Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility

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Objective: To compare the sperm chromatin dispersion (SCD) test and the terminal uridine nick-end labeling (TUNEL) assay for assessment of sperm DNA damage.

Design: Prospective comparative experimental study.

Setting: Andrology laboratory.

Patient(s): Twenty subfertile men with unexplained infertility.

Intervention(s): Sperm DNA damage was determined in the same semen samples using the TUNEL assay with fluorescence microscopy and the SCD test with bright-field microscopy.

Main Outcome Measure(s): Correlation coefficient and receiver operating characteristic analysis outcomes. The TUNEL assay was used as the reference standard to identify optimal cutoff points for assessing DNA damage by SCD.

Result(s): The SCD test detected a significantly higher proportion of sperm with damaged DNA (20.6% ± 14.0%) than the TUNEL assay (11.5% ± 7.3%). Spearman’s rank correlation showed that the methods were not comparable (r = 0.29). Receiver operating characteristic analysis revealed that 15% was the best SCD cutoff point to classify patients within the same levels of DNA fragmentation, normal or abnormal, as determined by the TUNEL assay, with an accuracy of 69%.

Conclusion(s): The SCD test is more sensitive than the TUNEL assay for the assessment of DNA damage in men with unexplained infertility. Although the methods are poorly correlated, SCD may discriminate men with normal and abnormal sperm DNA damage with moderate accuracy when compared with TUNEL. It is important to distinguish between the methods because they differently evaluate sperm DNA damage. (Fertil Steril 2013; )

Key Words: Sperm DNA damage, in situ nick-end labeling, sperm chromatin dispersion test, diagnosis, ROC curve

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The assessment of sperm chromatin integrity has emerged as an important biomarker for male infertility. Sperm DNA damage has been associated with several infertility phenotypes, including unexplained infertility, idiopathic infertility, repeated intrauterine and IVF failure, and recurrent miscarriage (1-4). Because ejaculates of infertile men harbor higher proportions of sperm with DNA damage compared with fertile counterparts (5, 6), different assays have been developed to evaluate DNA damage in sperm.

Among several tests, terminal uridine nick-end labeling (TUNEL) assay and sperm chromatin structure assay (SCSA) remain the gold standards for the identification of clinically significant sperm DNA damage (7-10). Although these methods have been implemented by many andrology laboratories, they cannot be performed routinely in the routine workup of male infertility because they are complex, difficult to implement, time-consuming, and expensive since they require fluorescent microscopy and flow cytometry, respectively (11). A less complex test would be desirable, and the sperm chromatin dispersion (SCD) test has reached technical
maturity to allow its widespread application with a standardized protocol using conventional bright-field microscopy (12–14). Whereas testing thresholds have been extensively reported for TUNEL and SCSA (15–19), there are few studies focusing on the diagnostic accuracy of SCD (20) and none in specific patient subsets, such as in unexplained male infertility. This condition accounts for 6%–27% of the male cases (21), and therefore a detailed evaluation of the diagnostic accuracy of SCD in such cases is warranted before shifting from the more complex and validated methods to SCD.

Therefore, we conducted a study to determine the accuracy of the SCD test using conventional bright-field microscopy in the evaluation of DNA damage in sperm. For this, we used the TUNEL assay carried out with fluorescence microscopy as the gold standard method for sperm DNA damage assessment.

MATERIALS AND METHODS

Patient Inclusion Criteria

A total of 20 consecutive patients, aged 18–43 years, presenting at the study center for infertility evaluation and who met the study criteria, was included. The inclusion criteria comprised the following: [1] presence of normal semen parameters, in accordance with the 2010 World Health Organization (WHO) guidelines (22), in a minimum of two separate previous semen analyses performed in our laboratory; [2] patients should be nonsmokers and not taking any medication with potential gonadotoxic effects for at least 3 months before the study; [3] all subjects should have completed an initial evaluation by the consulting urologists, and no obvious infertility problems noted in the medical history, physical examination, and endocrine profiles. As such, all subjects enrolled in the study were classified as having unexplained male infertility (23). In addition, data collection was planned before the tests were performed. The recruitment period ranged from March to July 2012. The study complied with the standards for the reporting of diagnostic accuracy studies (START statement). Institutional review board approval was obtained before the investigation.

