Original Article

Incorporating Sperm DNA Fragmentation Index with Computer-Assisted Semen Morphokinematic Parameters as a Better Window to Male Fertility

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Abstract

This study aimed to assess (1) the reproducibility of three sperm chromatin dispersion (SCD) assays for sperm DNA fragmentation, i.e., LensHooke R10® (R10), Halosperm G2® (G2), and BASO® (BA); (2) the correlation between computer-assisted semen analyzer (CASA) morphokinematic parameters and sperm DNA fragmentation index (DFI), and (3) the diagnostic value for male reproduction by combining semen morphokinematic parameters and DFI. Total 50 male participants were recruited, and all collected semen samples underwent semen analyses and SCD assays. Intra- and inter-observer variability of DFI data from different SCD measures was tested. In addition, the predictive ability of CASA parameters and DFI (with different cutoffs, i.e., 15% and 20%) for infertility was assessed using receiver operating characteristic curve analysis. We found that the G2 and R10 produced satisfactory variance coefficients (5.53%, 5.67%) compared to BA (14.8%). The DFI data from the R10 had lower intra-observer variability, in terms of higher intra-class coefficient (0.9615), than that of the G2 (0.8847) or BA (0.8824). Inter-observer variability of three SCD kits in scoring the DFI was comparable and satisfactory (concordance correlation coefficients ranging 0.9895–0.9630). The CASA parameters (i.e., total motility [$r = -0.57$], progression motility [$r = -0.55$], and rapidly progressive motility [$r = -0.55$]) were significantly correlated with DFI ($P < 0.001$). The predictive ability of the 15%-cutoff DFI data was better than that of the 20%-cutoff or continuous DFI data. The model comprising the CASA parameters, 15%-cutoff DFI, and 4%-cutoff normal morphology had the highest area under curve (0.8125) for infertility. For SCD assay, the R10 was the most reliable SCD assay to detect sperm DNA fragmentation. Combining the sperm DFI with CASA parameters might be a better diagnostic tool for male reproduction.

Keywords: Computer-assisted semen analyzer, conventional semen analysis, DNA fragmentation index, male infertility, sperm chromatin dispersion

Introduction

Approximately 15% of couples suffer from infertility after 1 year of unprotected intercourse. Patients with male factors which comprise a variety of causes contribute about half of all infertile cases.[1] According to the WHO 6th Edition,[2] the most common assessment tool for male fertility is conventional semen analysis (SA) in which the

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manual SA (MSA) assesses macroscopic and microscopic evaluations. With recently improved automation technology, the computer-assisted semen analyzer (CASA) system was introduced and has been proven to provide quick and accurate sperm kinematic data.[3]

Given the complex nature of human fertility, the morphokinematic parameters measured from the SA, however, might be insufficient to explain all reproductive outcomes.[4] Accumulating evidence has indicated that elevated sperm DNA damage might be associated with unexplained infertility, recurrent pregnancy loss, and offspring genetic diseases.[5-7] The extent of sperm DNA damage can be quantified with calculating the sperm DNA fragmentation index (DFI).[8,9] Recently, DFI has become a merging indicator in evaluating sperm quality besides conventional SA. However, the pathological cutoff point of DFI remains debated because the determining value may vary in different sperm DNA fragmentation assays and clinical circumstances.[10-12]

The sperm chromatin dispersion (SCD) test has been shown to be a simple and effective tool to determine sperm DNA integrity. This test is based on the concept that sperm with fragmented DNA cannot produce the characteristic halo of dispersed DNA loops after acid denaturation.[10] It is noteworthy that simplified biochemical steps and image-based interpretation make the SCD test clinically valuable. The Halosperm® kit (INDAS Laboratories, Madrid, Spain) was the first SCD assay to detect sperm dispersed DNA loops under bright-field microscopy.[13] Halosperm G2® (G2)[14] and BASO® (BA)[15] are two SCD kits on the market. Since SCD assays require high image quality to differentiate the sperm with or without DNA fragmentation, LensHooke R10® (R10) recently joined the market and claimed fewer biochemical steps with better image quality to categorize the halos surrounding the sperm head compared to G2.[16] However, the comparison of these three SCD assays for sperm DNA fragmentation has not been reported in detail.

Against these backgrounds, this study first assessed and compared the reproducibility (inter- and intra-observer variability) in scoring the DFI data from three available SCD kits (i.e., the G2, R10, and BA). Second, we evaluated the correlations between the CASA morphokinematic parameters and DFI measured from the most reliable SCD kit. Finally, the extent to which morphokinematic parameters, DFI, and normal morphology explain male fertility was assessed, in which the crucial predictors for infertility were identified, and the predictive ability of different DFI cutoffs was analyzed.

Materials and Methods

Study design and participants

Before commencement of the study, permission was obtained from the Institutional Review Board of National Cheng Kung University Hospital (NCKUH), Tainan, Taiwan (A-ER-110-017). Written consent was obtained from the participants.

Semen samples were obtained from 50 male donors (8 fertile males and 42 infertile males, with excluding the cases having female factor infertility and azoospermia) who were enrolled in this prospective study between January and June 2021 at the fertility center of NCKUH.

