Evaluation of DNA fragmentation in teratozoospermic infertile men compared with normozoospermic fertile men and its correlation with sperm morphology

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Purpose
This study aimed to examine the relationship between sperm morphology and sperm DNA fragmentation in infertile teratozoospermic men and fertile normozoospermic men.

Materials and methods
Semen samples were collected from 69 patients classified as infertile teratozoospermic men and 65 healthy fertile normozoospermic men (control). Semen parameters were assessed using WHO 2010 guidelines. Spermatozoa DNA integrity was assessed using the method of the sperm chromatin dispersion test.

Results
Sperm DNA fragmentation was statistically higher in infertile teratozoospermic men compared with fertile men ($P=0.001$) and the percentage of fragmented sperm had significant positive correlations with morphologically abnormal sperm ($P=0.001$).

Conclusion
On the basis of these findings, it is important to assess sperm DNA damage in the diagnosis of male infertility. Our data also showed a negative correlation between the degree of DNA fragmentation and the morphology of sperm in semen samples.

Keywords:
DNA fragmentation, male infertility diagnosis, sperm morphology, teratozoospermia

Introduction
Infertility is a reproductive health problem that affects approximately 15% of couples in the human population [1–3]. Male infertility solely accounts for 20–25% of infertile couples and is contributory in another 30% [4–6]. A conventional semen analysis test remains the most widely used predictor for male infertility diagnosis [7]. Routine semen analysis is mostly based on the descriptive microscopic evaluation of ejaculate sperm, including concentration, motility, and morphology. However, routine semen analysis is not the only way to discriminate effectively between the sperm of fertile and infertile men [8,9] because it does not reflect the integrity of the male genome [10,11]. Therefore, assessment of DNA integrity has been suggested as an independent additional marker of fertility [12–14] to provide the most conclusive information on sperm DNA.

The extraordinarily high percentage of polyunsaturated fatty acids of sperm plasma membrane makes them highly sensitive to oxidative processes [15,16], which leads to a destructive effect on sperm nucleus DNA [17,18]. When male damaged DNA is incorporated into the embryonic genome, it may disturb the biochemical events that occur during embryogenesis, such as DNA replication, transcription, and translation [19,20]. Clinically, sperm with high DNA fragmentation negatively affects both natural [21,22] and assisted pregnancy outcomes [23,24]. Many reports have shown that the increase in DNA fragmentation has a negative effect on fertilization rate [25,26], embryo development [27], clinical pregnancy rates [28,29], and embryo postimplantation development [30,31] in both in-vitro fertilization and ICSI. Therefore, the integrity of sperm DNA has been recognized as a useful contributing factor in differentiating between fertile and infertile men and in predicting ART outcomes [19]; thus, it should be incorporated into routine diagnostic investigations of infertile men before ART treatment.

Assessments of sperm DNA fragmentation are currently carried out using many different methods, including the sperm chromatin structure assay [32,33], the sperm chromatin dispersion (SCD) test [34,35], the DNA breakage detection-fluorescence in-situ hybridization test [36], the single-cell pulsed-field gel electrophoresis [37], the comet assay [38], and the TUNEL assay [39]. With the SCD test used in our study, DNA damage is quantified by treating sperm with an acid solution before lysis buffer; the sperm nuclei with fragmented DNA will fail to produce the characteristic DNA dispersion halos after the removal of nuclear proteins [40]. The SCD test is a powerful approach for sperm DNA fragmentation screening, enabling simple, fast, accurate, inexpensive, and highly reproducible procedures. However, the SCD test is a recently
developed technique; thus, little is known about its limitations and its clinical significance [41].

The present study aimed to investigate the correlation between the sperm morphology and DNA fragmentation in ejaculated semen using the modified SCD test.

**Materials and methods**

**Patients**

The present study was carried out from September 2012 to June 2014 at a specialized fertility and gynecology center. This study was carried out upon approval of the Institutional Review Board (IRB) and all patients involved provided written consent.