Initial Assessment of Semen Parameters

Subjects were asked to abstain from ejaculation for a fixed period of 3 days before collection. Semen specimens were collected by masturbation into sterile cups. All subjects used a collection room located in the same facility as the andrology laboratory. Semen was allowed to liquefy for 30 minutes, and an aliquot was taken for macroscopic and microscopic assessments. Specimens were assessed for volume, count, motility, vitality, morphology, and leukocytes, in accordance with the fifth edition of the WHO manual (22). We used the strict criterion (Tygerberg) for morphology evaluation. We assessed all specimens for the presence of rounded cells and used the Endtz test to determine the presence of polymorphonuclear leukocytes. The semen parameters of study subjects were above the fifth percentile, proposed as the lower reference limit by the WHO (22), and are presented in Supplemental Table 1 (available online). All tests were carried out in an International Organization for Standardization (ISO 9001:2008) certified andrology laboratory enrolled in both external and internal quality control programs (24, 25).

Sperm DNA Damage Assessment

After initial evaluation, semen specimens were split into two aliquots of equal volumes; one was tested by the TUNEL assay, set as the reference standard in the present study, and the other by the SCD test. Procedures were carried out in parallel.

TUNEL assay. The assay was performed using the Apo-Direct kit (Pharmingen) as described by Sharma et al. (15). A sperm aliquot containing 1 to 2 × 10^6 spermatozoa was washed in phosphate-buffered saline and resuspended in 3.7% paraformaldehyde. Thereafter, the suspension was placed on ice for 30–60 minutes at 4°C, washed again in phosphate-buffered saline to remove the paraformaldehyde, and then resuspended in 50 μL of freshly prepared staining solution for 60 minutes at 37°C. The staining solution was composed of terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein isothiocyanate–tagged deoxyuridine triphosphate nucleotides (FITC-dUTP), and distilled water. All specimens were further washed in rinse buffer and counterstained with 4,6-diamidino-2-phenylindole (DAPI, 2 micrograms/mL in vectashield) followed by analysis using fluorescence microscopy. A fluorescence microscope (Eclipse E600; Nikon) equipped with an epi-illumination module and a mercury ultraviolet source was used to examine the slides at ×1,000 magnification. The B2A filter cube was used for FITC-dUTP, which fluoresces apple-green. Sperm showing bright apple-green fluorescence represented damaged cells (TUNEL positive), in which dUTP was incorporated to DNA breaks, in contrast to nonstained cells representing nondamaged sperm (Supplemental Fig. 1).

The percentage of TUNEL-positive sperm was calculated as having unexplained male infertility (23). In addition, data collection was planned before the tests were performed. The recruitment period ranged from March to July 2012. The study complied with the standards for the reporting of diagnostic accuracy studies (START statement). Institutional review board approval was obtained before the investigation.
surrounding a compact and well-defined core. Sperm with damaged DNA (SCD-positive cells) showed small haloes of dispersed chromatin or no haloes (Supplemental Fig. 1). The percentage of SCD-positive sperm was calculated and reported as the percentage of cells exhibiting damaged DNA. A minimum of 400 sperm was assessed per specimen.