Manual semen analysis and computer-assisted semen analyzer

Each semen sample of study participant was collected after 2–3 days of abstinence by masturbation into a sterile container and placed in an incubator at 37°C until fully liquified. The semen preparation and examination were processed under the WHO 6th edition guidelines (2021).[2] Each liquefied semen sample was then aliquoted into four separates: one separate for conventional sperm analysis including the MSA and CASA and the other three separates for the SDF tests by three different SCD assay kits, R10, G2, and BA, respectively. Manual semen macroscopic parameters included liquefaction time, PH, appearance, and volume. Moreover, the microscopic sperm concentration and morphokinematic parameters such as morphology (strict Kruger criteria[17]), total motility (TM), progressive motility (PR), rapidly progressive motility (RP), slowly progressive motility (SP), non-progressive motility (NP), immotility (IM), linearity (LIN), straightness (STR), wobble (WOB), velocity along the average path (VAP), velocity along the straight-line path (VSL), velocity along the curvilinear path (VCL), lateral displacement of the head (ALH), and beat-cross frequency (BCF) were measured by LensHooke X1® CASA system (Bonraybio, Taichung, Taiwan).[18]

Sperm chromatin dispersion assays and DNA fragmentation index

Three different SCD assay kits (R10, G2, and BA) were performed to assess sperm DNA fragmentation. The stepwise method and necessary information were based on their individual instructions.[12-14] Briefly, the procedures consisted of (1) embedding the sperm sample into an agarose matrix to provide a languid suspension-like environment to manipulate the spermatozoa; (2) diluting the fresh liquefied semen sample by phosphate-buffered saline followed by DNA denaturant reagent to dissolve the DNA double helix, which only presented in damaged DNA; and (3) washing, dehydrating by ethanol baths, and staining with Wright-Giemsa dye for visualization under bright-field microscopy. All sample slides obtained after chromatin staining followed each SCD kit procedure were observed under 20X objective of Olympus® BX53 under the bright-field light source. These target images were then captured and converted to highly contrasting images equal to 100X objective of the microscope. There were generally five sperm head patterns to be distinguished (1) large halo: the halo width was equal or wider than the diameter of the core; (2) medium-size halo: the halo width was between these with large halo and with very small halo; (3) very small-size halo: the halo width was similar or shorter than one-third of the diameter of the core; (4) sperm cell without a halo;

and (5) degraded (weaky or irregularly stained) sperm cell without a halo. The DFI was defined by the percentage of very small-size halo, without a halo, and degraded sperm cells. At least 500 sperm cells typically need to be evaluated in each specimen to produce the DFI.[10]

Accessing the reproducibility of three sperm chromatin dispersion kits

To compare the reproducibility of three SCD kits, the agreement statistics were conducted, including (1) intra-observer variability which reflects that the amount observers vary from one another when reporting the DFI value on the same SCD kit and (2) inter-observer variability which indicates that the amount one observer varies between observations when reporting the DFI value more than once on the same kit. Specifically, a total of 10 semen samples from these 50 male donors were analyzed. Each sample was split into three aliquots and examined, respectively, by three different SCD kits (G2, R10, and BA), and in each kit, one semen sample was then equally divided into left (L) and right (R) test regions on the same slide for evaluation of DFI, respectively. Using each SCD kit, the same technician scored the DNA fragmentation (DFI value) three times for each sample on each test region (L and R). As a result, there were 60 DFI values from each SCD kit. The intra-observer variability of the DFI data from each SCD kit was determined based on the agreement of the DFI values from the same observer using intra-class correlation (ICC), while the inter-observer variability was evaluated based on the agreement of the DFI values between two technicians on the same kit and presented as concordance correlation coefficient (CCC). The ICC and CCC values are all in a range of 0–1, higher values indicating lower intra- or inter-observer variation.

Receiver operating characteristic curve

The SCD kit with the low observer's variation was chosen for the following analysis. First, the correlation between individual CASA parameter and DFI value (measured from the most reliable SCD kit) was assessed and the significance of correlation coefficient of parameter was determined using linear regression t-test. Second, all patients were divided into four subgroups according to the median value of DFI (with a cutoff point of 15% or 20%) and normal morphology (determined by LensHooke X1®) (with a cutoff point of 4%). The difference in patient characteristics (including age, infertility duration, MSA parameters, CASA parameters, and DFI values) across four subgroups was tested using Kruskal–Wallis test. Finally, the best predictive model that comprised CASA parameters, normal morphology (with a cutoff point of 4%), and DFI values for male infertility was identified using receiver operating characteristic (ROC) analysis. Of noted, the DFI data were treated as continuous variables or dichotomous variables (based on a cutoff of 15% or 20%). Moreover, the predictive ability of different types/cutoffs of DFI values was examined.

Statistical analysis

Data processing and statistical analyses were all performed using SAS software version 9.4 (SAS Institute, Cary, NC, USA). Descriptive statistical analyses were first performed and presented as mean ± standard deviation for continuous variables and frequency (proportion) for dichotomous and categorical variables.

Results

Intra- and inter-observer reliability of three sperm chromatin dispersion assays

Table 1 presents intra-observer variability of DNA fragmentation index data from three sperm chromatin dispersion kit assays (total of 60 DFI tests in each SCD method). Among the three kits, BA has the highest coefficient of variance in the assessment of sperm DNA fragmentation (i.e., 14.80%, 5.67%, and 5.53% for the BA, R10, and G2, respectively). Regarding to intra-class variability, the R10 has the highest ICC (0.9615) compared to that of G2 (0.8847) or BA (0.8824). Supplementary Table 1 shows the inter-rater reliability between the two technicians (i.e., readers) on the DFI data from the same kit and stratified by different operators (i.e., operators 1 and 2) who prepared the semen samples for observation. Higher CCC values between readers (0.9895 and 0.9874) were noted on the DFI data in the G2.

