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Novel sperm chromatin dispersion test with artificial intelligence-aided halo evaluation: A comparison study with existing modalities

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Abstract

Background: Sperm chromatin dispersion test is a common and inexpensive technique to assess sperm DNA fragmentation, but its subjectivity in assessing a small number of spermatozoa is a disadvantage.

Objectives: To study the efficacy of a new sperm chromatin dispersion test kit (R10) combined with an artificial intelligence-aided halo-evaluation platform (X12) and compare the results to those of existing sperm DNA fragmentation testing methods.

Materials and methods: Semen samples from normozoospermic donors ($n = 10$) and infertile men with abnormal semen parameters ($n = 10$) were enrolled. DNA fragmentation indices were examined by multiple assays, including R10, Halosperm G2 (G2), sperm chromatin structure assay, and terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling. In R10 assay, the DNA fragmentation indices were obtained both manually (manual R10) and by X12 (AI-R10). The obtained DNA fragmentation indices were analyzed by agreement analyses.

Results: The DNA fragmentation indices obtained by manual R10 and those obtained by AI-R10 showed a strong significant correlation ($r = 0.97$, $p < 0.001$) and agreement. The number of spermatozoa evaluated by AI-R10 was 2078 (680–5831). The DNA fragmentation indices obtained by manual R10 and AI-R10 both correlated with those of G2 ($r = 0.90$, $p < 0.001$; $r = 0.88$, $p < 0.001$). Between the AI-R10 and G2 results, Passing–Bablok regression showed no systematic or proportional difference, and Bland–Altman plots revealed overall agreement and a mean bias of 6.3% with an SD of 6.9% (95% limit of agreement: −7.2% to 19.9%). AI-R10 and sperm chromatin structure assays showed systematic differences with a mean bias of −1.9%, while AI-R10 and terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling revealed proportional differences with a mean bias of −10.7%.

Conclusions: The novel sperm chromatin dispersion kit and artificial intelligence-aided platform demonstrated significant correlation and agreement with existing sperm chromatin dispersion methods by assessing greater number of spermatozoa. This technique has the potential to provide a rapid and accurate assessment of sperm DNA fragmentation without technical expertise or flow cytometry.

KEYWORDS
artificial intelligence, male infertility, sperm chromatin dispersion, sperm DNA fragmentation

1 | INTRODUCTION

Up to 12% of the couples of reproductive age suffer from infertility worldwide, and male factors contribute to approximately 50% of these cases.[1] Despite a comprehensive evaluation using current testing, male infertility still remains idiopathic in 30%–50% of cases.[2] The evaluation of male fertility is primarily based on the analysis of conventional semen parameters, but this test has limitations in predicting male fertility potential and the success of assisted reproductive technology (ART).[3] Sperm DNA fragmentation (SDF) testing has become a useful tool in diagnosing male infertility.[4] Research suggests that high SDF can lead to male subfertility, in vitro fertilization (IVF) failure, and miscarriage.[5–10] SDF was also reported to be associated with several male infertility-related conditions, such as obesity, smoking, and clinical varicocele,[11–13] and some guidelines now recommend SDF measurement in specific conditions, such as recurrent pregnancy loss (RPL) and unexplained male infertility.[14,15] The recently published sixth edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen has also introduced SDF as an extended examination and recommends its use in these clinical settings.[16]

SDF is frequently assessed using laboratory techniques, such as terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling (TUNEL), sperm chromatin dispersion (SCD), comet and sperm chromatin structure assay (SCSA), and comet assay.[17] SCD is based on the principle that intact DNA loops in spermatozoa expand and form "halos" after denaturation and extraction of proteins from the nucleus.[16] The spermatozoa are embedded in an agarose gel on a slide and incubated in an acid solution that denatures the DNA exclusively in those spermatozoa with fragmented DNA. Then, the lysing solution removes the protamines so that the DNA loops tightly packed in the nucleus are spread, producing DNA halos emerging from a central core. On the other hand, halos do not form or are very small when DNA is fragmented.[18] SCD measurement is simple and does not necessitate expensive equipment such as flow cytometry.[19] However, the disadvantage of SCD is its subjectivity and intra-observer variability in judging halos under microscopes. Measurements are also limited to a few hundred spermatozoa because of time constraints with manual evaluation. Alternatively, flow cytometry in TUNEL or SCSA can count and analyze 5000–10,000 spermatozoa.[20,21]

To overcome the disadvantage of SCD, a new SCD testing kit (LensHooke R10® [R10], Bonraybio Co., Taichung, Taiwan) and a complementary automated halo-counting system (LensHooke X12PRO® [X12], Bonraybio Co.) have been developed. This R10 employs the same SCD measurement principle and staining methods as commercially available SCD kits, such as Halosperm G2® (G2; Halotech DNA, Madrid, Spain). However, the staining time was shorter in R10 than in G2 (40 and 75 min, respectively). A recent study compared the efficacy of R10 to other SCD assays.[22] Furthermore, spermatozoa with or without halo after staining can be counted manually or with the artificial intelligence (AI)-assisted X12 in R10. Thus, a larger number of spermatozoa can be evaluated technically while experiencing less inter-user variability. These kits may represent a simple and time-saving SDF assessment test that could be used in IVF clinics and andrology laboratories. This study compared the efficacy and accuracy of the new kit R10 in combination with X12 for traditional SDF assays.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study was approved by the Institutional Review Board of Cleveland Clinic (IRB No. 21-1224). Written informed consent was obtained from all participants.

