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Evaluation of sperm DNA fragmentation using multiple methods: a comparison of their predictive power for male infertility

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Objective: The usual seminal profile has been customarily used for diagnosing male infertility based on an examination of semen samples. However, sperm DNA fragmentation has also been causally linked to reproductive failure, suggesting that it should be evaluated as part of male infertility assessments. To compare the ability of the five most widely utilized methodologies of measuring DNA fragmentation to predict male infertility and reactive oxygen species by Oxisperm kit assay.

Methods: In this case-control study, which received ethical committee approval, the participants were divided into fertile and infertile groups (50 patients in each group).

Results: The alkaline comet test showed the best ability to predict male infertility, followed by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, the sperm chromatin dispersion (SCD) test, and the sperm chromatin structure assay (SCSA), while the neutral comet test had no predictive power. For our patient population, the projected cut-off point for the DNA fragmentation index was 22.08% using the TUNEL assay, 19.90% using SCSA, 24.74% using the SCD test, 48.47% using the alkaline comet test, and 36.37% using the neutral comet test. Significant correlations were found between the results of the SCD test and those obtained using SCSA and TUNEL ($r = 0.70$ and $r = 0.68$, respectively; $p < 0.001$), and a statistically significant correlation was also found between the results of SCSA and the TUNEL assay ($r = 0.77$, $p < 0.001$). Likewise, the results of the alkaline comet test showed significant correlations with those of the SCD, SCSA, and TUNEL tests ($r = 0.59$, $r = 0.57$, and $r = 0.72$, respectively; $p < 0.001$).

Conclusion: The TUNEL assay, SCSA, SCD, and the alkaline comet test were effective for distinguishing between fertile and infertile patients, and the alkaline comet test was the best predictor of male infertility.

Keywords: DNA fragmentation; Infertility; Sensitivity and specificity

Introduction

Sperm DNA fragmentation (SDF) has a major impact on fertility [1]. In the last decade, SDF has become a biomarker of male infertility, since it was discovered that spermatozoa with poor-quality or fragmented genetic material may hinder embryonic growth and development, increasing the risk of miscarriage in early pregnancy, issues involving fetal development [2,3]. High levels of SDF have been associated with repeated failure of assisted reproductive technology [4]. Various methods to gauge SDF have been assessed for clinical use in studies that sought to identify threshold values for conception and to investigate their significance, sensitivity, and specificity. The main purpose of our case-control study was to compare the five most widely used techniques of measuring DNA fragmentation index (DFI), to identify correlations among them, and to determine their

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sensitivity, specificity, and cut-off values for predicting male infertility [5]. More specifically, the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, the sperm chromatin dispersion (SCD) test, the sperm chromatin structure assay (SCSA), and the comet assay were compared. Of these methods, the SCD test and SCSA characterize SDF on basis of denatured chromatin in spermatozoa [6]. The SCSA utilizes acridine orange staining to label double- and single-stranded DNA with green and red fluorescence, respectively, after treatment with an acidic denaturing agent; higher levels of denaturation are associated with lower levels of sperm DNA integrity, and the clinical utility of this technique has been firmly established [7]. The SCD assay measures the extent to which chromatin in spermatozoa is dispersed based on the appearance of a radiant halo, and it enables non-divided (with a halo) spermatozoa to be distinguished from divided (without a halo) spermatozoa [8]. Although several clinical investigations have investigated the predictive power of these methods, few studies have explored correlations among the results obtained using TUNEL, SCD, and SCSA and the studies that have done so have not investigated the sensitivity and specificity of each test [9]. However, studies have shown a relationship between embryo quality and SDF based on SCSA, while other studies have not found relationships between SDF and embryo growth or the outcomes of assisted reproductive technology [10]. However, the design of such studies does not allow the role of SCSA to be accurately determined, since their results could have resulted from female factor infertility, manifesting in the form of factors such as differences between oocytes in the function of the DNA repair system that ensures successful fertilization [10]. We have discovered that extensive single-strand SDF may prevent conception [10].