Image quality of three sperm chromatin dispersion assays

Figure 1 illustrates the images of five categories of SCD dying patterns processed by each SCD diagnostic reagent. All images were captured by a 20 million pixels charge-coupled device camera under 20X objective of Olympus® BX53 with the bright-field light source and converted to highly contrasting images equal to 100X objective without image editing. Five categories of SCD patterns processed by the R10 revealed the highest structured resolution in defining sperm core from halo width. Besides, the characteristics of dispersing halo, sperm core, and tail were clearly identified by the R10 dying slides. However, the property of poorly differentiated background color from the border of dispersing halo processed by the G2 kits often presented poorly contrasting images with misinterpretation of the halo sizes. In the BA SCD dying slides, although they had the property of clearly recognizing background color from the border of dispersing halo, these unstable qualities of dying agents made poorly differentiated levels of identifying halo sizes and demonstrated gaps in

Table 1: Intra-observer variability of DNA fragmentation index data from three sperm chromatin dispersion kit assays

SCD kit	*n*	DFI (%), mean±SD	DFI (%) median	DFI (%) range	CV (%)	ICC
Halosperm G2®	60	13.10±2.96	14	6-18	5.53	0.8847
LensHooke R10®	60	13.32±5.3	14	3-25	5.67	0.9615
BASO BA®	60	2.42±1.74	2	1-8	14.8	0.8824

"*n*" refers to the number of semen samples. ICC ranges from 0~1, with higher values indicating lower intra-observer variation. DFI: DNA fragmentation index, SCD: Sperm chromatin dispersion, CV: Coefficient of variance, ICC: Intra-class correlation, SD: Standard deviation

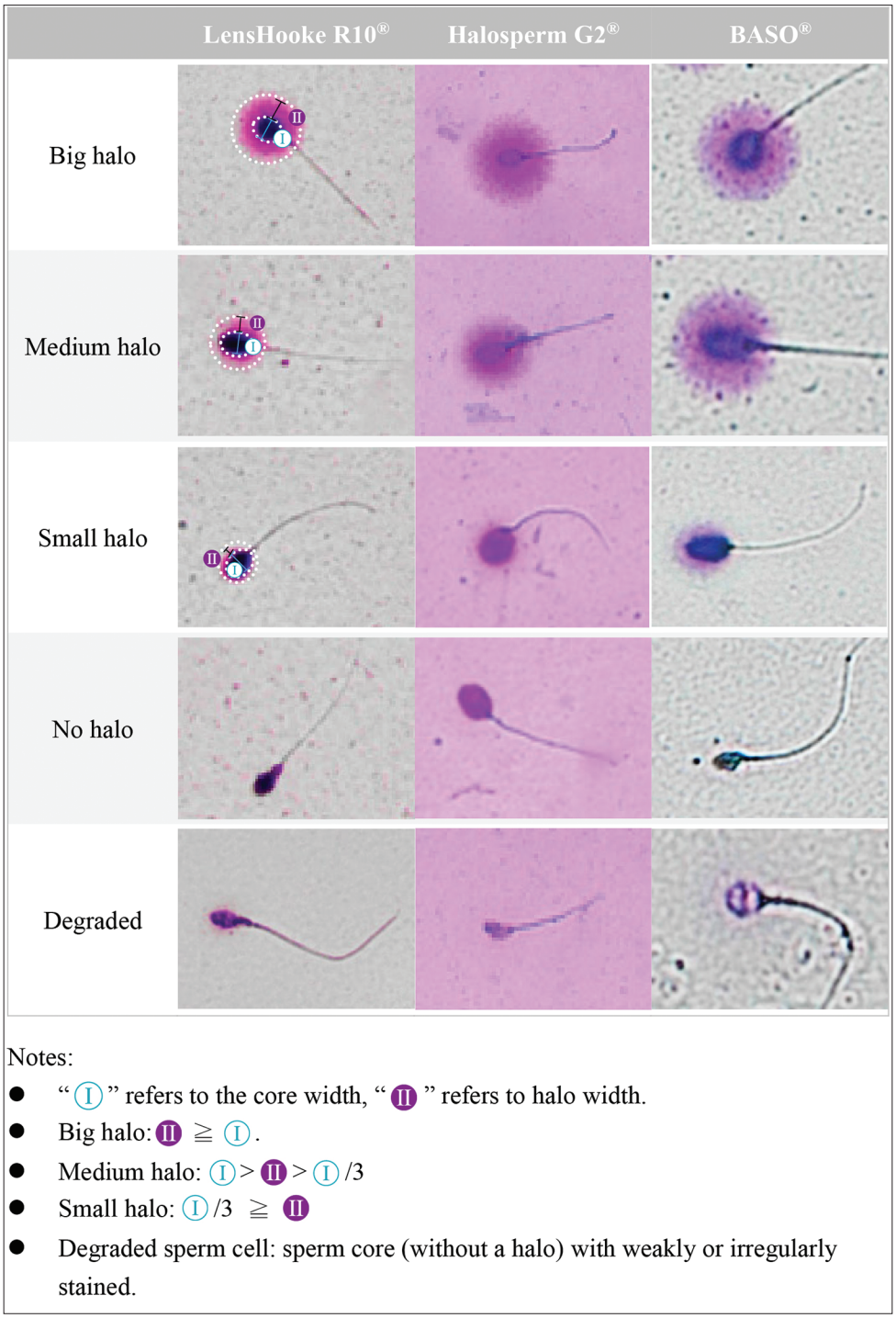

Figure 1: Comparison of three SCD diagnostic reagents in the definition of five categories of halo size. SCD: Sperm chromatin dispersion.