2.2 | Study design

Between March 2022 and July 2022, healthy normozoospermic donors ($n = 10$) and infertile men with abnormal sperm parameters ($n = 10$) were enrolled. The control group included healthy male volunteers with normal semen parameters according to WHO 2010 guidelines.[23] Infertile men with abnormal semen parameters were enrolled at the time of their clinically indicated semen analysis. To conduct all the assays and semen analysis for each sample, we excluded the samples from patients with total sperm count of <5 million per sample, including azoospermia samples. The obtained semen parameters, oxidation–reduction potential (ORP) level, and percent of DNA fragmentation index (%DFI) from every SDF assay were analyzed in parallel (Figure 1). In the R10 assay, the same stained slides of R10 were used for both manual and X12 evaluation. Semen analysis, ORP measurement, and R10 and G2 assessments were conducted consequently

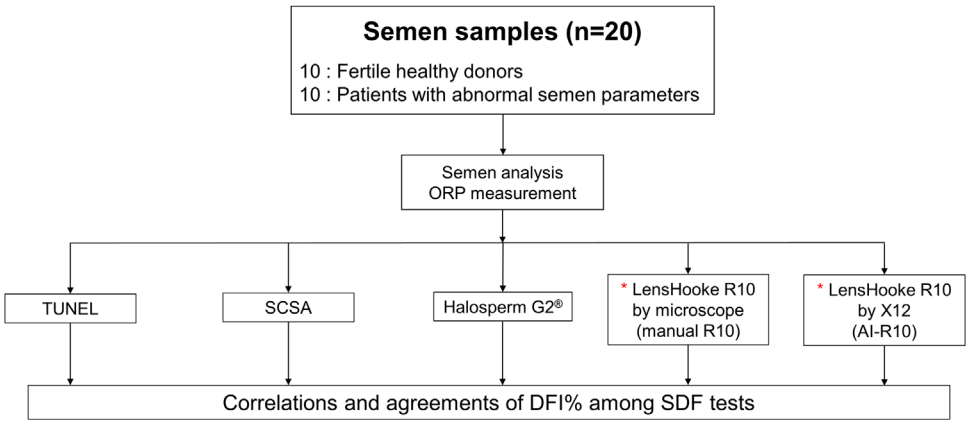

FIGURE 1 Flow diagram of the study's design. *In R10 assay, the same stained slides were examined for both manual evaluation of sperm DNA fragmentation (SDF) (manual-R10) and X12 evaluation (AI-10). DFI, DNA fragmentation index; ORP, oxidation–reduction potential; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling.

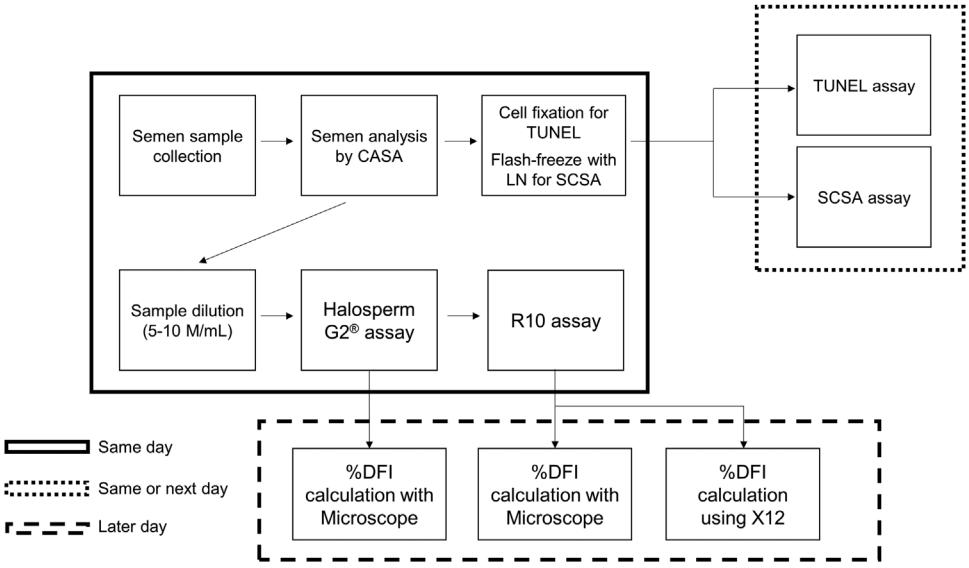

FIGURE 2 Experimental procedure flow. CASA, computer-assisted semen analysis; DFI, DNA fragmentation index; LN, liquid nitrogen; SCSA, sperm chromatin structure assay; SDF, sperm DNA fragmentation; TUNEL, terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling.

right after sample collection. Regarding TUNEL and SCSA, samples were partially aliquoted for storage at the time of semen collection and analyzed on a later day. Counting and calculation of %DFI in G2 and R10 both manually (manual R10) and by X12 (AI-R10) were performed on the same day or the next day. The procedure is shown in Figure 2.

2.3 | Semen analysis

Semen analysis was performed according to the WHO fifth edition 2010 guidelines.[23] The sperm samples were collected from the participants who had been abstinent for 2–7 days. Sperm samples were incubated at 37°C and allowed to liquefy. After complete liquefaction, semen analysis was performed by the computer-assisted semen analysis system LensHooke X1PRO® (Bonraybio Co.). By analyzing 40 μL of the semen sample, LensHooke X1PRO® can provide the semen volume, pH, concentration, motility (total, progressive, and non-progressive), and normal morphology.

2.4 | ORP

To evaluate the status of oxidative stress in semen, the ORP was measured in all study samples. ORP level was measured using MiOXSYS® (Caerus Biotech, Geneva, Switzerland) according to the manufacturer's

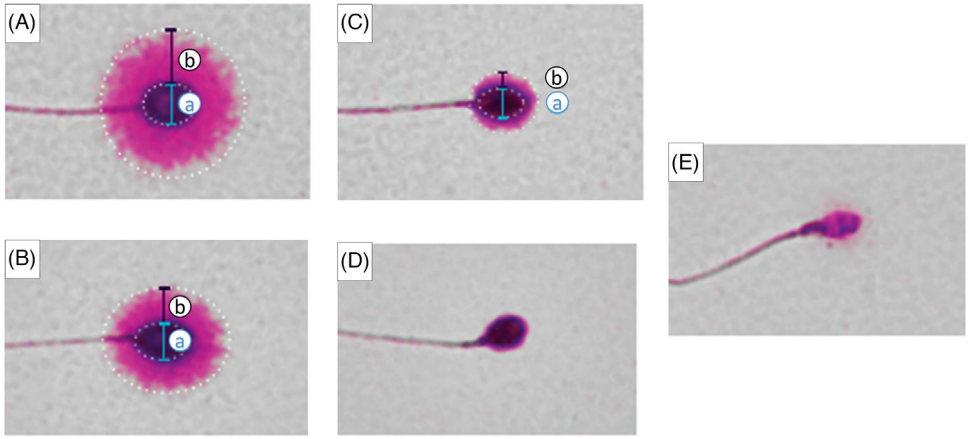

FIGURE 3 Classification criteria of the halo size in G2 and R10. (A) Spermatozoa with large halos. The halo width (*b*) is equal or larger than the minor diameter of the sperm head (*a*). (B) Spermatozoa with medium halos. The halo width (*b*) is less than the minor diameter of the sperm head (*a*) but also larger than one-third of (*a*). (C) Spermatozoa with small halos. The halo width (*b*) is similar or smaller than one-third of the minor diameter of the sperm head (*a*). (D) Spermatozoa without halos. (E) Degraded spermatozoa. Spermatozoa with an irregularly shaped head or a weakly stained head.

recommendation using 30 μL of the semen sample.[24] The absolute ORP values (mV) displayed on the screen were then noted and divided by sperm concentration (10^6 spermatozoa/mL) to obtain the normalized ORP value (mV/10^6 spermatozoa/mL).