1. Oxidative stress

Reactive oxygen species (ROS) contribute to SDF and male infertility. Oxidative stress occurs when ROS generation surpasses the body's own normal antioxidant defenses, bringing about cellular damage. Seminal discharge consists of various types of cells, such as mature and immature spermatozoa, round cells at various phases of spermatogenesis, pus cells, leukocytes, and epithelial cells [11]. The three noteworthy sources of ROS in semen are pus cells, leukocytes, and spermatozoa themselves. However, it has been suggested that pus cells and leukocytes contribute the most to oxidative stress based on a comparison to the effects of spermatozoa. The rate of ROS production was observed to be almost 800 to 1,000 times higher in pus cells and leukocytes than in spermatozoa. Furthermore, ROS generation is higher in patients who smoke and drink. However some medical conditions also result in ROS generation, such as varicocele, genital tract infections, and spinal cord injury. Age and infertility also play an important role in ROS generation [12]. It has been observed that ROS generation was higher in semen samples of infertile patients than in those of fertile patients, and that SDF increased as a result of the increased concentration of ROS in semen samples. Nonetheless, ROS play a vital role in sperm physiological and biochemical processes, such as activation, capacitation, acrosome reaction and signaling for fertilization. As described above, oxidative stress has been linked to poor sperm motility and sperm function, leading to poor embryo formation, miscarriage, and infertility. Therefore, in this study, we examined ROS formation using the Oxisperm kit in the fertile and infertile samples [13].

Methods

1. Test collection

Raw semen samples from 50 fertile and 50 infertile subjects were collected from patients receiving care at assisted reproductive center facilities in Base Fertility Medical Science Pvt Ltd. Infertility was defined as the inability of a sexually active couple not using contraception to achieve pregnancy in 1 year. Samples were gathered using the antegrade technique with a period of sexual abstinence of 2–6 days and were analyzed using the World Health Organization criteria (2010). A small amount of semen from each subject was frozen in liquid nitrogen for SCSA, and the other five tests were carried out using the fresh sample on the same day. Informed lawful consent (ICMR BASED-NMAS-77-97) was procured from all participants. Applicable approval was received from the ethical committee of the Base Fertility Medical Science, Department of Infertility and Reproductive Medicine (approval No. BFM/ivf-80RI-78). The datasets used and/or analyzed during the current study are available from the corresponding author upon a reasonable request.

2. TUNEL assay

The TUNEL evaluation was carried out using an *in situ* cell death detection kit. For 30 seconds, the air-dried smeared sample was fixed in 3.9% paraformaldehyde at 28°C and further washed with phosphate-buffered saline (PBS) at a pH of 7.4 and then permeabilized with 2% Triton X-100. Under sterile conditions, the nucleotide mixtures labelled with TdT were layered onto individual slides and incubated in a humidified chamber at 37°C for 58 minutes in the absence of light. Subsequently, the humidified slides were washed three times and stained with 8 mg/mL diamidino-2-phenylindole (DAPI), and negative controls without the TdT-tagged enzyme were run in each duplicate for each sample. A total of 300 sperm per entity were examined using fluorescence microscopy by the same surveyor. The spermatozoa stained with DAPI (blue) were counted first, followed by the spermatozoa dyed green (TUNEL-positive), and then the percentage of these cells in the total sample was calculated [14].

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3. SCSA

Our lab modified protocol used for SCSA, and accordingly, each semen sample was diluted to a concentration of 2×10^6 spermatozoa/mL in TE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) in a total volume of 250 µL. The sample was treated with an acid solution for 35 seconds and restaining was completed with acridine orange (5 µg/mL) for 150 seconds. A total of 1,000 spermatozoa were analyzed in each examination, and the results are presented as the DFI (%) calculated utilizing FAC scan program (BD Biosciences, Franklin Lakes, NJ, USA). The proportion of spermatozoa with DNA fragmentation was shown by red fluorescence, in comparison to green fluorescence in the non-fragmented sample [15].

4. SCD

The SCD test was carried out using the Halo sperm kit-Parque Tecnológico de Madrid Spain according to the manufacturer's protocol. The samples were smeared using a standard kit; 300 spermatozoa were examined and identified as fragmented or non-fragmented based on whether they formed a halo. More specifically, in this technique, whole spermatozoa (fresh or frozen) are immersed in an inert agarose microgel on a pretreated slide. The initial treatment of spermatozoa with non-fragmented DNA with dilute acid denatures the DNA, and the lysing buffer subsequently expels the vast majority of the nuclear proteins. In the absence of colossal DNA breakage, nucleoids are produced with outsized coronas of spreading DNA coils, ascending from a focal center. Visualization can be performed under bright field microscopy; however, if the staining is too concentrated, the prestained slide can be gently washed with tap water [16].

