Fragmentation of sperm DNA using the TUNEL method


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KEYWORDS
Sperm nucleus fragmentation; TUNEL; Test validity; Sperm parameters; Swim-up; Susceptibility to damage in the laboratory

Abstract
Objectives: To establish the validity of the TUNEL assay in determining sperm DNA fragmentation, the relationship between the degree of fragmentation and the seminal parameters and the sample needed to conduct the test.
Material and methods: We used semen samples from healthy fertile men \( n = 33 \), patients who consulted for infertility with a prescription for the TUNEL assay \( n = 77 \) and patients with intracytoplasmic sperm injection failure \( n = 20 \), analyzed according to the 2010 WHO. The TUNEL/propidium iodide test was performed by flow cytometry, on baseline and post-swim-up samples.
Results: The cutoff value for the TUNEL assay (ROC curves) was 26\%, with a sensitivity and specificity of 85\% and 89\%, respectively. The pre-swim-up and post-swim-up medians of the results from the TUNEL assay showed no significant differences (17.0\% vs. 12.9\%, respectively). However, 39.1\% of the samples showed a difference greater than 15 in absolute value between the results of the baseline and post-swim-up TUNEL assays. The linear correlation study of the morphology, mobility and vitality using the post-swim-up TUNEL assay showed a greater correlation than preselection, with significant results \( r: -0.394, p < .0001; r: -0.461, p < .0001; r: -0.526, p < .0001 \).
Conclusions: The TUNEL assay is a valid test for clinical use. DNA fragmentation is a factor independent from traditional semen tests. We found a greater susceptibility to damage generated in the laboratory procedures in the samples with lower quality. The sample of choice for evaluating DNA fragmentation will depend on whether the clinician is treating a natural or assisted fertilization.

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Fragmentación del ADN espermático empleando el método de TUNEL

Resumen

**Objetivos:** Establecer la validez del test de TUNEL en la determinación de la fragmentación del ADN espermático, la relación entre el grado de fragmentación con los parámetros seminales y la muestra a emplear para efectuar la prueba.

**Materiales y métodos:** Se emplearon muestras de semen de varones sanos fértiles (n = 33), pacientes que consultaron por infertilidad con prescripción de test de TUNEL (n = 77) y pacientes con fracaso en ICSI (n = 20), analizadas según OMS 2010. Se efectuó el test de TUNEL/ioduro de propidio por citometría de flujo, en muestras basales y post swim up.

**Resultados:** El valor de corte de TUNEL (curvas ROC) fue de 26% con sensibilidad y especificidad de 85% y 89% respectivamente. Las medianas de los resultados de TUNEL pre y post swim up no mostró diferencia significativa (17.0% vs. 12.9%). Sin embargo, el 39.1% expuso una diferencia superior a 15 en valor absoluto entre los resultados del TUNEL basal y post swim up. El estudio de correlación lineal de la morfología, movilidad y vitalidad con el TUNEL post swim up mostró una correlación mayor que el pre selección, con resultados significativos (r: -0,394, p < 0,0001; r: -0,461, p < 0,0001; r: -0,526, p < 0,0001).

**Conclusiones:** La prueba de TUNEL es una prueba válida para su empleo en la clínica. La fragmentación del ADN es un factor independiente de las pruebas tradicionales del semen. Hallamos mayor susceptibilidad al daño generado en los procedimientos de laboratorio en las muestras de peor calidad. La muestra de elección para evaluar la fragmentación del ADN dependerá de si se tratará de una fertilización natural o asistida.