2.5 | SDF assessment protocol by LensHooke R10® and LensHooke X12PRO®

2.5.1 | Staining

The R10 staining technique is based on the SCD assay and is similar to G2. Positive and negative controls were stained simultaneously. The semen sample was diluted with phosphate-buffered saline (PBS) to a concentration of 5–10 million/mL. The raw sample was used at concentrations <10 million/mL. Agarose gel in eppendorf tube was melted in a 95°C hot water bath for 1.5 min and incubated for 5 min at 37°C. Then, 25 μL of DNA denaturant solution (0.28 N HCl) was added to the melted agarose gel. Then, 25 μL of the diluted semen sample was added. Twenty-five microliters of the above mixture was then dripped down to the pre-treated microscope slide and covered by a coverslip. The slide was cooled in a refrigerator at 4°C for 5 min to allow the agarose to solidify and embed the sperm cells within. The lysis solution (2.5 M NaCl, 50 mM Tris(2-chloroethyl) phosphate, 1% Triton X-100, 1 M urea, 0.05% SDS, 50 mM Ethylenediaminetetraacetic acid (EDTA), and pH 8.0) was added to the test area, and the slide was incubated at room temperature for 10 min. The slide was washed with distilled water at room temperature for 5 min and dehydrated in the staining tray by flooding with 95% methanol for 1 min. Four hundred microliters of staining solution A (Wright–Giemsa stain) was added using a pipette to fully cover the slide and blown for 1 min by a dust blower. Without draining, staining solution B (phosphate buffer, pH 7.4) was added to fully cover the slide and blown for 1 min to mix the solutions, and the slide was placed horizontally for 2 min. The slide was rinsed with distilled water for 20–30 s and then set aside for drying at room temperature. After drying completely, the slide was then subsequently evaluated.

2.5.2 | Evaluation of spermatozoa and halo

The criteria for the evaluation of the halo size were identical to those used with G2. Spermatozoa with big halos (Figure 3A: the halo width is equal or larger than the minor diameter of the sperm head) and medium halos (Figure 3B: the width is between large and small halo) were considered non-fragmented spermatozoa. Spermatozoa with small halos (Figure 3C: the halo width is similar or smaller than one-third of the minor diameter of the sperm head) or without halos (Figure 3D) or degraded spermatozoa (Figure 3E: spermatozoa with an irregularly shaped head or a weakly stained head) were considered fragmented. Spermatozoa without a clear head and tail were excluded from the evaluation.

2.5.3 | Manual counting

When the slides were completely dried, they were examined under a bright-field microscope (×200). Leica DM2000 Phase Contrast Microscope™ combined with HD monitor Excelis™ AU-600-HDS (ACCU-SCOPE Inc., Commack, NY, USA) were used in this study. In manual counting, 500 spermatozoa were counted and examined for the halo size. As the slides had two wells, the better-stained well was chosen for the counting. Both wells were used if one well was insufficient to evaluate the required number of spermatozoa. %DFI

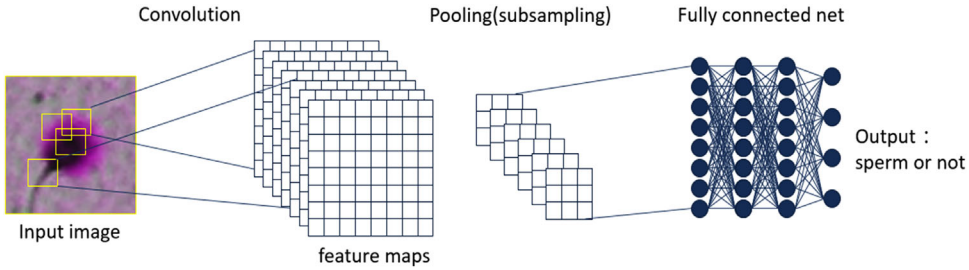

FIGURE 4 Deep learning model of X12 based on convolutional neural networks.