Sperm DNA evaluation using the studied methods was carried out by a single experienced senior technician, with more than 4 years’ expertise in performing sperm DNA damage using the TUNEL assay. This same technician has undergone prior training with the SCD test, as provided by Halotech DNA. Each run was carried out in duplicate with an appropriate experimental positive and negative control using patient specimens. For SCD, positive controls were made by treating specimens with 50 μL of H2O2 before immersing slides into the denaturation solution. Negative controls were created by omitting the lysis step from the protocol. For TUNEL, positive control slides were made by adding 1 μL of DNase I, which generates DNA breaks in most cells, to the staining solution. In TUNEL-negative controls, the TdT enzyme was omitted from the labeling solution. In addition, frozen-thawed specimens from patients with negative and positive DNA damage were included as internal controls in each run. Categorization of SCD and TUNEL-positive sperm was performed on the fixed specimens in a blind manner (ie, a technician who was not involved in conducting the experiment masked the slides, to withhold information from the reader).

### Statistical Analysis

Sperm DNA damage data were assessed for homogeneity and normal distribution by the Kolgomorov-Smirnov test, and results were expressed as mean ± standard deviation, median, and minimum and maximum values. Comparison of means was performed by the paired t test. The TUNEL assay and SCD test were compared for the detection of DNA damage using Spearman’s rank correlation. For these tests, the significance level was .05. Receiver operating characteristic (ROC) analysis was performed using four different TUNEL thresholds (4%, 10%, 12%, and 19%) to select the optimal cutoff points of SCD to discriminate between patients with normal and abnormal levels of sperm DNA damage, as determined by the TUNEL assay. Accuracy of all possible SCD cutoff points using the TUNEL thresholds, as determined by ROC analysis, are presented in Table 2.

Using the cutoff points of 15% and 10% for SCD and TUNEL, respectively, 60% (12 of 20) and 45% (9 of 20) of the patients had ejaculates with an elevated proportion of sperm with DNA damage. In six patients, the results of TUNEL and SCD differed with regard to the diagnostic category assessed, normal or abnormal. In four patients, the results of TUNEL were below the cutoff point, whereas those of SCD were above it. In two patients, contrary results were observed. None of the patients were excluded because of inadequate or failed staining and undetermined or missing results. The tabulation of the results of the SCD test and the TUNEL assay is shown in Supplemental Table 2.

### RESULTS

The proportion of sperm with damaged DNA, as assessed by SCD and TUNEL, is shown in Table 1. The SCD test detected a significantly higher proportion of sperm with damaged DNA (20.6% ± 14.0%) than the TUNEL assay (11.5% ± 7.3%; P<.001). The relationship between the SCD test and the TUNEL assay, as assessed by Spearman’s rank correlation, was 0.29 (P=.25; Fig. 1).

Receiver operating characteristic analysis showed that the SCD cutoff point of 15% yielded the largest AUC (0.687; 95% CI 0.522–0.753) when the TUNEL threshold was set to 10%, with a sensitivity, specificity, PPV, and NPV of 82%, 56%, 69%, and 71%, respectively (Fig. 2). Sensitivity, specificity, PPV, NPV, and accuracy of the optimal SCD cutoff points, as determined by ROC analysis, are presented in Table 2.

Using the cutoff points of 15% and 10% for SCD and TUNEL, respectively, 60% (12 of 20) and 45% (9 of 20) of the patients had ejaculates with an elevated proportion of sperm with DNA damage. In six patients, the results of TUNEL and SCD differed with regard to the diagnostic category assessed, normal or abnormal. In four patients, the results of TUNEL were below the cutoff point, whereas those of SCD were above it. In two patients, contrary results were observed. None of the patients were excluded because of inadequate or failed staining and undetermined or missing results. The tabulation of the results of the SCD test and the TUNEL assay is shown in Supplemental Table 2.

### DISCUSSION

The SCD test and the TUNEL assay are methods to assess sperm DNA integrity. When the SCD test was used in our
Our results indicate that the SCD method is more sensitive than TUNEL in the evaluation of sperm DNA damage. Additionally, increases in the proportion of DNA-damaged sperm assessed by one method will not necessarily be confirmed by the other. It means that the results obtained by the methods are not interchangeable because they differently assess DNA damage. As a result, andrology laboratories willing to switch from one method to the other should exercise caution because the methods are not comparable. However, logistic regression analysis showed that SCD can be used with moderate accuracy to discriminate between men with abnormal and normal levels of sperm DNA damage, as established by the reference standard, the TUNEL assay. The clinical implication of our findings is that SCD, at a cutoff point of 15%, will correctly identify ejaculates with normal or abnormal levels of DNA damage in up to 69% of the cases, as established by TUNEL using a threshold of 10%. Nevertheless, lower accuracy would be expected if different TUNEL thresholds were adopted.