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## Introduction

The aim of andrology laboratory procedures is to predict the fertility potential of males, either in a natural or assisted way. So far, the 3 diagnostic foundations have been mobility, morphology and sperm count; however, the literature refers to a low predictive power of these tests, especially regarding highly complex assisted fertilization procedures. Thus, new trials emerged aimed at exploring the state of the genetic material to be transferred, the most widespread ones being those determining sperm DNA integrity. These tests can be carried out through different methodologies such as the sperm chromatin structure assay (SCSA), the sperm chromatin dispersion (SCD) test, the single-cell gel electrophoresis (COMET) assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The first two are indirect measures of the state of DNA fragmentation, whereas the latter two enable the identification of actual damage. In our experience, the TUNEL assay, both through flow cytometry or fluorescence microscopy, could be validated with appropriate analytical parameters, which allowed us to transfer it to clinical practice. In order to define a diagnostic test as appropriate it is necessary to consider its validity, which is expressed by its sensitivity, specificity and predictive values. The clinical validity of the TUNEL study has been recently questioned with regard to their capacity to predict the success of pregnancy, both through natural fertilization or instrumental procedures.

On the other hand, the literature is contradictory when the attempt is made to correlate the TUNEL assay with traditional sperm parameters. Another issue under discussion in our clinical practice is whether the TUNEL assay should be carried out on the ejaculated sample or after the procedures performed in the laboratory, such as enrichment techniques, so as to highlight the actual state of the gamete to be used in case assisted fertilization is chosen.

The aim of this study was to establish the validity of the TUNEL assay when determining sperm DNA fragmentation as a diagnosis of reproductive capacity, to determine the association between the degree of sperm DNA fragmentation and sperm parameters: mobility, morphology, sperm count and vitality, and to establish on which sample type the test should be conducted in order to obtain better information on the state of sperm DNA involved in the fertilization process.

## Materials and methods

The samples were collected by masturbation after a 3- to 5-day sexual abstinence. After liquefaction (30–60 min), basic semen analysis was performed according to WHO 2010 guidelines. A CASA system (SCA Microptic, Spain), which had been previously validated by our group, was used for sperm count and motility.

In order to establish the diagnostic validity, sperm DNA fragmentation was determined using the TUNEL/propidium iodide for flow cytometry on 40 semen samples, which corresponded to 20 healthy donors of proven fertility in the previous 12 months and between the ages of 20 and 45 years, and to 20 patients treated at the Gynecology Division, Area of Marital Infertility at Hospital de Clínicas José de San Martín, who had failed intracytoplasmic sperm injection (ICSI) with women under 35 years of age and who provided healthy oocytes.

In order to establish the material to be used in the test, so that more detailed information could be provided on the
state of the DNA involved in the fertilization process, sperm selection was performed using the swim-up technique, with Ham’s F-10 (Gibco) and serum supplement (Irvine Scientific), on 110 samples corresponding to fertile men (n = 33) and patients consulting for infertility (n = 77). The TUNEL test was assessed in the basal semen sample and in spermatozoa selected after swim-up. A relationship was found between the degree of sperm DNA fragmentation and sperm parameters, mobility, morphology, vitality and sperm count in both sample types.

In order to perform the TUNEL assay, the spermatozoa from the semen sample and those from the post-selection sample were washed with phosphate-buffered saline (PBS) pH 7.2) supplemented with 0.3% albumin and adjusted to a concentration of 20 million/ml; 100 μl of each sample was fixed with 100 μl 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Once the fixative was removed, it was washed twice with PBS-albumin, and permeated with 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate for 2 min in ice bath and was subsequently washed twice with PBS. Before incubation with the TUNEL solution, an additional sample was incubated for 20 min at 37°C with 50 μl (8 U/μl) of recombinant DNase I (Roche; 10,000 U). All the samples, including the positive control, were incubated in the dark for 1 h at 37°C, with 50 μl of a TUNEL reaction solution (In Situ Cell Death Detection Kit, Fluorescein-Roche) comprising a deoxyuridine triphosphate (dUTP) solution marked with fluorescein isothiocyanate (FITC) plus the terminal enzyme deoxyxynucleotidyl transferase (TdT) which enables the specific attachment of uracil to the 3'-OH terminal end of a DNA fragment. Samples which had only been incubated with the staining solution (fluorescein-dUTP) without enzyme aggregates were used as negative controls. Once staining was complete, each sample was washed twice with PBS to remove the non-attached solution and resuspended in a final volume of 400 μl, and 100 μl of propidium iodide was added at 0.1% in PBS to remove the events with no DNA from the analysis. The samples were read in a FACScalibur flow cytometer and 10,000 events were analyzed for each sample. The results were obtained using the Winmdi program. In the case of severe oligozoospermic samples or those with very low recovery, suspensions were concentrated by centrifugation and readings were made using fluorescence microscopy (Olympus CX31), evaluating at least 100 spermatozoa in duplicate using 2 different operators.