the DFI values compared with the other SCD methods while processing the same semen samples. Overall, our data suggested that R10 provides a significantly higher resolution of dispersed chromatin and sperm core images than G2 or BA.

Four subgroups stratified by DNA fragmentation index and sperm morphology

Total semen samples were obtained from 50 male participants (8 fertile men versus 42 infertile men). The comparison of semen parameters and DFI values is illustrated in Supplementary Table 2. A total of 50 participants (with 50 semen samples) were subdivided into four groups with the DFI (15% or 20% as cutoff values) measured from with the R10 and sperm morphology (with a cutoff point 4% assessed by the CASA). As shown in Table 2, there are 7, 10, 9, and 24 participants in the DFI <15% and normal morphology <4%, DFI < 15% and normal morphology ≥4%, DFI ≥15% and normal morphology <4%, and DFI ≥15% and normal morphology ≥4% groups, respectively.

Table 2: Clinical characteristics of four subgroups categorized by the DNA fragmentation index (with a cutoff point of 15%) and normal morphology (with a cutoff point of 4%)

Characteristics	Overall		DFI <15% and normal morphology <4%		DFI <15% and normal morphology ≥4%		DFI ≥15% and normal morphology <4%		DFI ≥15% and normal morphology ≥4%		*P*
	n	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	
Age at study enrollment (year)	50	38.56±5.55	7	39.00±8.52	10	36.40±4.27	9	40.67±6.16	24	38.54±4.74	0.6019
Infertility duration at study enrollment (year)	50	2.67±2.60	7	2.57±3.05	10	2.83±3.40	9	3.38±2.19	24	2.37±2.34	0.3548
Fertile (%)	50	2.67±2.60	7	2.57±3.05	10	2.83±3.4	9	3.38±2.19	24	2.37±2.34	0.6764
MSA											
Volume (mL)	50	3.11±1.72	7	3.14±1.35	10	3.01±1.7	9	3±1.94	24	3.18±1.83	0.9742
Concentration (×10^6/mL)	50	53.31±46.58	7	45.86±41.01	10	56.3±30.83	9	14.03±17.31	24	68.97±53.17	0.0047
CASA											
Concentration (×10^6/mL)	50	59.06±45	7	49.81±50.21	10	74.01±31.86	9	22.02±28.98	24	69.42±46.85	0.0091
TM (%)	50	53.05%	7	54.86%	10	79.56%	9	23.89%	24	52.42%	0.0008
PR (%)	50	40.76%	7	39.29%	10	63.10%	9	16.67%	24	40.92%	0.0009
RP (%)	50	15.04%	7	12.14%	10	28.40%	9	5.67%	24	13.83%	0.0007
SP (%)	50	25.90%	7	27.29%	10	34.70%	9	11.44%	24	27.25%	0.0102
NP (%)	50	12.04%	7	15.57%	10	14.70%	9	7.56%	24	11.58%	0.0811
IM (%)	50	47.34%	7	45.14%	10	22.20%	9	76.33%	24	47.58%	0.0011
LIN (%)	50	42.22%	7	41.86%	10	47.30%	9	27.11%	24	45.88%	0.0045
STR (%)	50	66.62%	7	65.29%	10	71.50%	9	50.67%	24	70.96%	0.0301
WOB (%)	50	57.14%	7	59.00%	10	61.20%	9	43.78%	24	59.92%	0.0249
VAP (μm/s)	50	14.57±4.66	7	15±1.09	10	18.41±3.28	9	9.6±6.11	24	14.7±3.56	0.0039
VSL (μm/s)	50	11.61±4.30	7	11.83±1.89	10	15.17±3.2	9	6.68±4.99	24	11.92±3.27	0.002
VCL (μm/s)	50	23.39±7.05	7	23.83±1.59	10	28.27±4.89	9	18.19±11.19	24	23.18±5.61	0.0429
ALH (μm)	50	1.8±0.58	7	1.74±0.22	10	2.07±0.5	9	1.53±0.93	24	1.8±0.49	0.3846
BCF (Hz)	50	5.88±1.29	7	6.14±0.97	10	6.38±0.62	9	4.76±2.56	24	6.01±0.49	0.1355
Normal morphology (%)	50	4%	7	3%	10	5%	9	2%	24	5%	<.0001
DFI (%)											
LensHooke R10®	50	22%	7	10%	10	10%	9	32%	24	26%	<.0001
Halosperm G2®	50	20.58%	7	13.79%	10	9.95%	9	28.83%	24	23.90%	0.0003
BASO BA®	50	5.87%	7	3.36%	10	2.70%	9	7.56%	24	7.29%	0.0069