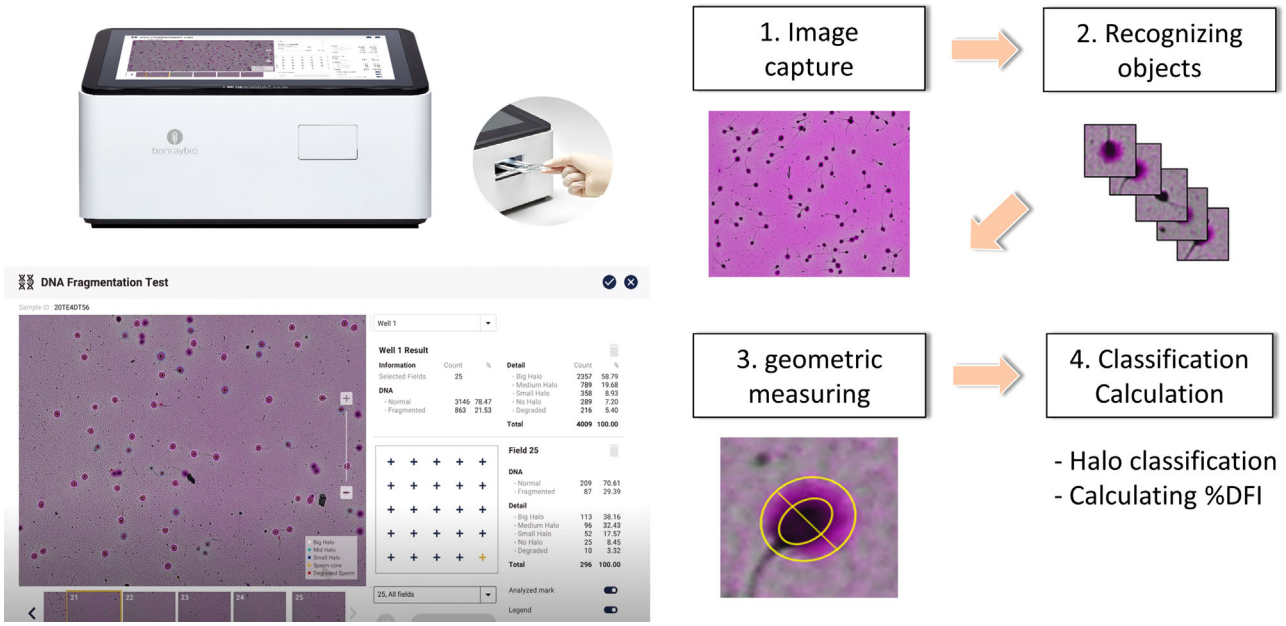

FIGURE 5 Image and measurement system in X12. %DFI, percent of DNA fragmentation index.

was calculated as (number of fragmented spermatozoa)/(number of total spermatozoa) × 100 (%).

2.5.4 | AI-guided analysis platform

The AI-aided device X12 used in this study is based on convolutional neural network models. It recognizes spermatozoa while excluding impurities, abnormal staining, and spermatozoa that lack fundamental components such as the tail. Dataset training was conducted using >1000 R10 slides for capturing and detecting spermatozoa and halos (Figure 4). X12 captured images of R10-stained slides and automatically divided them into 25 small fields. In each region, the system recognizes objects as spermatozoa and then detects sperm head and halo expansion as an approximate ellipse to measure the core and halo. After the measurement, it classifies the halo size into five categories as described above. The classification and calculation of %DFI took 3–6 min depending on the staining condition. The approximate evaluation time for each well was 3–6 min. The average %DFI of the two wells was considered the final result (Figure 5). X12 works only with R10-stained slides and cannot be combined with any other SCD-stained slides, including G2.

2.6 | SDF assessment by other assays

2.6.1 | Halosperm G2®

A previous publication described the detailed protocol of G2,[19] the manufacturer's instructions for which are included in Supporting Information S1. To summarize, the sperm sample was diluted in PBS to a concentration of 5–10 million/mL. Eight microliters of diluted sperm sample was fixed with melted agarose gel and solidified at 4°C. The slides were tilted while the DNA denaturant reagent and DNA lysis solution were added and incubated. Following a wash with diluted water and ethanol, staining solutions were added, incubated, and the slides were tilted. After the slide had dried completely, 300 spermatozoa and their halo sizes were examined under bright-field microscopy ×200. The same microscope and HD monitor as in R10 were used. The %DFI was calculated as (number of fragmented spermatozoa)/(number

of total spermatozoa) × 100 (%), following the same protocol as R10. In general, male infertility thresholds ranging from 16% to 30% are used, and 20% is considered the best value for distinguishing infertile men from fertile men.[25]

2.6.2 | TUNEL assay

For each sample, two tubes with 1 mL of PBS were prepared. The semen sample was aliquoted by calculating a total of 2.5 million spermatozoa in each tube. These tubes were centrifuged for 7 min at 1600 rpm. The supernatant was discarded, and the pellet was suspended in 1 mL of PBS and centrifuged again. After the removal of the supernatant again, the pellet was suspended in 1 mL of 3.7% paraformaldehyde and stored at 4°C. After all the samples were collected, the test was run at one time for every sample. The Apo-DIRECT™ kit (BD Biosciences, Franklin, NJ, USA) was used to conduct the test. Paraformaldehyde was removed by centrifugation from the stored samples. The pellet was suspended in 1 mL of 70% ice-cold ethanol. The sample was stored at 4°C for 15–30 min. Kit controls (negative and positive controls), test samples, and internal controls (spermatozoa with a known amount of SDF) were washed twice using a wash buffer. Samples were stained with 50 μL of the staining reagent and incubated for 60 min in the dark, 1 mL of the rinse buffer was added, and the sample was centrifuged twice. Propidium iodide/RNase solution was added, and the samples were analyzed after 30 min by flow cytometry (BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences). In total, 5000–10,000 spermatozoa were counted and evaluated for each sample. The software provided by the manufacturer generated the plots and calculated the percentage of TUNEL-positive cells as %DFI. The final %DFI was calculated using the average results of the two tubes per sample. A previous study has indicated that 17% of %DFI was the best cut-off value to diagnose infertile men.[26]

2.6.3 | SCSA

The assay measures the susceptibility of sperm DNA to acid-induced DNA denaturation in situ, followed by staining with the fluorescent dye acridine orange.[27] For each sample, 0.5 mL (0.2–0.4 mL in low-volume case) of the semen sample was aliquoted into a cryo-tube and flash-frozen with liquid nitrogen. The samples were shipped with a dry shipper to an external lab and analyzed (SCSA Diagnostics Inc., Brookings, SD, USA). Although these tests were performed in the external lab in this study, the detailed protocol was described in previous publications.[27,28] Sample flash-freezing with liquid nitrogen for shipping was officially recommended by the laboratory. Briefly, the raw semen was thawed and diluted to a concentration of 1–2 million spermatozoa per mL with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA, pH 7.4, 4°C). The diluted sample was mixed with a low pH buffer (400 μL of 0.08 N HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2, 4°C) to potentially denature the sperm DNA, and stained with acridine orange solution. The mixture was placed in the flow cytometer. Acridine orange-stained normal double-stranded DNA appeared green, and denatured, single-stranded DNA stained red. In flow cytometry, 5000–10,000 spermatozoa in each sample were analyzed, and %DFI was calculated as (number of fragmented spermatozoa)/(number of total spermatozoa) × 100 (%). Twenty-five percent is often used %DFI as thresholds to indicate to have poor pregnancy outcomes.[28]

2.7 | Statistical analysis

The sample size was determined to obtain 80% power and alpha = 0.05 with predicted correlation coefficient = 0.7. Based on this calculation, 13 or more samples were needed. Statistical analyses were performed using MedCalc® (Medcalc Software Ltd., Ostend, Belgium). Correlations of %DFI among each assay and with semen parameters were analyzed using Pearson's correlation test, or Spearman's rank correlation test was used depending on its distribution. Linear regression analyses were also performed. The Mann–Whitney *U*-test was performed to compare the semen parameters and %DFI between donors and patients. To determine the agreements among different techniques, Passing–Bablok regression analyses[29] and Bland–Altman plotting[30] were performed. Passing–Bablok regression analysis fits parameters *A* and *B* of the linear equation $y = A + Bx$. Deviation from linearity, systematic difference, and proportional difference between assays were examined. Significance was indicated by the *p*-value of the test <0.05. In Bland–Altman plotting, the limit of agreement (LOA) is defined as the mean difference ± 1.96 SD. Inter-observer variability of manual readings in R10 and G2 was also assessed by Bland–Altman plotting.