5. Comet assays

Single- and double-stranded SDF can be measured using the alkaline and neutral comet assays. The procedure was simultaneously performed in semen samples on two distinct slides. Frozen semen samples were liquefied and washed with HEPES/MOPS buffer, and the sperm concentration was diluted to 10×10^6 spermatozoa/mL. Then, 25-µL aliquots of spermatozoa were mixed with 50 µL of 1% low-liquefying-point agarose in double-distilled water. Immediately, 10 µL of the combination was arranged on two pretreated slides of agarose gel, encased with cover slips, and placed on a cold plate at 5°C for 4 minutes. Next, the cover slips were carefully isolated, and the two slides were washed with an excess of lysing buffer for 30 minutes, followed by a 10-minute wash in Tris-borate EDTA (TBE). In the neutral comet assay, electrophoresis was performed with a TBE arrangement of 20 V (1 V/cm) for 12 minutes and 30 seconds, with a subsequent wash with 0.9% NaCl for 2 minutes. For the alkaline comet assay, which measures the extent of denaturing, the slide was washed for 3 minutes at 5°C, and electrophoresis was then performed in 0.03 M NaOH at 20 V (1 V/cm) for 4 minutes. Then, both slides were incubated in a neutralizing solution for 5 minutes and with TBE for 2 minutes. The slides were dried in a graded series of ethyl alcohol solutions (75%, 85%, and 100%) for 2 minutes each. Finally, 500 spermatozoa were assessed in terms of whether they showed divided or non-divided chromatin according to previously published criteria [17].

Table 1. Reactive oxygen species levels from the Oxisperm kit assay for infertile and fertile patients

Color scheme-Oxisperm Kit	Intensity (%)	
	Fertile (n=45)	Infertile (n=45)
L1 (Low)	39	16
L2 (Low–medium)	24	11
L3 (Medium)	11	31
L4 (High)	26	42

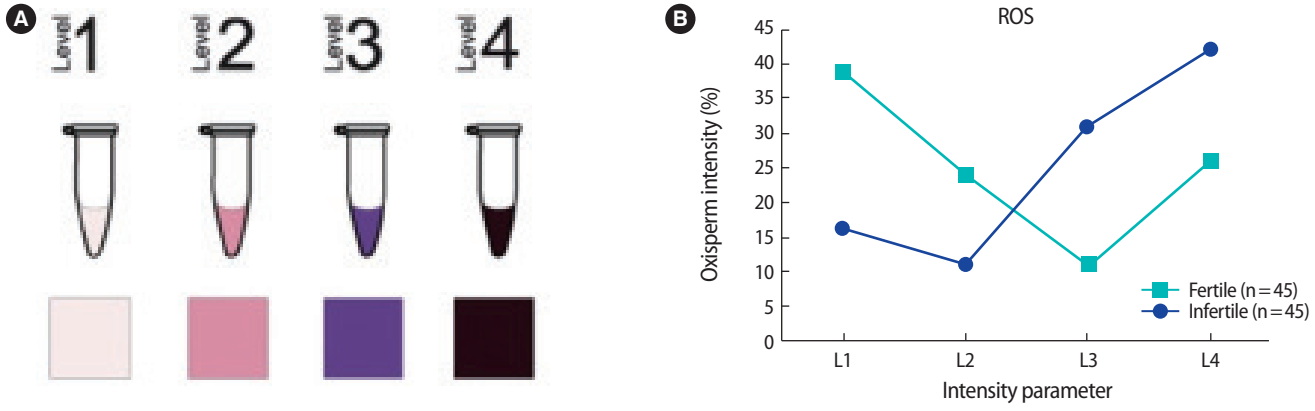

Figure 1. (A) Reactive oxygen species (ROS) intensity color scheme for semen samples using the Oxisperm kit assay. The figure was supplied by halotech (Madrid, Spain). (B) ROS intensity plot for the infertile and fertile samples using the Oxisperm kit assay. L (level) 1, low; L2, low–medium; L3, medium; L4, high.

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6. Oxisperm kit

The human spermatozoon is highly vulnerable to oxidative stress. Peroxidative injuries directly influence the lipid component of the membrane and generate breaks in the strands of both nuclear and mitochondrial DNA. Nitroblue tetrazolium (NBT; 0.1%) was combined with PBS by adding 100 mg of NBT powder to 1,000 mL of PBS (pH 7.2) and mixed at 28°C for 60 minutes. The NBT solution was sieved with a 0.2-μm filter channel. NBT (0.1%) was added to 0.5 mL of dilute semen and incubated for 45 minutes at 37°C. The sterile tubes were centrifuged at 800 rpm for 5 minutes, and smears were set up from the pellet and air-dried. The slide was recolored with Wright's stain, and an aggregate of 100 spermatozoa was scored under ×100 intensification. Two experienced examiners (AJ, STM) scored the NBT-recolored slides in a blinded manner, using the following four predefined levels of intensity: L1, low; L2, low-medium; L3, medium; L4, high. The color of the sample was compared with the reclassified color scheme (Table 1, Figure 1).