The TUNEL assay and SCD test are based on different principles and therefore differ in their ability to detect DNA damage. The TUNEL assay relies on a terminal transferase that catalyzes the incorporation of modified nucleotides at the site of damage (5’-3’ ends) in a fixed specimen, and it is therefore a method that directly measures DNA damage. In contrast, SCD measures the susceptibility of DNA to denaturation, that is, its principle is to first promote DNA denaturation and then measure DNA damage by detecting the formation of DNA strand breaks. The difference in the pattern of forming a loop around lysed and acid-treated nuclear membrane carcass reflects the overall chromatin structure and is used to indirectly measure DNA breaks in the SCD test. Given the fact that probe incorporation in TUNEL depends on the amount of chromatin that is partially freed from the proteins protecting the DNA, it is possible that existing breaks are not detected owing to chromatin compaction, thus explaining the observed different levels of sperm DNA damage by the method and the poor correlation between TUNEL and SCD. In fact, modified protocols for TUNEL have been proposed aiming to increase the accessibility of terminal transferase to the sites of DNA breaks. In a recent study, Antonucci et al. proposed a novel protocol to improve sperm chromatin accessibility, which involves the use of 0.5% paraformaldehyde, 5 mM 1,4-dithiothreitol, 100 U/mL heparin, and 0.1% Triton-X 100, that might be useful if used in conjunction with the TUNEL assay.

Whereas the denaturation step results in the production of single-strand DNA motifs from pre-existing single- or double-strand breaks, the lysis step differentially removes proteins linked to single- or double-strand DNA stretches.

TABLE 2

<table>
<thead>
<tr>
<th>Optimal SCD cutoff (%)</th>
<th>Accuracy (AUC)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>TUNEL threshold (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥9</td>
<td>0.597 (0.441–0.736)</td>
<td>88.9 (32.5–99.1)</td>
<td>50.0 (35.2–88.6)</td>
<td>94.1</td>
<td>33.3</td>
<td>≥4</td>
</tr>
<tr>
<td>≥15</td>
<td>0.687 (0.522–0.753)</td>
<td>81.8 (59.0–92.6)</td>
<td>55.6 (23.1–77.3)</td>
<td>69.2</td>
<td>71.4</td>
<td>≥10</td>
</tr>
<tr>
<td>≥17</td>
<td>0.631 (0.508–0.724)</td>
<td>66.7 (35.9–97.5)</td>
<td>63.6 (35.2–92.1)</td>
<td>60.0</td>
<td>70.0</td>
<td>≥12</td>
</tr>
<tr>
<td>≥17</td>
<td>0.555 (0.31–0.795)</td>
<td>100.0 (100.0–100.0)</td>
<td>55.6 (32.6–78.5)</td>
<td>20.0</td>
<td>100.0</td>
<td>≥19</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are 95% CIs.

Feijó. SCD accuracy in unexplained male infertility. Fert Stéril 2013.
thus facilitating the detection of DNA damage using the SCD assay (13, 26). These features also explain the higher sensitivity of SCD compared with TUNEL.