Correlation and agreement analyses were performed to determine whether (1) automated halo evaluation by X12 for R10-stained slides (AI-R10) produced comparable results than manual evaluation (manual R10), (2) manual R10 and AI-R10 results correlated and agreed with G2 as the SCD standard technique, and (3) AI-R10 results agreed with SCSA and TUNEL assay results as different SDF testing modalities.

3 | RESULTS

The comparison of semen parameters, ORP values, and %DFIs obtained by each SDF test between donors and patients is shown in Table 1. Concentration and total motility were significantly lower in the infertile group (45.3 ± 46.9 × 10^6/mL vs. 104.0 ± 57.6 × 10^6/mL, $p = 0.016$; 38.6 ± 17.9% vs. 57.1 ± 19.4%, $p = 0.034$). The ORP was significantly higher in the infertile group (2.20 ± 3.36 mV/10^6 spermatozoa/mL vs. 0.06 ± 0.23 mV/10^6 spermatozoa/mL, $p = 0.001$). Other semen parameters did not show a significant difference between donors and patients. In the comparison between donors and patients, G2 and SCSA showed significantly higher %DFI in patients (32.6 ± 16.5 vs. 17.5 ± 7.2, $p = 0.034$; 23.8 ± 14.9 vs. 9.8 ± 5.6, $p = 0.010$). This difference was not significant in R10 or TUNEL although %DFIs tended to be higher in patients. Motility showed mild inverse correlation between R10 ($r = -0.54$, $p = 0.007$), G2 ($r = -0.58$, $p = 0.007$), and SCSA ($r = -0.54$, $p = 0.015$) values. The ORP level

TABLE 1 The semen characteristics %DNA fragmentation index by each sperm DNA fragmentation (SDF) test of study subjects ($n = 20$)

		Donors ($n = 10$)	Patients ($n = 10$)	p-Value
Semen parameters	Semen volume (mL)	2.4 ± 1.2	3.3 ± 2.1	0.31
	Semen pH	7.6 ± 0.1	7.6 ± 0.2	0.94
	Concentration ($\times 10^6$/mL)	104.0 ± 57.6	45.3 ± 46.9	0.016*
	Total motility (%)	57.1 ± 19.4	38.6 ± 17.9	0.034*
	Progressive motility (%)	43.1 ± 18.4	27.2 ± 14.1	0.066
	Non-progressive motility (%)	14.0 ± 5.5	11.3 ± 5.5	0.22
	Normal morphology (%)	4.8 ± 1.0	4.0 ± 1.2	0.18
	ORP (mV/10^6 spermatozoa/mL)	0.06 ± 0.23	2.20 ± 3.36	0.001*
%DNA fragmentation index by each SDF test	Manual R10	15.8 ± 5.6	25.1 ± 16.5	0.33
	AI-R10	15.0 ± 7.4	22.4 ± 15.3	0.36
	Halosperm G2	17.5 ± 7.2	32.6 ± 16.5	0.034*
	SCSA	9.8 ± 5.6	23.8 ± 14.9	0.010*
	TUNEL	6.1 ± 3.3	9.9 ± 10.7	0.94

Note: Values denote mean ± SD.

Abbreviations: ORP, oxidation–reduction potential; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling.

*Statistically significant ($p < 0.05$).

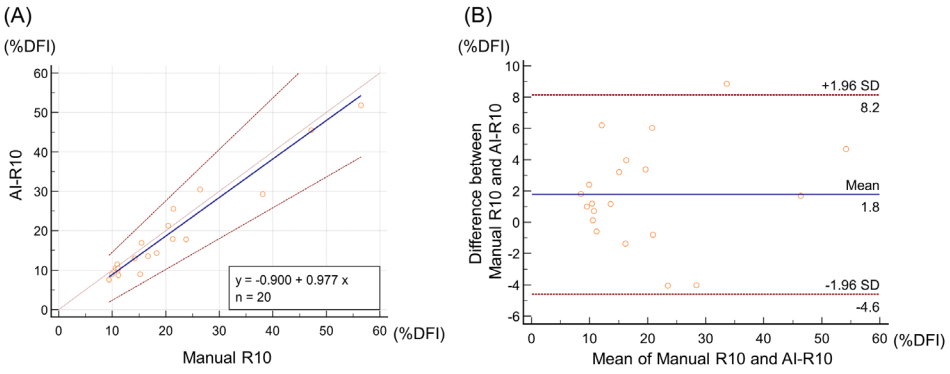

FIGURE 6 Agreement analyses between percent of DNA fragmentation indices (%DFIs) obtained by manual R10 and AI-R10. (A) Passing–Bablok regression between manual R10 and AI-R10. The solid blue line depicts the regression line, and the brown dashed line shows a confidence band. (B) Bland–Altman plots between manual R10 and AI-R10. The solid blue line indicates the mean of the two methods and the red dashed lines are the 95% confidence ranges.

showed mild correlation between G2 (rho = 0.474, $p = 0.035$) and SCSA (rho = 0.517, $p = 0.02$), whereas R10 and TUNEL were not significantly correlated. Concentration, morphology, and progressive motility did not show a significant correlation with any of the SDF assays.