P<0.05 indicates a statistical difference across four subgroups stratified by the DFI and normal morphology. "*n*" refers to the number of participants. DFI data were measured using LensHooke R10®. DFI: DNA fragmentation index, SD: Standard deviation, MSA: Manual semen analysis, CASA: Computer-assisted semen analysis, TM: Total motility, PR: Progressive, RP: Rapidly progressive, SP: Slowly progressive, NP: Nonprogressive, IM: Immotile, LIN: Linearity, STR: Straightness, WOB: Wobble, VAP: Velocity along the average path, VSL: Velocity along the straight-line path, VCL: Velocity along the curvilinear path, ALH: Amplitude of the lateral displacement of the head, BCF: Beat-cross frequency

There were no differences among the four subgroups regarding the baseline characteristics such as age, infertility duration, and the proportion of fertile patients. However, the MSA parameters (including sperm concentration) and CASA morphokinematic parameters (including concentration, TM, PR, RP, SP, IM, LIN, STR, WOB, VAP, VSL, and VCL) were significantly different across the subgroups ($P < 0.05$). Supplementary Table 3 provides the clinical characteristics of four subgroups according to the DFI (with a cutoff value 20%) and normal sperm morphology (with a cutoff 4%) data. Similarly, the MSA and CASA parameters were significantly different across the subgroups ($P < 0.05$).

Correlation between the semen parameters and DNA fragmentation index

As shown in Table 3, the DFI was significantly and negatively associated with the concentration, SP, NP, VAP, VSK, VCL, ALH ($P < 0.05$), TM, PR, and RP ($P < 0.0001$) while positively associated with the IM ($P < 0.0001$).

Prediction of semen parameters, normal sperm morphology, and DNA fragmentation index for male infertility

Figure 2 suggests that the model with the DFI data based on a cutoff of 15% (area under curve [AUC = 0.6172]) provided better prediction than that of DFI with a cutoff of 20% (AUC = 0.5500) or continuous DFI data (AUC = 0.6141). We further examined different combinations of the DFI (with a cutoff of 15%), CASA parameters, and normal morphology (with a cutoff point of 4%) and found that the model which comprised three different types of information (DFI, CASA, and normal morphology) yielded the best prediction for male infertility (AUC: 0.8125) [Figure 3]. Of noted, seven CASA

parameters (i.e., RP, IM, STR, WOB, VSL, ALH, and BCF) included in this final model were identified from model selection procedures.

Discussion

This prospective study provides the first preliminary evidence on the comparative reliability of the three available SCD kit assays in the assessment of sperm DNA fragmentation, the correlation between the DFI data and CASA kinematic parameters, and the best prediction model for male infertility.

Table 3: Correlations between semen morphokinematic parameters and the DNA fragmentation index (measured using the LensHooke R10®)

	Correlation Coefficient	*P*
MSA		
Volume (mL)	−0.04	0.81
Concentration (×10⁶/mL)	−0.28	0.05
CASA		
TM (%)	−0.57	<0.0001
PR (%)	−0.55	<0.0001
RP (%)	−0.55	<0.0001
SP (%)	−0.47	0.0005
NP (%)	−0.42	0.0005
IM (%)	0.56	<0.0001
LIN (%)	−0.24	0.09
STR (%)	−0.13	0.35
WOB (%)	−0.27	0.06
VAP (μm/s)	−0.50	0.0005
VSL (μm/s)	−0.46	0.0005
VCL (μm/s)	−0.40	0.0005
ALH (μm)	−0.30	0.04
BCF (Hz)	−0.23	0.11

DFI: DNA fragmentation index, MSA: Manual semen analysis, CASA: Computer-assisted semen analysis, TM: Total motility, PR: Progressive, RP: Rapidly progressive, SP: Slowly progressive, NP: Nonprogressive, IM: Immotile, LIN: Linearity, STR: Straightness, WOB: Wobble, VAP: Velocity along the average path, VSL: Velocity along the straight-line path, VCL: Velocity along the curvilinear path, ALH: Amplitude of the lateral displacement of the head, BCF: Beat-cross frequency

Specifically, according to the intra- and inter-observation agreement testing, the R10 was suggested as the most reliable compared to the other two assays. The G2 reagent, as the most commonly used SCD kit today, has been proven its reliability to determine sperm DNA fragmentation.[13] However, it generally takes more than 1 h to process one semen sample and may yield the poorly contrasting images due to undifferentiated background color. That is, the G2 might be problematic to measure the halo size under bright-field microscopy as shown in [Figure 1], leading a difficulty in determining the DNA integrity. In contrast, the R10 assay provides a high resolution of dispersed chromatin and sperm core images. In terms of time efficiency, it took much less time for SDF testing by the R10 than the G2 or BA to process one semen sample, which echoes the report of Chang *et al*. in 2021.[16] The comparison of time needed and costs for three SCD assays is illustrated in Supplementary Table 4.

