Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients

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Objective: To investigate DNA fragmentation by using terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling in relation to World Health Organization parameters and computer-aided sperm analysis (CASA) in sperm to determine the possibility of obtaining a correlation among CASA parameters, sperm morphology, and DNA fragmentation.

Design: Sperm analysis according to World Health Organization parameters, terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) for sperm DNA fragmentation, and CASA for sperm movement. Prospective study.

Setting: All the patients were under clinical management, consulting for hypofertility at a fertility center in France.

Patient(s): One thousand six hundred thirty-three men who were referred for infertility investigation, including a complete sperm analysis.

Intervention(s): Sperm analysis and DNA damage testing.

Main Outcome Measure(s): Sperm morphology, DNA fragmentation, and movement characteristics.

Result(s): One third of the patients had a TUNEL rate of >30%. Analysis of the 21 semen parameters tested revealed that 7 of them were significantly correlated with the TUNEL results.

Conclusion(s): World Health Organization sperm parameters and DNA damage are complementary, rather than strongly linked. This should be considered to more fully understand the paternal contribution in assisted reproductive technologies failures. (Fertil Steril 2009;91:1801–5. ©2009 by American Society for Reproductive Medicine.)

Key Words: Sperm, WHO parameters, DNA fragmentation, TUNEL, CASA

In reproductive medicine, sperm DNA integrity now is considered to be a highly limiting factor that affects both fertilization and embryo development (1–5). Furthermore, sperm DNA fragmentation analysis is a potentially valuable tool to reveal the paternal origin of some unexplained repeated intracytoplasmic sperm injection (ICSI) failures in term of fertilization and implantation failure (6). It may even help to decide the choice of the most efficient assisted reproductive technologies procedure (7), to reduce the paternal negative contribution: DNA fragmentation can be, in part, overcome by the use of ICSI. Sperm selection for ICSI on the basis of morphological aspects and by using high magnification can be of benefit in clinical-pregnancy outcome. Bartoov et al. (8), Berkovitz et al. (9), and Hazout et al. (10) reported no clear correlation between sperm morphological aspects on the basis of strict criteria, sperm count, and chromatin condensation. A prospective analysis of semen parameters and sperm chromatin structure assay showed a nonsignificant correlation between sperm concentration and DNA fragmentation (11). Sakkas et al. (12) reported a high percentage of fragmented sperm DNA linked to a high expression of apoptosis markers such as p53, Bcl-X, and Fas in semen samples with low spermatozoa concentration and high morphological abnormalities. Huang et al. (13) reported that sperm DNA apoptosis rates were significantly higher in patients with abnormal sperm parameters than in those with normal sperm. In patients with unexplained recurrent pregnancy loss, aneuploidy, abnormal morphology, and apoptosis rate are significantly linked (14). The literature is controversial, and thus we undertook a prospective study in >1,600 couples to determine the correlations between the semen parameters, including CASA ones and the fragmentation rates as determined by terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL).

MATERIALS AND METHODS

Semen parameters and sperm DNA fragmentation were investigated in 1,633 patients in a prospective study. The work received approval from the ethics committees of the three clinics and the two laboratories involved. Patients were informed and signed a consent form. Semen analysis was performed according to World Health Organization guidelines (15). Length of abstinence, total number of spermatozoa in whole ejaculate, viability, motility, detailed morphology, and survival rate after a 24-hour incubation period were recorded.
For detection and quantification of DNA strand breaks, terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate-biotin nick-end labeling (TUNEL) was performed. A fluorescein isothiocyanate–labeled deoxyuridine triphosphate (dUTP) kit (Roche Diagnostics Corporation, Mannheim, Germany) was used according to the instructions of the manufacturer for in situ or flow cytometry technique. Briefly, after centrifugation and washing (10 min at 750 g) to remove seminal plasma, sperm were treated for 20 minutes with trypsin–ethylenediaminetetraacetic acid at 37°C, followed by 20 minutes of treatment at 37°C with 1% of trisodium citrate for hypotonic shock to facilitate a fragmented DNA TUNEL procedure, after which sperm are fixed with 3:1 methanol–ethanol solution.

Flow cytometry was used to detect TUNEL staining of sperm from patients with >1 million cells per milliliter. A Beckman Coulter (Villepinte, France) cytomter at 488 nm, with a 15-mW argon–ion laser as the light source, detected a minimum of 5,000 events. Debris was eliminated by establishing a region around the population of interest. The percentage of labeled sperm was characterized by identifying a region that included >90% of events in the frequency histogram of the positive control (26). The data were processed on a computer using LYISIS (Becton-Dickenson, Mountain View, CA) software.