3.1 | Correlation and agreement analyses among SDF assays

3.1.1 | Manual R10 versus AI-R10

In manual R10, the mean inter-observer difference was −0.6% with SD of 2.0% (LOA: −4.7% to 3.4%). The %DFIs obtained by manual R10 and by AI-R10 showed a strong significant correlation ($r = 0.97$, $p < 0.001$). The mean numbers of spermatozoa evaluated by manual R10 and by AI-R10 were 500 and 2078 (680–5831), respectively. In Passing–Bablok regression between manual R10 and AI-R10, no significant deviation from linearity was found ($p = 0.98$). The intercept A and slope B values were −0.9002 (95% CI: −5.4708 to 1.4946) and 0.9770 (95% CI: 0.7819–1.3041), respectively, indicating the absence of proportional and systematic differences (Figure 6A). Bland–Altman plots revealed overall agreement and a mean bias of 1.8% with SD of 3.3% (95% LOA: −4.6% to 8.2%) (Figure 6B).

3.1.2 | Manual R10, AI-R10 versus Halosperm G2

In G2, the mean inter-observer difference was −0.4% with SD of 3.3% (LOA: −7.0% to 6.1%). Manual R10 and by AI-R10 %DFIs were both correlated with G2 ($r = 0.90$, $p < 0.001$; $r = 0.88$, $p < 0.001$,

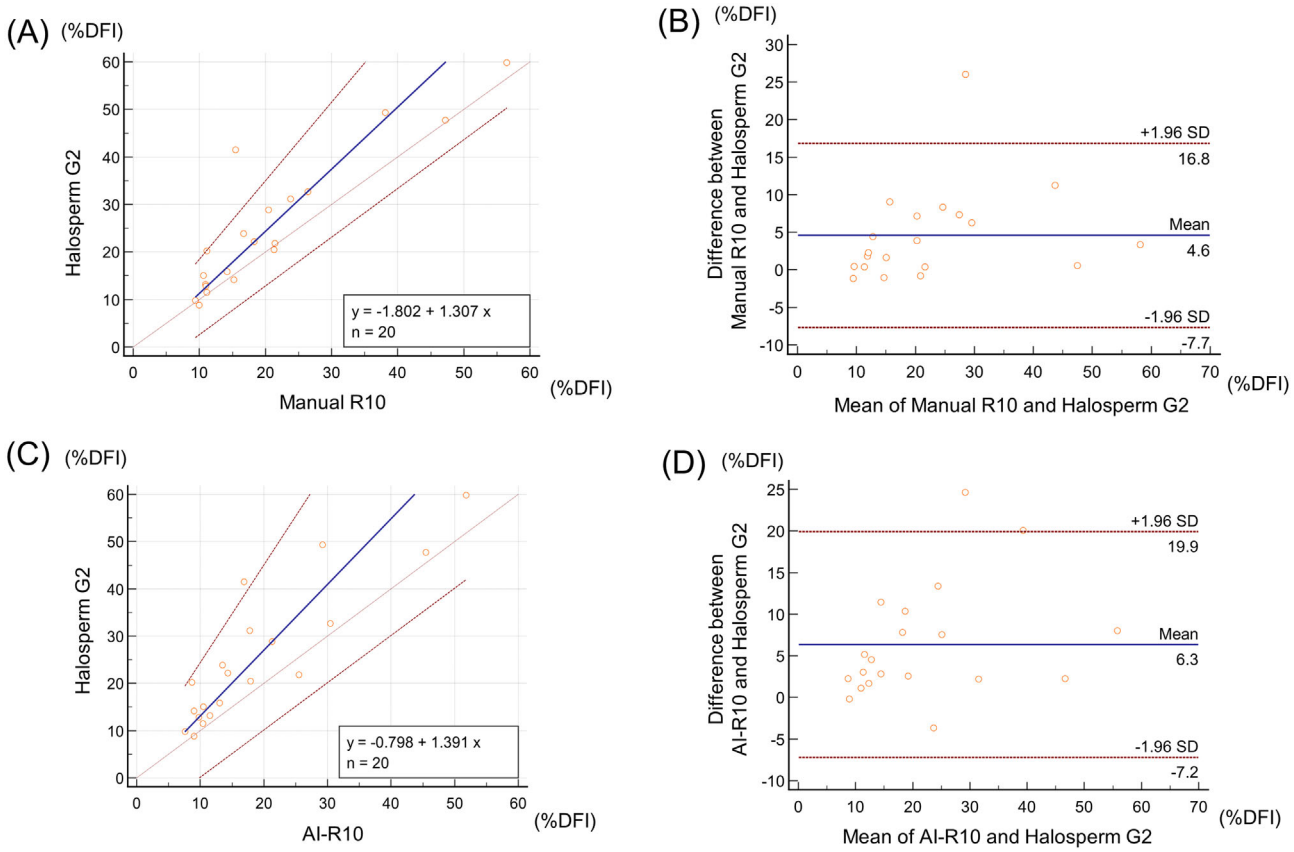

FIGURE 7 Agreement analyses between percent of DNA fragmentation indices (%DFIs) collected by G2 and R10. (A) Passing–Bablok regression between G2 and manual R10. The solid blue line depicts the regression line, and the brown dashed line shows a confidence band. (B) Bland–Altman plots between G2 and manual R10. The solid blue line indicates the mean of the two methods and the red dashed lines are the 95% confidence ranges. (C) Passing–Bablok regression between G2 and AI-R10. The solid blue line denotes the regression line, and the brown dashed line shows a confidence band. (D) Bland–Altman plots between G2 and AI-R10. The solid blue line indicates the mean of the two methods and the red dashed lines indicates the 95% confidence ranges.

respectively). Between %DFIs by manual R10 and G2, Passing–Bablok regression found no significant deviation from linearity ($p = 0.72$). The intercept *A* and slope *B* values were −1.8024 (95% CI: −7.6634 to 1.9307) and 1.3073 (95% CI: 1.0256–1.6526), respectively (Figure 7A). There was no systematic difference, but there was a significant proportional difference between the two methods. The Bland–Altman plots revealed overall agreement and a mean bias of 4.6% with SD of 6.2% (95% LOA: −7.7% to 16.8%) (Figure 7B). Between %DFIs by AI-R10 and G2, Passing–Bablok regression found no significant deviation from linearity ($p = 0.98$). The intercept *A* and slope *B* values were −0.798 (95% CI: −9.8652 to 3.7644) and 1.391 (95% CI: 1.0000–2.0641), respectively indicating no systematic difference or proportional difference (Figure 7C). Bland–Altman plots showed overall agreement and a mean bias of 6.3% with SD of 6.9% (95% LOA: −7.2% to 19.9%) (Figure 7D).