7. Statistical analysis

The statistical analysis was conducted utilizing the IBM SPSS ver. 20.0 (IBM SPSS Corp., Armonk, NY, USA). The Mann-Whitney *U*-test was used to analyze the five different SDF measurement techniques. The Spearman test was used to evaluate the correlations between the techniques, and receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity, specificity, and cut-off values for each test.

Results

1. SDF and male infertility

Statistically significant differences were observed between the fertile and infertile patients using TUNEL, SCD, SCSA, and the alkaline comet test ($p < 0.001$), but not when the neutral comet test was used ($p = 0.865$).

2. Oxidative stress (ROS) and male infertility

The presence of ROS was analyzed in 45 patients each from the fertile and infertile groups. The color scheme pattern for the Oxisperm kit assay represents the level of ROS in the semen sample. The distribution of observed intensities in the fertile group was as follows: L1 (low), 39%; L2 (low–medium); 24%; L3 (medium), 11%; and L4 (high), 36%. In the infertile group, the distribution was as follows: L1 (low), 16%; L2 (low-medium), 11%; L3 (medium), 31%; and L4 (high), 42%. Thus, ROS levels were higher in the samples from infertile men than in those from fertile men (Table 1, Figure 1A).

3. Correlations between procedures

Spearman correlation analysis was used to assess the correlations between all methods. Strong and significant correlations were observed between the SCD test and SCSA ($r = 0.70$, $p < 0.001$), between the SCD test and the TUNEL assay ($r = 0.68$, $p < 0.001$), and between SCSA and the TUNEL assay ($r = 0.77$, $p < 0.001$). Reasonably strong and significant connections were detected between the alkaline comet assay and the SCD test ($r = 0.59$, $p < 0.001$), between the alkaline comet test and SCSA ($r = 0.57$, $p < 0.001$), and between the alkaline comet test and the TUNEL assay ($r = 0.72$, $p < 0.001$). No meaningful correlation was found between the neutral comet assay and the other four techniques.

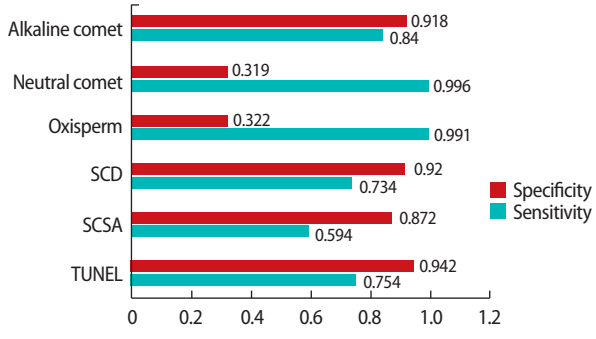

Figure 2. Sensitivity and specificity under the receiver operating characteristic curve for the six independent observations (95% confidence interval). SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

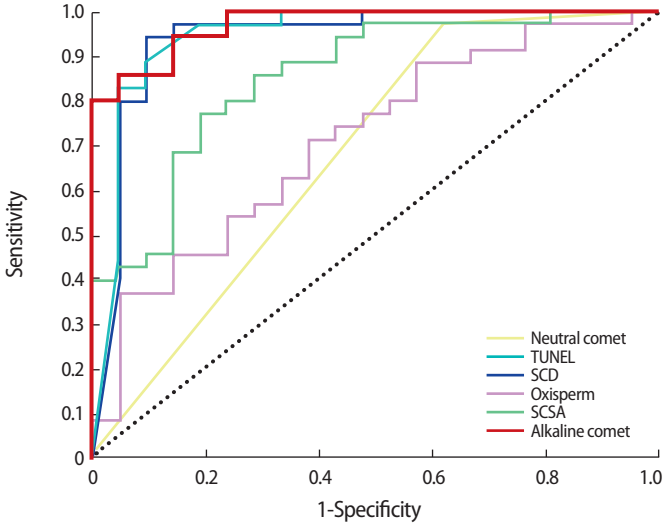

Figure 3. Receiver operating characteristic curve for the sperm DNA fragmentation assays. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay.