In our settings, we take approximately 7.3 hours to conduct a single test, from specimen collection to reporting results, using the TUNEL assay. A maximum of two assessments is scheduled per day, because this represents the limit of tests that can be run in parallel by a single technician. In contrast, we take only 1.7 hours to perform an integral evaluation of sperm DNA damage using the SCD test, and up to four tests can be run concomitantly. As such, it would be advantageous to use SCD as a surrogate for the more complex methods in the evaluation of sperm DNA damage. Nevertheless, conflicting data exist on the correlation among different tests in reporting abnormal levels of sperm DNA fragmentation. In one study, Chohan et al. (29) have shown that SCSA, TUNEL, and SCD techniques had similar predictive values for detecting DNA damage (29). In contrast, Henkel et al. (30) could not corroborate the aforesaid findings and concluded that the assays were not comparable. Our data are in agreement with the latter, which seem sound owing to the different nature of DNA damage assessed by each method.

Thresholds for the SCD test have not yet been determined in men with unexplained infertility. However, a cutoff point of 17% was shown to discriminate pregnant and nonpregnant couples in IVF treatment, with sensitivity and specificity close to 75% (20). To our best knowledge, this study is the first to use a logistic regression model and the TUNEL assay, as the reference standard method, to determine the diagnostic accuracy of SCD to assess sperm DNA damage in men with unexplained infertility. Because different threshold values of 4%, 10%, 12%, and 19% have been reported for the TUNEL assay (15–19), we included these cutoff points in our model to establish the best accuracy of SCD as a diagnostic method. Of note, we observed that 60% and 45% of our study group had elevated levels of sperm DNA damage using the optimal SCD and TUNEL cutoff points of 15% and 10%, respectively, despite normal semen analysis according to the WHO criteria. Men with unexplained infertility have no obvious history of fertility problems, and physical examination, endocrine laboratory testing, and semen analysis results are normal (21, 23). It has been shown that these men have significantly higher levels of DNA damage compared with fertile individuals, which may explain their inability to conceive (5, 6). Hence, assessment of sperm function is of utmost importance in this category of male infertility.

Sperm DNA damage is a broad term that accounts for many defects in the DNA structure, including [1] single or double DNA strand breaks, [2] base deletion or modification, [3] interstrand or intrastrand cross-linkage, and [4] DNA-protein cross-linkage (31). Sperm DNA damage can occur at any level during spermatogenesis, spermiogenesis, and epididymal transit (11). Postmeiotically initiated abortive apoptosis, unresolved strand breaks during spermiogenesis, and oxidative stress have all been implicated as potential sources of this damage (21). Sperm with damaged DNA are released in the semen, and despite the likely result of infertility, these defective cells may still retain the ability to fertilize. Elevated levels of sperm DNA damage have been associated with infertility and repeated intrauterine and IVF failure (1–4). Additionally, offspring generated from such defective sperm may harbor an increased risk of imprinting defects and cancer (32, 33). Notwithstanding, there seems to be an association with increased DNA fragmentation and pregnancy loss after IVF and intracytoplasmic sperm injection (risk ratio 2.16; 95% CI 1.54–3.03; P<.00001) (34). Altogether, these considerations argue in favor of using sperm DNA damage testing in the workup of infertile males. Yet at present no single test seems to be reliable enough to detect clinically significant DNA damage with high accuracy to predict the sperm reproductive profile in natural conception, IUI, IVF, or intracytoplasmic sperm injection, thus preventing recommendation of their routine use in the evaluation and treatment of infertile males. Hence there is an urgent need to refine the methods for assessing the sperm DNA integrity and validating their cutoff points in different subsets of patients, as well as in different interventions. Despite these limitations, the results provided by sperm DNA damage assays can be used as additional markers of sperm quality in men with unexplained infertility, because they have been associated with better diagnostic and prognostic value than routine semen analysis (7, 10, 21, 26).

In conclusion, our data indicate that the SCD test is more sensitive than the TUNEL assay for the assessment of DNA damage in men with unexplained infertility. Although the methods are poorly correlated, SCD may discriminate men with normal and abnormal sperm DNA damage with up to 70% accuracy, considering the TUNEL assay as the gold standard for sperm DNA evaluation. Whereas TUNEL depends on a terminal transferase to directly incorporate fluorescent UTP at single and double 3’-OH-free ends, SCD involves the combination of DNA denaturation and depletion of proteins protecting the DNA. Andrology laboratories should distinguish between the methods because they differently evaluate sperm DNA damage.