Impairment of sperm DNA integrity has been recognized as one of the reasons for unexplained infertility and repeated pregnancy loss.[19] However, an optimal pathological DFI cutoff value for infertility in the clinical guidelines is yet to be determined. The DFI cutoff values vary widely in different study cohorts, including 15%,[20,21] 20%,[10,22] and 30%.[23,24] DFI exceeding 30% is often considered a worse reproductive outcome, even with ART. The various DFI cutoff values may result from different sperm DNA fragmentation tests, inconsistent semen processing protocols, and small sample-size study cohorts. Interestingly, different SCD assay kits recommend different DFI thresholds. LensHooke R10® (R10) recommends sperm DFI at a cutoff point of 26.1% suggested by Wiweko and Utami in 2017.[25] Halosperm G2® (G2) suggested a threshold of 30% based on the study by Evenson and Wixon in 2006[26] On the contrary, BASO® (BA) does not offer any recommendation regarding the DFI threshold. Our data suggested that the DFI with a cutoff of 15% yielded a better prediction (greater AUC) for male infertility than that of DFI with a cutoff of 20% or continuous DFI data [Figure 2]. Our present study recruited only 50 male participants. The statistical power would be limited if set with an extreme 30% of cutoff value. Nevertheless, future large prospective research remains warranted to corroborate our findings.

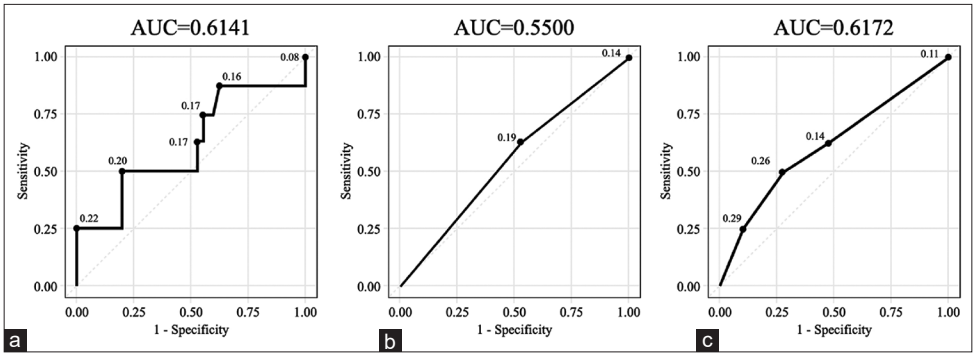

Figure 2: ROC curve analysis for male infertility using different DFI cutoff values. (a) The model with DFI as continuous variable, (b) the model with DFI as dichotomous variable based on a cutoff point of 20%, and (c) the model with DFI as dichotomous variable based on a cutoff point of 15%. ROC: receiver operating characteristic, AUC: area under receiver operating characteristic curve, DFI: DNA fragmentation index.

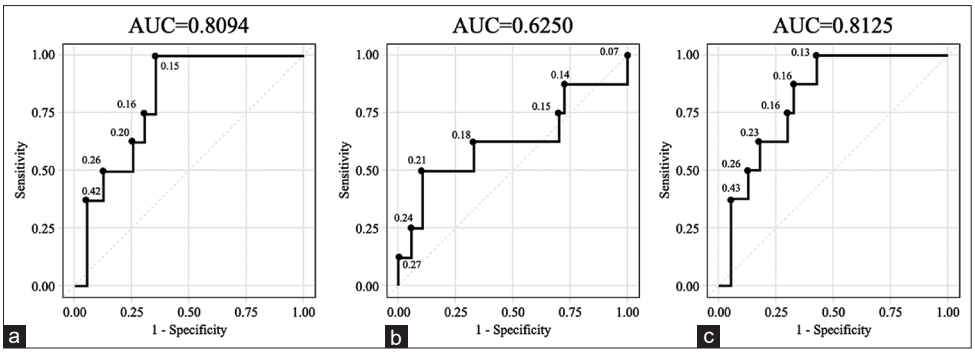

Figure 3: ROC curve analysis for male infertility. (a) The model comprising the DFI (a cutoff point of 15%) and CASA parameters, (b) the model comprising the DFI (a cutoff point of 15%) and normal morphology (a cutoff point of 4%), and (c) the model comprising the DFI (a cutoff point of 15%), CASA parameters, and normal morphology (a cutoff point of 4%). Note: CASA parameters included in the models were RP, IM, STR, WOB, VSL, ALH, and BCF. ROC: receiver operating characteristic, AUC: area under receiver operating characteristic curve, DFI: DNA fragmentation index, CASA: computer-assisted semen analysis, IM: immotile, STR: straightness, WOB: wobble, VSL: velocity along the straight-line path, ALH: amplitude of the lateral displacement of the head, BCF: beat-cross frequency.

Cissen *et al*.[27] have published one meta-analysis regarding four different sperm DNA fragmentation measurements and clinical reproductive outcomes of ART (IVF and ICSI). The result suggests that current sperm DNA fragmentation tests have limited capacity to predict the chance of pregnancy in the context of ART. In addition, sperm DNA fragmentation tests have little or no difference in predictive value between IVF and ICSI. This meta-analysis incorporated five studies using the SCD test; four of them used Halosperm dying kits, and only one used SpermFunc™ DNAf kit (BRED Life Science, Shenzhen, China). The result showed that predictive accuracy for pregnancy with ART (IVF and ICSI) of the SCD test was poor (AUC: 0.49) under ROC curve analysis.