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Table 2. Cut-off values with sensitivity and specificity for each assay

Technique	Number	Area[a)]	Cut-off value (%)	Sensitivity	Specificity
TUNEL	95	0.901	22.08	0.754	0.942
SCSA	100	0.790	19.90	0.594	0.872
SCD	100	0.871	24.74	0.734	0.920
Oxisperm	90	0.504	35.38	0.991	0.322
Neutral Comet	100	0.511	36.37	0.996	0.319
Alkaline Comet	100	0.977	48.47	0.840	0.918

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; SCSA, sperm chromatin structure assay; SCD, sperm chromatin dispersion.
[a)]Area under the receiver operating characteristic curve.

4. Sensitivity, specificity, cut-off values, and ROC analysis

The ROC curve analysis of the five different assays assessed their sensitivity, specificity, and cut-off values for predicting male infertility. The largest area under the curve 0.977 was observed for the alkaline comet assay, with an SDF value of 48.74% yielding a sensitivity and specificity of 0.840 and 0.918, respectively (Figure 2). Next, the TUNEL assay resulted in an area under the curve of 0.901, with an SDF cut-off value of 22.08% yielding a sensitivity and specificity of 0.754 and 0.942, respectively. For the SCD test, the area under the curve of was 0.871 with an SDF cut-off value of 24.74%, yielding a sensitivity and specificity of 0.734 and 0.920, respectively. However, for SCSA, less predictive power was found, with an area under the curve of 0.790 for an SDF cut-off value of 19.90%, with a sensitivity of 0.594 and a specificity of 0.872, respectively (Figure 3). Moreover, the neutral comet assay and Oxisperm assay showed no correlations with male infertility, with areas under the curve of 0.511 and 0.504, SDF cut-off values of 36.37% and 35.38%, and sensitivity and specificity values of 0.996, 0.991, 0.319, and 0.322, respectively (Table 2).

Discussion

Although the utilization of various approaches to survey sperm DNA damage has been broadly examined, few reports have studied the clinical utility and relationships between the most widely recognized techniques in a comprehensive manner [5]. Therefore, we performed this comparative investigation to evaluate correlations among the most utilized procedures and to establish their clinical cut-off values.

Significant differences in SDF were found between fertile and infertile patients using the TUNEL test, SCSA, the SCD test, and the alkaline comet test, as previous studies have reported [18]. However, no significant distinctions were found between fertile and infertile patients using the neutral comet test. In previous studies, a bimodal distribution was found in fertile donors, showing that fertile men are a heterogeneous group in this regard [19]. The neutral comet assay also demonstrated a normal distribution among the infertile samples, yielding generally high estimations of double-stranded SDF. A difference was found in SDF in fertile patients depending on whether the alkaline comet test or the SCD test, SCSA, or the TUNEL assay was used, which may have been a direct result of the electrophoresis step, since this step could amplify the sensitivity of the identification of DNA, breaks [20].

In infertile patients, the estimations of SDF made using the alkaline comet test were statistically significantly higher than those obtained with the SCD test, SCSA, and TUNEL techniques, demonstrating that the comet assay appeared to have higher sensitivity for identifying sperm DNA breaks, identifying that up to 100% of spermatozoa had DNA fragmentation in some infertile patients. SCSA yielded statistically significantly lower SDF values than the SCD and TUNEL assays, which did not have significant differences between their values. These findings imply that different techniques may recognize different aspects of SDF, as SCD and SCSA focus on chromatin fragmentation, while the comet test and the TUNEL assay directly identify DNA breaks [21].

The closest correlation was found between the cytometric measures (TUNEL and SCSA), as previously reported [22]. This finding is intriguing given that the two tests are believed to gauge distinct aspects of SDF. Additionally, it is important to standardize the TUNEL technique, as minor technical variations in this method lead to variation in SDF. Nevertheless, despite the contrast between the TUNEL assay and SCSA and the requirement for standardization of the former, both assays yielded fundamentally similar results for SDF. In addition, the Oxisperm kit showed higher levels of ROS in infertile patients than in fertile patients, which is directly related to sperm DNA damage [22]. Additionally, the alkaline comet assay demonstrated a moderate correlation with the SCD test, the TUNEL test, and SCSA, as previously identified in various studies [22]. However, this correlation was not as robust as the relationships found among the last three techniques, which may have been a direct result of the higher sensitivity of the alkaline comet test in comparison to alternate methods. Interestingly, the neutral comet test did not demonstrate any correlations with the other four techniques used to evaluate SDF. As pro-