For patients with <1 million sperm cells per milliliter, an in situ TUNEL technique was used, and a minimum of 200 spermatozoa were analyzed by epifluorescence microscope. Positive and negative controls were used daily. For positive control, sperm was treated with 0.2 IU of deoxyribonuclease (DNase, RNase free; Roche) for 20 minutes at room temperature, whereas the enzyme was omitted from the reaction mixture and replaced with distilled water for negative control.

To assess the efficiency and concordance of flow cytometry and in situ technique, 68 semen samples were analyzed simultaneously with both techniques.

Sperm morphology analysis was performed after Harris-Schorr coloration. Movement characteristics of sperm were assessed by CASA by using the Hamilton Thorne Integrated Visual Optical System. Global velocity was not tested, because with the advent of ICSI, this parameter is less and less required. All of the experiments were performed in a double-blinded manner.

**Statistical Analysis**

All the data and information from 1,633 patients were collected; they were ranked in four categories according to level of fragmentation, the limits being defined according to our experience. The significance and the accuracy of our limits were controlled by using the Mac Nemar test (κ = 0.94). All other statistical tests were performed by using SAS software (Windows version 8.2; SAS). Tests with a P value of <.05 were considered to have statistically significant results for the considered population. Quantitative parameters were described on the basis of patient number, average, median, quartile, and maximum and minimum.

The variance analysis was performed with the test of fixed effect and was considered for each parameter and based on numerator degrees of freedom, denominator degrees of freedom, freedom value of the test, and the probability of freedom.

**RESULTS**

The feasibility and efficiency of the TUNEL procedure using both flow cytometry and in situ techniques was demonstrated. A significant correlation was observed in the 68 patients with a high sperm count as analyzed by both techniques (Fig. 1; r = 0.94, P <.001).

For our population of 1,633 patients, 41% showed a TUNEL-positive rate of <20%, and 28%, of between 20% and 30%. The last third of the patients had a TUNEL rate of >30%. Of these patients, 216 (13%) had a rate of >40% (Table 1). Analysis of the 21 semen parameters tested here revealed that 7 parameters are significantly correlated with the TUNEL rate in the ejaculate (see the details of the different parameters in Table 2).

A correlation was found between TUNEL and the following variables:

- Patient age;
- Length of abstinence (positive correlation), P =.006;
- Total motile sperm count (inverse correlation), P <.0001;
- Rapid progression (inverse correlation), P <.0001;
- Vitality (negative correlation), P <.0001;
- Percentage of atypical forms (positive correlation), P <.0006;
- Abnormal necks (positive correlation), P =.016;
- Coiled tails (positive correlation), P <.0001.

No correlation was observed between the TUNEL rate and the following variables:

- Number of motile and atypical spermatozoa per ejaculate;
- Progressive motility after 1 hour and amplitude of lateral head displacement;
- Number of spermatozoa with abnormal basal piece or circular flagella;
- Semen volume and sperm concentration;
- Semen pH;
- Morphology of head, acrosome, and intermediate piece;
- Variation of sperm agglutination factors;
- Presence of leukocytes and polynuclear cells in the ejaculated sperm.

**DISCUSSION**

It is becoming more clear that the classical sperm parameters, as defined by the World Health Organization, are not informative enough for the prediction of assisted reproductive technologies outcome. Sperm DNA fragmentation and decondensation now are more and more clearly designated as obvious parameters in early or late failures of assisted
reproductive technologies. Several procedures have been designed to evaluate DNA strand breaks. Comet assay, sperm chromatin structure assay, and TUNEL results have been shown to be highly correlated (16, 17), even if it is not clear whether they detect exactly the same types of DNA decays (i.e., apoptosis, reactive oxygen species–related decays, and so on). These three techniques have demonstrated that DNA fragmentation is a highly limiting factor for pregnancy, especially for intrauterine insemination and IVF (2–4, 17).

Results from DNA fragmentation analysis can help direct toward the best assisted reproductive technique to use. There has been some controversy (5, 7), but when DNA fragmentation is elevated, the use of artificial insemination is useless but ICSI appears to be the best choice, avoiding useless delays (7). This can be explained in two ways: first, that during normal fertilization, capacitation (occurring as well in vitro and in vivo) is associated with a burst of reactive oxygen species, potentially increasing the sperm DNA decay. The second explanation is that ICSI allows more rapid access to the DNA repair processing of the oocyte. On the contrary, cumulus oophorus may have a positive function for selecting the best spermatozoon (18). Nevertheless, the consensus threshold appears to be more or less 30%. We have demonstrated here that there is a good correlation for TUNEL between the in situ technique and TUNEL flow cytometry: TUNEL technology can be used with the flow cytometry technique for patients having >1 million sperm cells per milliliter and can be used with the in situ technique for patients with <1 million sperm cells per milliliter in the ejaculate. Sperm concentration usually is a problem when using the sperm chromatin structure assay, for which a minimum concentration is required. In our population consulting for infertility, the critical threshold of 30% sperm DNA fragmentation was reached by one third of the patients.