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REFERENCES


ORIGINAL ARTICLE: ANDROLOGY
Assessment of DNA damage in sperm. (A) TUNEL assay. Sperm showing bright apple-green fluorescence represent cells with damaged DNA (red arrows), in which dUTP is incorporated to DNA breaks, in contrast to non–DNA-damaged sperm (white arrows) that have been first identified using DAPI staining (not shown). Sperm photomicrographs obtained at ×1,000 using a fluorescence microscope (Eclipse E600; Nikon) equipped with an epi-illumination module and a mercury ultraviolet source. Filter B2A was used for FITC-dUTP. (B) SCD test. Nucleoids obtained with the improved SCD procedure (Halosperm; Halotech DNA) under bright-field microscopy and Wright’s stain. The red arrow indicates a spermatozoon containing a normal DNA molecule (a halo of dispersed chromatin is seen). The blue arrow indicates a spermatozoon with damaged DNA molecule (an absent halo of dispersed chromatin is seen). Microphotographs obtained at ×1,000 using bright-field microscopy (Alphaphot-2 YS; Nikon).

**SUPPLEMENTAL TABLE 1**

Distribution of semen characteristics of 20 men with unexplained infertility.

<table>
<thead>
<tr>
<th>Semen characteristic</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>3.0</td>
<td>1.5</td>
<td>2.6</td>
<td>1.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Sperm count ((\times 10^9)/mL)</td>
<td>49.5</td>
<td>26.8</td>
<td>41.8</td>
<td>15.5</td>
<td>109.0</td>
</tr>
<tr>
<td>Total sperm count ((\times 10^9))</td>
<td>147.5</td>
<td>115.8</td>
<td>94.8</td>
<td>52.7</td>
<td>442.5</td>
</tr>
<tr>
<td>Total motility (%)</td>
<td>70.9</td>
<td>6.8</td>
<td>71.0</td>
<td>46.0</td>
<td>79.0</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>57.1</td>
<td>9.4</td>
<td>57.5</td>
<td>33.0</td>
<td>68.0</td>
</tr>
<tr>
<td>Vitality (% alive)</td>
<td>71.2</td>
<td>7.5</td>
<td>72.0</td>
<td>59.0</td>
<td>78.2</td>
</tr>
<tr>
<td>Morphology (% normal forms)</td>
<td>7.0</td>
<td>3.0</td>
<td>6.0</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Leukocyte count ((\times 10^6))</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Tabulation of the results of the SCD test (index test) and the TUNEL assay (reference standard).

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Age (y)</th>
<th>Chart no.</th>
<th>TUNEL (% positive-sperm)</th>
<th>SCD (% positive-sperm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,125</td>
<td>39</td>
<td>2,602</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>1,165</td>
<td>27</td>
<td>External</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>1,223</td>
<td>38</td>
<td>1,525</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>1,224</td>
<td>41</td>
<td>958</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>1,225</td>
<td>26</td>
<td>2,806</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>1,271</td>
<td>40</td>
<td>280</td>
<td>4</td>
<td>23</td>
</tr>
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<td>1,272</td>
<td>18</td>
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<td>7</td>
<td>11</td>
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<td>081</td>
<td>39</td>
<td>3,156</td>
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<td>2,884</td>
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<td>108</td>
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<td>109</td>
<td>31</td>
<td>2,941</td>
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<td>16</td>
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<td>134</td>
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<td>2,790</td>
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<td>652</td>
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<td>1,464</td>
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<tr>
<td>710</td>
<td>41</td>
<td>2,972</td>
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<td>738</td>
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<td>2,385</td>
<td>19</td>
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</tr>
<tr>
<td>739</td>
<td>35</td>
<td>2,557</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

* Positive sperm represent the cells with DNA damage.