

Due to the retrospective study design, the requirement for informed consent was waived.

Conflicts of Interest

The authors declare no conflicts of interest to report regarding the present study.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work the authors used the proofread feature in Microsoft Word to improve readability and correct grammatical errors. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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