Sperm DNA fragmentation index appears to be a more stable criterion than do classical semen parameters (19). It generally is admitted that the relation between fragmentation and World Health Organization sperm parameters is weak (1). However, a clear difference in molecular markers of apoptosis was shown between males with normal and abnormal sperm parameters (20). In a small cohort of patients consulting for infertility investigation, a prospective analysis showed a significant inverse correlation between sperm concentration and DNA fragmentation (11, 21). In these studies, both progressive motility and normal morphology also were inversely correlated to the rate of DNA damage. The use of DNA diffusion assay for apoptosis analysis (21) determined inverse associations between the apoptosis rate and sperm motility, progressive motility, and morphology (strict criteria) (22). All these observations, using different techniques for DNA damage analysis, are in concordance with our observations. Concerning the vitality and necrospermia, a very strong correlation between necrospermia and sperm chromatin structure assay has been described (23). This also is in agreement with our inverse correlation between TUNEL and vitality (P < .0001). In agreement with data from sperm chromatin structure assays or TUNEL, our results demonstrate that abstinence affects the TUNEL-positive rate (24–26).

Our data do not show any correlation between the presence of leukocytes and DNA damage, in concordance with the results of Ricci et al. (27) but not with those of Erenpreiss et al. (28), who reported a negative effect of leukocytes that was supposed to provoke potential DNA damage in a cascade-like manner, particularly in sperm with poor morphology and motility. However, no highly elevated level of leukocytes was present in our patients. A correlation between degraded morphology and the presence of leukocytes and associated increased reactive oxygen species has been described elsewhere (29, 30).

Injection of highly abnormal sperm DNA is a matter of concern in severe male infertility (31), especially regarding transmission to the next generations of mutations that lead to carcinogenesis, when DNA repair capacity of the oocyte is bypassed. New tools for sperm selection on the basis of sperm head morphology recently have been introduced in routine ICSI programs. Selection of morphologically normal sperm using high magnification before injection leads to

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**TABLE 1**

Distribution of sperm DNA fragmentation in 1,633 ejaculates.

<table>
<thead>
<tr>
<th>% Fragmentation</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–20</td>
<td>668 (40.9)</td>
</tr>
<tr>
<td>20–30</td>
<td>463 (28.4)</td>
</tr>
<tr>
<td>30–40</td>
<td>286 (17.5)</td>
</tr>
<tr>
<td>≥40</td>
<td>216 (13.2)</td>
</tr>
</tbody>
</table>

a significant improvement in pregnancy outcome (32). As a result of intracytoplasmic injection of morphologically selected sperm, the injection of spermatozoa with better nuclear morphology improved clinical outcomes in couples who had previous repeated conventional ICSI failures (8, 10, 32). The so-called improvement, which remains to be confirmed, probably is related in part to the relation between some parameters of morphology and DNA fragmentation. This aspect probably is redundant with our observation concerning the relation between atypical forms and fragmentation.

Three parameters more or less related to the midpiece, that is, broken necks, abnormal necks, and curled tails, show a positive correlation with the DNA fragmentation. This observation may be related to degradation of mitochondrial structure, including mitochondrial DNA.

Therapy with antioxidant vitamins (A, C, and E associated with selenium) has been proposed to reduce sperm DNA fragmentation, but a careful analysis of the literature shows wide discrepancies (33). This may be linked to the other aspect of DNA decay, that is, sperm decondensation (34), which obviously leads to chromosomal abnormalities in the embryos.

This aspect should not be overlooked in assisted reproductive technologies.

In conclusion, paternal factors are multifactorial in early embryogenesis. These factors generally are poorly linked to each other. Our data confirmed that in the case of sperm of good quality, as defined by the World Health Organization guidelines and CASA parameters, a careful analysis of chromatin structure and DNA decays is mandatory. It is becoming clear, with the use of new tools such as proteomic analysis (35), that good oocyte quality is not necessarily able to compensate for poor sperm DNA and protamine status (36).