3.1.3 | AI-R10 versus SCSA, TUNEL

AI-R10 had a significant and positive correlation with SCSA ($r = 0.90$, $p < 0.001$). In Passing–Bablok regression, there was no significant deviation from linearity between %DFIs by AI-R10 and SCSA ($p = 1.00$). The intercept *A* and slope *B* values were −3.935 (95% CI: −10.8607 to −0.6674) and 1.035 (95% CI: 0.7743–1.6311), indicating a significant systematic difference but no proportional difference (Figure 8A). The Bland–Altman plots revealed overall agreement and a mean bias of −1.9% with SD of 5.7% (95% LOA: −13.0% to 9.2%) (Figure 8B).

AI-R10 had a moderately significant correlation with TUNEL ($r = 0.66$, $p < 0.001$). In Passing–Bablok regression, there was no significant deviation from linearity between AI-R10 and TUNEL ($p = 0.14$). The intercept *A* and slope *B* values were 0.0894 (95% CI: −7.5217 to 2.4062) and 0.3449 (95% CI: 0.1539–0.9827), indicating a significant proportional difference and the absence of a systematic difference, respectively (Figure 8C). The Bland–Altman plots revealed overall agreement and a mean bias of −10.7% with SD of 9.2% (95% LOA: −7.2% to 19.9%) (Figure 8D).

4 | DISCUSSION

Despite its limitations, SDF has become a widely used test in the diagnosis of advanced male infertility, and higher levels of SDF have

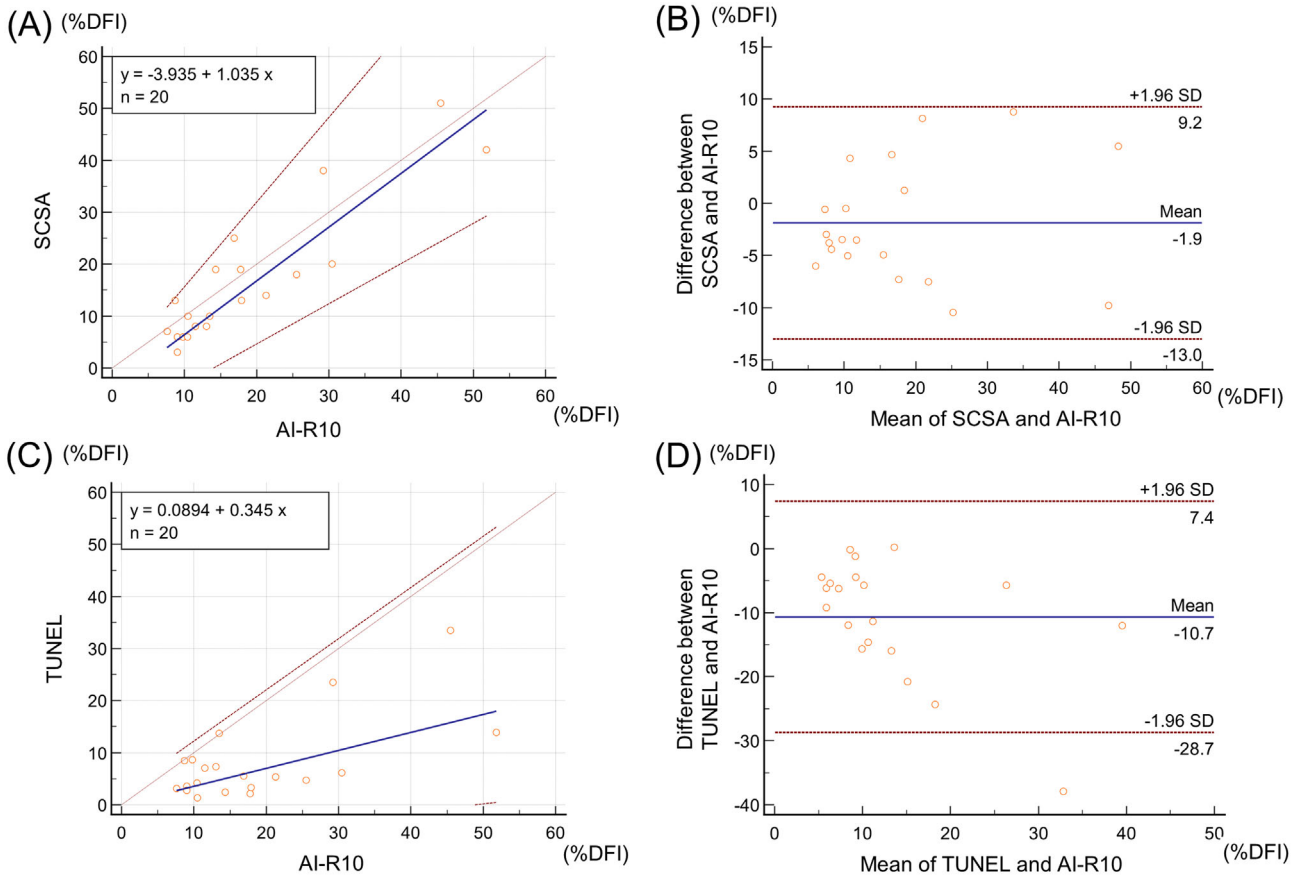

FIGURE 8 Agreement analyses between percent of DNA fragmentation indices (%DFIs) obtained by AI-R10 and sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase deoxynucleotidyl transferase nick end labeling (TUNEL). (A) Passing–Bablok regression between SCSA and AI-R10. The solid blue line demonstrates the regression line, and the brown dashed line shows a confidence band. (B) Bland–Altman plots between SCSA and AI-R10. The solid blue line indicates the mean of the two methods and the red dashed lines indicates the 95% confidence ranges. (C) Passing–Bablok regression between TUNEL and AI-R10. The solid blue line depicts the regression line, and the brown dashed line shows a confidence band. (D) Bland–Altman plots between TUNEL and AI-R10. The solid blue line indicates the mean of the two methods and the red dashed lines are the 95% confidence ranges.

been reported in infertile men with abnormal semen parameters and normozoospermic male partners of infertile couples (i.e., unexplained infertility).[31] Several studies have found a link between a high SDF level and RPL and ART failure.[32,33] The results of SDF tests may aid clinical practitioners in developing treatment strategies for infertile couples seeking to conceive. The American Society for Reproductive Medicine guidelines acknowledged that SDF testing is clinically useful in intrauterine insemination, IVF, and intracytoplasmic sperm injection outcomes. Furthermore, SDF testing was recommended by the European Association of Urology guidelines and the 2017 Society for Translational Medicine guidelines for infertile couples with RPL, unexplained infertility, and ART failure.[15,34] SCD is one of the novel and efficient methods for SDF detection based on the failure of fragmented sperm DNA to form the characteristic halo. Inter-observer variability, labor-intensiveness if a microscope is being used, and longtime requirements are the main disadvantages of SCD.[35] This study sought to overcome the drawbacks of SCD testing by using new SCD testing kits and an automated halo-counting system.