It is controversial regarding the correlation between sperm DFI and semen parameters, and many studies have focused on conventional SA parameters. Sivanarayana *et al*.[22] reported negative correlations between the sperm DFI and conventional SA parameters (i.e., count, motility, and morphology). Muriel *et al*.[28] showed the negative correlations between DFI and sperm morphology and motility. In contrast, some studies also concluded no significant correlations between conventional SA parameters and the sperm DFI.[29-31] The CASA generally provides more detailed kinematic characteristics than the MSA. Standard CASA parameters ordinary includes nine variables: VCL, VSL, VAP, ALH, BCF, MAD, WOB, straightness (STR, VSL/VAP), and linearity (LIN, VSL/VCL).[2] These variables categorize the subgroups of spermatozoa, which have been linked to treatment according to motility change and genetic perturbations.[32,33] The CASA has been used to evaluate sperm hyperactivation in clinical practice and correlated to subsequent fertility.[34] This study was the first to assess the correlations between semen morphokinematic parameters measured from the CASA and DFI. The CASA kinematic parameters, including RP and SP, which have been known as important predictors for pregnancy outcomes,[35,36] were negatively correlated with sperm DFI in this study. The VCL, LIN, and ALH, which are three CASA parameters related to sperm motility, all showed a significant negative correlation with the sperm DFI in our cohort. Furthermore, the TM, PR, and RP, which are known as prognostic factors for male fertility outcomes,[37,38] were highly correlated with the DFI data.

According to the ROC analysis, the combination of DFI (a cutoff 15%), seven selected CASA kinematic parameters (i.e., RP, IM, STR, WOB, VSL, ALH, and BCF), and normal morphology (a cutoff of 4%) generated the best prediction for male infertility (explaining the infertility up to 81.25%). Our result indicates that the MSA, CASA, or SCD test alone had a limited ability to discriminate male infertility status (in terms of low AUC values). The integration of semen parameters, sperm DNA fragmentation, and normal morphology thus may offer a comprehensive picture to describe male reproductive health. However, due to the small sample size (n = 50) in this study, future research with larger sample sizes of patients is needed to confirm our findings.

Conclusion

Our result indicates that the R10 may be the most reliable SCD assay to determine sperm DNA fragmentation. Besides the common sperm motility parameters such as TM, PR, and RP, the other CASA generating kinematic parameters (including SP, NP, LIN, STR, WOB, VCL, ALH, and BCF) were negatively associated with the DFI in our study cohort. The combination of sperm DFI with CASA morphokinematic parameters might be a better diagnostic tool for male infertility in clinical practice.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: DNA fragmentation index values by study operators and readers, and inter-observer variability in scoring the DNA fragmentation index data between readers, stratified by the operators who prepared semen samples and by three sperm chromatin dispersion kit assays

	n	DFI (%), mean±SD	DFI (%) median	DFI (%) range	CCC between readers on DFI from Halosperm G2®	CCC between readers on DFI from LensHooke R10®	CCC between readers on DFI from BASO BA®
Operator 1							
Reader 1	60	18.18±18.84	12	2-94	0.9895	0.9630	0.9751
Reader 2	60	18.22±18.68	13	0-96			
Operator 2							
Reader 1	60	19.36±20.76	12	0.6-94	0.9874	0.9807	0.9692
Reader 2	60	20.36±21.1	12.5	0-95			

"*n*" refers to the number of semen samples. CCC ranges from 0~1, with higher values indicating lower inter-observer variability. DFI: DNA fragmentation index, SD: Standard deviation, CCC: Concordance correlation coefficient

Supplementary Table 2: The comparison of semen parameters and DNA fragmentation index values among fertile and infertile groups

Characteristics	Mean±SD or %		*P*
	Fertile group (*n*=8)	Infertile group (*n*=42)	
Age at study enrollment (year)	40.05±3.15	38.62±4.95	0.027
MSA			
Volume (mL)	3.12±1.40	2.99±1.45	0.6
Concentration (×10⁶/mL)	72.23±52.24	53.05±46.39	0.03
CASA			
Concentration (×10⁶/mL)	70.63±24.58	60.41±46.18	0.076
TM (%)	74.4%	53.77%	<0.0001
PR (%)	59.2%	41.05%	<0.0001
RP (%)	21%	16.2%	0.012
SP (%)	38.25%	25.02%	<0.0001
NP (%)	15.2%	12.19%	0.0013
IM (%)	25.6%	46.96%	<0.0001
LIN (%)	45.75%	42.62%	0.034
STR (%)	71.2%	67.41%	0.0056
WOB (%)	59.15%	57.35%	0.1502
VAP (μm/s)	16.49±2.64	14.57±5.01	0.0012
VSL (μm/s)	13.62±2.69	11.75±4.55	0.0012
VCL (μm/s)	26.19±3.41	23.15±7.36	0.0002
ALH (μm)	1.97±0.24	1.76±0.6	0.0008
BCF (Hz)	6.39±0.54	5.78±1.48	<0.0001
Normal morphology (%)	5.2%	4.06%	0.0059
DFI (%)			
LensHooke R10®	12.8%	21.62%	<0.0001
Halosperm G2®	14.75%	23.22%	<0.0001
BASO BA®	3.42%	6.55%	<0.0001

P<0.05 indicates a statistical difference. "*n*" refers to the number of participants. TM: Total motility, PR: Progressive, RP: Rapidly progressive, SP: Slowly progressive, NP: Nonprogressive, IM: Immotile, LIN: Linearity, STR: Straightness, WOB: Wobble, VAP: Velocity along the average path, VSL: Velocity along the straight-line path, VCL: Velocity along the curvilinear path, ALH: Amplitude of the lateral displacement of the head, BCF: Beat-cross frequency, DFI: DNA fragmentation index, SD: Standard deviation, MSA: Manual semen analysis, CASA: Computer-assisted semen analysis