### REFERENCES


### TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(0–20%)</th>
<th>(20%–30%)</th>
<th>(30%–40%)</th>
<th>≥40%</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>668</td>
<td>463</td>
<td>286</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>36.6 ± 6.1</td>
<td>37.1 ± 6.4</td>
<td>38.2 ± 6.8</td>
<td>39.3 ± 9.6</td>
<td>.001</td>
</tr>
<tr>
<td>Abstinence (d)</td>
<td>3.5 ± 1.37</td>
<td>3.8 ± 2.33</td>
<td>3.8 ± 1.91</td>
<td>3.9 ± 1.89</td>
<td>.006a</td>
</tr>
<tr>
<td>Total sperm count</td>
<td>114.07 ± 128.187</td>
<td>117.73 ± 126.967</td>
<td>117.78 ± 112.574</td>
<td>105.97 ± 92.220</td>
<td>NS</td>
</tr>
<tr>
<td>Total motile sperm count</td>
<td>32.69 ± 49.69</td>
<td>30.06 ± 52.1</td>
<td>23.73 ± 40.42</td>
<td>16.66 ± 28.59</td>
<td>.&lt;.0001a</td>
</tr>
<tr>
<td>Rapid progression (%)</td>
<td>32.2 ± 20.70</td>
<td>28.6 ± 18.48</td>
<td>25.3 ± 16.82</td>
<td>21.3 ± 16.92</td>
<td>&lt;.0001a</td>
</tr>
<tr>
<td>Velocity (ALH), µm/s</td>
<td>3.16 ± 0.607</td>
<td>3.12 ± 0.568</td>
<td>3.07 ± 0.587</td>
<td>3.01 ± 0.692</td>
<td>NS</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>74.3 ± 13.09</td>
<td>71.5 ± 12.54</td>
<td>68.9 ± 12.86</td>
<td>62.3 ± 14.82</td>
<td>&lt;.0001a</td>
</tr>
<tr>
<td>Atypical forms (%)</td>
<td>72.8 ± 14.91</td>
<td>74.0 ± 14.47</td>
<td>76.5 ± 14.50</td>
<td>76.3 ± 13.91</td>
<td>.0006a</td>
</tr>
<tr>
<td>Elongated head</td>
<td>6.3 ± 9.59</td>
<td>7.2 ± 10.99</td>
<td>6.1 ± 8.25</td>
<td>6.9 ± 10.82</td>
<td>NS</td>
</tr>
<tr>
<td>Microcephalic</td>
<td>4.0 ± 6.24</td>
<td>3.9 ± 6.30</td>
<td>3.2 ± 5.12</td>
<td>4.1 ± 6.49</td>
<td>NS</td>
</tr>
<tr>
<td>Macrocephalic</td>
<td>2.3 ± 5.02</td>
<td>2.5 ± 3.32</td>
<td>2.2 ± 2.68</td>
<td>2.2 ± 2.78</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple heads</td>
<td>0.9 ± 1.55</td>
<td>0.9 ± 1.64</td>
<td>1.0 ± 1.85</td>
<td>0.9 ± 1.86</td>
<td>NS</td>
</tr>
<tr>
<td>Abnormal acrosome</td>
<td>47.0 ± 19.11</td>
<td>47.4 ± 19.65</td>
<td>48.3 ± 19.28</td>
<td>47.0 ± 20.16</td>
<td>NS</td>
</tr>
<tr>
<td>Abnormal neck (base)</td>
<td>19.0 ± 12.43</td>
<td>20.8 ± 13.91</td>
<td>21.7 ± 14.69</td>
<td>19.3 ± 13.13</td>
<td>.0157</td>
</tr>
<tr>
<td>Cytoplasmic droplets</td>
<td>1.2 ± 2.52</td>
<td>1.3 ± 2.74</td>
<td>1.4 ± 2.98</td>
<td>1.1 ± 2.67</td>
<td>NS</td>
</tr>
<tr>
<td>Broken neck (angulation)</td>
<td>20.4 ± 9.51</td>
<td>19.8 ± 9.51</td>
<td>20.4 ± 9.22</td>
<td>19.1 ± 9.34</td>
<td>NS</td>
</tr>
<tr>
<td>Curled tail</td>
<td>11.3 ± 8.47</td>
<td>12.3 ± 8.82</td>
<td>14.2 ± 10.00</td>
<td>18.5 ± 13.16</td>
<td>&lt;.0001a</td>
</tr>
<tr>
<td>Short tail</td>
<td>7.1 ± 8.61</td>
<td>6.4 ± 8.18</td>
<td>7.2 ± 9.94</td>
<td>6.8 ± 8.68</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple tails</td>
<td>1.1 ± 2.23</td>
<td>1.0 ± 1.61</td>
<td>1.0 ± 1.66</td>
<td>0.9 ± 1.52</td>
<td>NS</td>
</tr>
<tr>
<td>Index of Multiple</td>
<td>1.9 ± 0.39</td>
<td>1.9 ± 0.31</td>
<td>1.9 ± 0.30</td>
<td>1.8 ± 0.34</td>
<td>NS</td>
</tr>
<tr>
<td>Abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>0.4 ± 1.01</td>
<td>0.3 ± 0.57</td>
<td>0.3 ± 0.45</td>
<td>0.4 ± 0.78</td>
<td>NS</td>
</tr>
<tr>
<td>Polynuclear</td>
<td>3.1 ± 9.13</td>
<td>2.5 ± 8.25</td>
<td>2.1 ± 4.68</td>
<td>2.0 ± 4.25</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: ALH = amplitude of lateral head displacement; NS = not statistically significant.

*a Statistically significant.