AI could play a critical role in male infertility treatment by reducing variability and standardizing sperm analysis, thereby overcoming subjectivity in motility and morphology assessment.[36] AI research on sperm DNA integrity, however, is limited. The comet assay and SCD test are the only existing SDF assays that require manual evaluation under a microscope. Because this process is subjective and time consuming, AI may be able to improve these techniques to make them more objective. In this preliminary study, %DFIs by manual R10 and AI-R10 were strongly correlated ($r = 0.97$) and agreed by Passing–Bablok regression and Bland–Altman plots. Because manual halo evaluation was performed by well-trained observers, this result indicated that AI evaluation of stained slides was accurate and consistent with manual technique in R10. The AI-R10 could evaluate a much larger number of spermatozoa (an average of 2078, ranging from 680 to 5831), whereas the manual protocol could only evaluate 500 spermatozoa.

Halosperm® and G2 are commercially available and widely used SCD assay kits, whereas R10 is a brand-new SCD assay kit that was just released in the market. A recent study found that R10 manual

procedure produced more reliable DFI results than other SCD assays (G2 and BASO®) with higher intra-class coefficient.[22] To the best of our knowledge, this is the first study to examine the efficacy of combining R10 and an AI-aided halo-evaluation system. Our findings revealed that %DFIs by manual R10 and AI-R10 correlated strongly ($r = 0.90$ and 0.88) and agreed with G2. Although Passing–Bablok and Bland–Altman revealed significant overall agreement among the techniques, the former revealed a proportional difference between manual R10 and G2. Particularly between AI-R10 and G2, no systematic or proportional difference was found in Passing–Bablok regression. Although the Bland–Altman plots showed a 6.3% difference, which was considered fixed bias, the agreement results suggested that the AI-R10 combination has the potential to replace G2, the current gold standard of SCD assay. Furthermore, at least 300 spermatozoa must be strictly evaluated to obtain %DFI in G2, which requires approximately 10—15 min of expert reader time per sample. Alternatively, AI-R10 could evaluate a larger number of spermatozoa in 6—12 min per slide. Therefore, we assume that this method has the potential to save human resources and effort in andrology labs. In comparison to SCSA, %DFI by AI-R10 had a significant correlation ($r = 0.90$). Although Passing–Bablok indicated systematic difference between them, Bland–Altman plots revealed overall agreement with a small amount of mean difference (−1.9%). Because SCSA and SCD are founded on different principles, complete agreement cannot be expected. In this study, however, we were able to demonstrate a strong correlation with a small difference and agreement between them. However, the correlation between AI-R10 and TUNEL was modest ($r = 0.66$), and Passing–Bablok and Bland–Altman plots revealed lower power of agreement. Although there may be some differences or biases between because they are based on different principles, analysis of multiple samples may produce more robust correlation and agreement between them. AI-R10 has the potential to evaluate >3000 spermatozoa per manufacturer's instructions. According to our findings, it can evaluate up to 5831 spermatozoa. Although this number is less than 5000—10,000 spermatozoa that flow cytometry analyzes in TUNEL and SCSA, AI-R10 has the potential to overcome this limitation because traditional SCD assays only evaluate 300—500 spermatozoa.

This study has some limitations. First, we validated the combination of AI-R10 using very small sample size as a preliminary report ($n = 20$) although the number met the minimum requirement by power calculation. A larger number of cases in a multicenter setting should be analyzed. Second, samples from patients with severe oligozoospermia of total sperm count <5 million/mL were not included as certain amount of spermatozoa was needed to conduct multiple SDF assays for each sample in the study design. Third, the ability of X12 to detect and evaluate spermatozoa differed depending on the manual staining quality of R10 slides. In several cases, X12 could detect and evaluate <1000 spermatozoa, although it can potentially evaluate >3000 spermatozoa. It is considered the learning curve for this methodology, especially in staining process. Thus, we assumed that this can be improved by analyzing more procedures. In addition, the X12 function in detecting spermatozoa can be improved by learning. By checking the results manually, inappropriately recognized spermatozoa can be remarked or excluded; this input can provide X12 better evaluation in the future. Fourth, although %DFIs tended to be higher in patients than in donors, R10 could not differentiate donors and patients probably because of small number of cases. As it is well known that infertile patients present higher SDF than healthy donors, larger sample size may show significant difference between the two groups. To set cut-off value for this technique by evaluating higher number of healthy donors and patients is needed in future studies. Fifth, R10 did not show a significant correlation between ORP. A correlation is expected given that oxidative stress likely causes SDF.[37] In this study, only SCSA and G2 demonstrated very mild correlation with ORP. This might be because SDF is generated not only by ORP but also by multiple causes. Further evaluation with a larger population may show a significant correlation between R10 and ORP.

In conclusion, this is the first report of a validation study for SCD combined with the AI-aided halo-evaluation system. Although the overall sample size is small, obtained %DFIs by AI-R10 correlated and agreed significantly with G2 and other SDF testing modalities. AI-R10 could evaluate a higher number of spermatozoa in a short time compared with the conventional SCD test, and is cost-saving than flow cytometry-based methods. This AI-combined technique was considered objective and robust without flow cytometry. Further validation with larger number of cases is needed.

AUTHOR CONTRIBUTIONS

Conceptualization: Shinnosuke Kuroda, Keshab Kumar Karna, and Ashok Agarwal. *Data curation*: Shinnosuke Kuroda, Keshab Kumar Karna, and Raneen Sawaid Kaiyal. *Formal analysis*: Shinnosuke Kuroda. *Writing—original draft*: Shinnosuke Kuroda, Keshab Kumar Karna, and Raneen Sawaid Kaiyal. *Writing—review and editing*: all authors.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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