Participants were given a fact sheet and signed consent forms which had the support of the hospital ethics committee, with no conflict of interests in the present study.

Statistical models

The area under the receiver operating characteristic (ROC) curve was determined and the cut-off value, its sensitivity and specificity were calculated on the basis of the test results for fertile and infertile individuals. The Wilcoxon test for paired samples was used for the comparisons between pre- and post swim-up results, and the Mann-Whitney test (independent samples) was used to establish differences among the patient groups. The linear correlation between sperm parameters and the TUNEL results was estimated using the MedCalc program.
Table 1  Characterization of the semen samples in the 3 groups of males analyzed.

<table>
<thead>
<tr>
<th>Age, Median (Interquartile ranges)</th>
<th>Men with fertility in the past year (n=31)</th>
<th>Men presenting with infertility (n=77)</th>
<th>Infertile men with failure in ICSI (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, Median (Interquartile ranges)</td>
<td>28 (25.00–34.00)</td>
<td>38 (33.75–41.00)</td>
<td>40 (35.00–43.50)</td>
</tr>
<tr>
<td>Ez/ey (E 10⁶)</td>
<td>172.85 (96.955–301.680)</td>
<td>127.71 (69.145–250.390)</td>
<td>–</td>
</tr>
<tr>
<td>Ez/ml (E 10⁹)</td>
<td>59.80 (33.450–105.950)</td>
<td>52.30 (31.750–99.000)</td>
<td>17.5 (2.070–96.750)</td>
</tr>
<tr>
<td>Total mobile (%)</td>
<td>48.10 (39.100–60.900)</td>
<td>42.50 (30.000–51.900)</td>
<td>26 (17.500–42.000)</td>
</tr>
<tr>
<td>Progressive mobile (%)</td>
<td>45.00 (34.450–51.550)</td>
<td>33.10 (22.475–43.400)</td>
<td>–</td>
</tr>
<tr>
<td>Alive (%)</td>
<td>90.00 (80.000–95.000)</td>
<td>82.00 (71.500–90.000)</td>
<td>–</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>16.50 (8.000–22.500)</td>
<td>9.00b (4.250–14.750)</td>
<td>3.00c (2.000–9.500)</td>
</tr>
<tr>
<td>TUNEL (%)</td>
<td>17.35 (9.600–24.000)</td>
<td>16.00 (7.750–24.750)</td>
<td>32.00d (24.250–36.750)</td>
</tr>
<tr>
<td>Post swim up TUNEL (%)</td>
<td>7.55a (4.000–12.900)</td>
<td>15.00 (6.750–28.000)</td>
<td>–</td>
</tr>
</tbody>
</table>

Significant differences (Mann–Whitney test, independent samples).

- a p = 0.0011 versus basal TUNEL (Wilcoxon test, paired samples).
- b p = 0.0008 vs fertile.
- c p = 0.0087 vs fertile.
- d p < 0.0001 vs fertile.
- e p < 0.0001 vs fertile.
- f p < 0.0001 vs fertile.