Supplementary Table 3: Clinical characteristics of four groups categorized by the DNA fragmentation index (with a cutoff point of 20%) and normal morphology (with a cutoff point of 4%)

Characteristics	Overall		DFI <20% and normal morphology <4%		DFI <20% and normal morphology ≥4%		DFI ≥20% and normal morphology <4%		DFI ≥20% and normal morphology ≥4%		*P*
	n	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	*n*	Mean±SD or %	
Age (year)	50	38.56±5.55	8	39.13±7.90	20	36.75±3.92	8	40.75±6.58	14	39.57±5.23	0.1282
Infertility duration (year)	50	2.67±2.60	8	2.25±2.96	20	2.33±2.60	8	3.80±1.91	14	2.75±2.79	0.2073
Fertile (%)	50	2.67±2.60	8	2.25±2.96	20	2.33±2.60	8	3.8±1.91	14	2.75±2.79	0.294
MSA											
Volume (mL)	50	3.11±1.72	8	3.63±1.85	20	3.11±1.45	8	2.5±1.31	14	3.17±2.21	0.5854
Concentration (×10^6/mL)	50	53.31±46.58	8	40.33±41.07	20	79.2±46.69	8	15.59±17.82	14	45.31±42.83	0.001
CASA											
Concentration (×10^6/mL)	50	59.06±45	8	43.86±49.44	20	84.73±36.22	8	24.5±29.94	14	50.82±44.17	0.0022
TM (%)	50	53.05%	8	52.50%	20	78.83%	8	22.38%	14	34.07%	<0.0001
PR (%)	50	40.76%	8	37.75%	20	62.50%	8	15.38%	14	25.93%	<0.0001
RP (%)	50	15.04%	8	11.75%	20	26.45%	8	5.25%	14	6.21%	<0.0001
SP (%)	50	25.90%	8	26.13%	20	36.05%	8	10.63%	14	20.00%	0.0003
NP (%)	50	12.04%	8	14.75%	20	15.45%	8	7.38%	14	8.29%	0.0055
IM (%)	50	47.34%	8	47.50%	20	22.05%	8	77.88%	14	65.93%	<0.0001
LIN (%)	50	42.22%	8	40.63%	20	46.90%	8	26.50%	14	45.43%	0.0064
STR (%)	50	66.62%	8	65.50%	20	70.95%	8	48.63%	14	71.36%	0.0248
WOB (%)	50	57.14%	8	57.00%	20	61.20%	8	43.88%	14	59.00%	0.0381
VAP (μm/s)	50	14.57±4.66	8	14.91±1.04	20	17.96±2.81	8	9.01±6.26	14	12.69±2.86	<0.0001
VSL (μm/s)	50	11.61±4.30	8	11.78±1.75	20	14.73±2.84	8	6.09±4.99	14	10.24±2.69	<0.0001
VCL (μm/s)	50	23.39±7.05	8	24.53±2.46	20	27.62±4.18	8	16.79±11.08	14	20.49±5.34	0.0005
ALH (μm)	50	1.8±0.58	8	1.85±0.37	20	2.08±0.39	8	1.4±0.90	14	1.59±0.50	0.0228
BCF (Hz)	50	5.88±1.29	8	6.16±0.90	20	6.22±0.52	8	4.56±2.66	14	5.98±0.57	0.124
Normal morphology (%)	50	4%	8	2%	20	5%	8	3%	14	5%	<0.0001
DFI (%)											
LensHooke R10®	50	22%	8	11%	20	13%	8	34%	14	33%	<0.0001
Halosperm G2®	50	20.58%	8	16.75%	20	11.98%	8	27.75%	14	30.96%	0.0001
BASO BA®	50	5.87%	8	4.13%	20	2.98%	8	7.31%	14	10.18%	0.0002

P<0.05 indicates a statistical difference across four subgroups stratified by the DFI and normal morphology. "*n*" refers to the number of participants. DFI data were measured using LensHooke R10®. DFI: DNA fragmentation index, SD: Standard deviation, TM: Total motility, PR: Progressive, RP: Rapidly progressive, SP: Slowly progressive, NP: Nonprogressive, IM: Immotile, LIN: Linearity, STR: Straightness, WOB: Wobble, VAP: Velocity along the average path, VSL: Velocity along the straight-line path, VCL: Velocity along the curvilinear path, ALH: Amplitude of the lateral displacement of the head, BCF: Beat-cross frequency, MSA: Manual semen analysis, CASA: Computer-assisted semen analysis

Supplementary Table 4: The comparison of time needed and costs for three sperm chromatin dispersion assays

	Halosperm G2®	LensHooke R10®	BASO®
Average time needed[a] (min)	72.3±2.24	40.7±1.85	63.9±3.33
Cost per test[b] (USD)	60	40	30

[a]The time is recorded with timer when one well-trained technician performing SCD assays. The average time has been calculated with mean±SD for ten tests. Total time does not include the observation and scoring under microscope, [b]The cost is based on the price in Taiwan in 2022 and converted to USD. SD: Standard deviation, SCD: Sperm chromatin dispersion