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Table 3. SDF values for infertile and fertile patients with each assay

Technique	Fertile		Infertile	
	No. of patents	Mean ± SD (range)	No. of patents	Mean ± SD (range)
TUNEL	50	12.65 ± 5.65 (6.2–31.4)	45	27.55 ± 11.65 (7.8–75.3)
SCSA	50	11.41 ± 5.11 (4.9–27.5)	50	22.1 ± 12.18 (7.8–75.6)
SCD	50	16.04 ± 6.12 (4.2–31.2)	50	32.1 ± 14.84 (6.1–79.1)
Neutral comet	50	62.23 ± 30.24 (14.2–99.1)	50	67.21 ± 17.02 (28.3–100.0)
Alkaline comet	50	26.32 ± 10.25 (9.5–68.0)	50	61.25 ± 16.45 (17.5–98.6)

SDF, sperm DNA fragmentation; SD, standard deviation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; SCSA, sperm chromatin structure assay; SCD, sperm chromatin dispersion.

posed previously, the results of the neutral comet assay are associated with the risk of unsuccessful implantation, as the double-stranded DNA breaks might reflect a non-extensive type of DNA damage found only in a few loci along the genome, in the matrix connection districts between toroids; such breaks may result from intense or fractionated elucidation to radiation, as previously shown in tumor cells [23]. Although the TUNEL test and SCSA identify both single- and double-stranded DNA damage, our findings indicate that both the TUNEL assay and SCSA showed correlations with the alkaline comet test, which principally distinguishes single-stranded SDF. However, these assays did not show a correlation with the neutral comet assay, which has been demonstrated to primarily survey for double-stranded DNA breaks [24]. Additionally, the neutral and alkaline comet tests demonstrated a moderate correlation in infertile patients, which could have been associated with the likelihood that many single-stranded DNA breaks in the same vicinity could prompt double-stranded DNA breaks.

An ROC curve analysis was performed to assess the predictive power of these tests for male infertility. The alkaline comet test showed the highest area under the curve, followed by the TUNEL test, the SCD test, SCSA, and the neutral comet test (Table 3, Figure 3). The alkaline comet examination showed an area under the curve of 0.977 with an SDF threshold of 48.74%, demonstrating high sensitivity and specificity. This finding is not directly comparable to those of past investigations, in which the percentage of damaged DNA was evaluated, not the percentage of fragmented sperm cells [25]. The TUNEL test demonstrated a cut-off SDF value for male infertility of 22.08%, with high values for the area under the curve and specificity (0.901 and 0.942, respectively); however, it was markedly less sensitive than the alkaline comet test (0.754). These outcomes are practically identical to those obtained by Sharma et al. [26], who reported a cut-off estimation of 19.25%, with an area under the curve, sensitivity, and specificity of 0.890, 0.649, and 1.000, respectively. Our results show an estimated limit of 19.9% for SDF, which is on the low end of the distribution; however, this finding is consistent with those of other studies that have reported values of approximately 20%. Additionally, it is worth noting that SCSA is the most standardized procedure across various research centers [26]. Furthermore, the neutral comet assay demonstrated an exceptionally weak relationship with male infertility, with fertile participants showing either low or high estimated levels of double-stranded DNA fragmentation using this technique [27]. However, infertile patients dependably demonstrated a high SDF. Hence, the cut-off SDF value of showed high sensitivity and low specificity, due to the bimodal distribution in fertile participants, as has been reported previously [28]. This finding is noteworthy because high values of SDF are related to miscarriage [29]. For further evaluation, as various methods may gauge distinct aspects of chromatin integrity, a double investigation focusing on a single method for measuring SDF would confirm these results.

This study presents clinical information from the five methods that are most often used to assess SDF in the same set of patients. Based on these results, it can be concluded that, aside from the neutral comet test, the remaining four strategies are productive for distinguishing between fertile and infertile patients, with the alkaline comet test being the best predictor of male infertility. In addition, the Oxisperm kit assay showed that the semen samples from infertile men had more ROS than those obtained from fertile men, which is directly related to the greater SDF in the infertile samples.

Conflict of interest

No potential conflict of interest relevant to this article was reported. Base Fertility Medical Science Pvt. Ltd. holds no conflict of interest.

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Conceptualization: AJ. Data curation: AJ, MST. Formal analysis: MKR, MST. Methodology: AJ, MKR. Project administration: MST, MKR. Visualization: AJ. Writing - original draft: AJ, MKR, MST. Writing - review & editing: MST, MKR.

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