Discussion

The TUNEL assay, through the enzymatic incorporation of a marked uracil at the 3'-OH terminal end of a DNA fragment, directly measures damage, unlike other tests used in studies on sperm chromatin. On the other hand, the use of flow cytometry enables us to assess over 10,000 events per sample, objectify the study and document the results. The test was validated for clinical practice at the laboratory, using semen samples from healthy men with natural fertility rates over the last year and from men with failure in ICSI and whose partners had no female-factor infertility. A cut-off value of 26% was established with a sensitivity and specificity of 85 and 89% respectively. These data are similar to those reported by other authors. The TUNEL results of 77 patients who had consulted for fertility at our hospital in the period 2012–2013, who had been asked for study participation, showed a range from 0.6% to 71.2%, it being so wide as that of traditional sperm parameters. These results show which percentage of spermatozoa shows DNA fragmentation in the original semen sample, but do not indicate the fragmentation degree of the spermatozoa involved in the assisted fertilization process, in case it is decided to carry it out. One of the semen preparation techniques for assisted fertilization procedures is the swim-up method, in which motile sperm is separated from the rest of the semen, that is, from the other cells, immotile necrotic sperm, and from seminal plasma with its antioxidant content. This technique involves a subsequent centrifugation in order to gather the material for insemination, which is known to possibly cause oxidative stress. When the TUNEL assay was carried out on this material we obtained again a really wide range in the results, it being from 1.0% to 89.0%. The median of the TUNEL assay in the basal and post-swim-up sample showed no statistical differences; however, not all the samples behaved in the same way. In 39% of the studied cases a difference exceeding 15 was found between both results, either in excess or defect. The increase in DNA fragmentation following the screening test in our experience was 14.5% of the samples of the men studied, and this would highlight an increased susceptibility of their spermatozoa to an in vitro procedure. This response has led to the questioning of the diagnostic value of the test conducted on the basal sample, especially in patients who resort to assisted fertilization techniques and who will inevitably undergo these procedures. Those samples in which a significant increase in DNA fragmentation was observed were confirmed with a second sample, what we cannot say with certainty is that such behavior will occur with other selection techniques or other culture media. The decrease in the TUNEL assay following selection, especially in those samples with an initial pathological value, can be attributed to samples with a high necrotic spermatozoa population which increase the percentage of spermatozoa with fragmented DNA from the basal sample, but that will not be present or involved in the fertilization process after selection. In our experience, this occurred in 13.6% of the men studied.

The correlations between the damage in DNA sperm and the traditional spermogram parameters have been the subject of studies by numerous authors. They have all reported high significance, but with varying correlation coefficients (r), probably because there is no consensus on which sample the study was conducted on, or on the methodology used to assess it. On the other hand, it should be made clear that a correlation can be statistically significant, and that does not imply clinical relevance. The fact that "r" is statistically significant means that the data are adjusted to a linear model, so that the slope of the line is different from 0, but it does not indicate how different it is from 0, even if p < 0.0001. The clinical relevance of this association is determined by the coefficient of determination ($R^2 \times 100$), which indicates which percentage of the dependent variable’s variance is explained by the independent variable. Typically, a $R^2$ value of at least 0.5 ($r > 0.7$) is considered clinically significant, since X would explain around 50% of the Y-variance. In our study, we found...
a highly significant linear correlation (p < 0.0001) between morphology, progressive mobility, sperm count and vitality and the post-sperm-selection TUNEL results, with negative correlation coefficients, similar to those reported by Borini et al.,

19 but without high clinical relevance. We can conclude that DNA fragmentation is a factor independent from traditional semen tests. Nonetheless, we can talk about a susceptibility to damage after treatment which is generated in the laboratory, such as the preparation of the sperm sample for assisted fertilization techniques, which might be partially predictable due to poor semen quality.

Finally, we conclude that the TUNEL assay is a valid technique for clinical use, that DNA fragmentation is a factor independent from traditional semen tests, and with regard to the sample of choice for assessing the state of sperm DNA we consider that the results obtained in the basal and post-swim-up samples are of interest, since they help characterize infertile patients.

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Conflict of interest

The authors declare that they have no conflict of interest.

References