A total of 134 men were included in the present study. Men were divided into two groups. Group A included 65 men with proven fertility with normal semen parameters and group B included 69 infertile teratozoospermic men according to the WHO 2010 criteria [42] of semen analysis. All infertile men included in this study had engaged in regular unprotected intercourse for at least 1 year, with no pregnancy achieved. All fertile men included proven to be fertile were able to conceive and bear children through normal sexual activity a year ago.

**Semen analysis**

Semen samples were produced by masturbation into sterile cups following 2–4 days of sexual abstinence and after 20 min, when liquefaction was complete, conventional semen analysis was carried out according to the recommendations of WHO 2010 [42].

**Measurement of leukocytes**

To differentiate white blood cells from other round cells, a drop of 10 μl of semen was mixed with 10 μl of working solution of leukocytes stain (LeucoScreen; Ferti Pro M.V., Aalter, Belgium). The slide was examined at a magnification of ×400. Yellow-stained or brown-stained cells were counted as peroxidase-positive cells.

**DNA fragmentation analysis**

DNA fragmentation was measured using the method of the SCD test using the Halosperm Kit (HalotechDNA, Spain) according to the manufacturer’s instructions. Raw or diluted semen samples were adjusted to a concentration ranging between 5 and 20 million/ml. Then, 25 μl of the sperm sample was transferred to the 50 μl of melted agarose tube at 37°C. A drop of 8 μl of the cell suspension was placed onto a slide and then covered with a coverslip. After solidification, the coverslip was removed gently and was applied to solution 1 for 7 min. Then, solution 2 was applied for 20 min, followed by washing the slide for 5 min with abundant distilled water. Finally, dehydration was induced by flooding with 70% ethanol for 2 min and then 100% ethanol for another 2 min. After drying, staining was performed by applying solution 3 for 6 min, followed by solution 4 for 7 min. The ratio of sperm without halo or sperm with small halo yields the percentage of DNA fragmentation.

**Statistical analysis**

Data were analyzed using the computer program SPSS (Statistical Package for Social Science) version 16. In the statistical comparison between the different groups, the significance of difference was tested using the Student t-test.

A P value of less than 0.05 was considered statistically significant (S), whereas a P value greater than 0.05 was considered statistically insignificant. A P value of less than 0.01 was considered highly significant (HS) in all analyses.

**Results**

The summary statistics of all analyzed parameters are compiled in Table 1. The age of the men in groups A and B was not statistically different (age = 33.92 ± 8.09 and 32.7 ± 7.37 years, respectively, P = 0.36). Also, the mean BMI of fertile men was not statistically different from infertile men (BMI = 24.46 ± 0.61 and 24.62 ± 0.63 kg/m², respectively, P = 0.145). There was no statistically significant difference in semen volume or pus cells’ concentration between patients of both groups.

The percentage of abnormal forms was statistically significantly different in group A and group B (A vs. B = 95.54 ± 3.1 vs. 99.02 ± 1.32%; P = 0.001) and the percentage of damaged DNA sperm was statistically higher in teratozoospermic infertile men of group B compared with fertile men of group A (A vs. B = 10.55 ± 4.85 vs. 30.53 ± 6.62%; P = 0.001). Also, as shown in Table 1, sperm concentration, percentage of sperm total motility, and progressive motility were not statistically different between group A and group B.

Table 2 summarizes the results of a correlation analysis between sperm morphology and sperm DNA fragmentation in both fertile and teratozoospermic infertile men. A highly significant positive correlation (P = 0.001) was observed between sperm DNA fragmentation and abnormal sperm morphology.

**Discussion**

Several studies have been suggested the percentage of sperm DNA fragmentation as a complementary parameter to the other routine semen analysis parameters in predicting sperm quality [43–45]. In our study, sperm DNA damage was evaluated using the SCD test, performed on some of the semen samples of 180 patients after conventional semen analysis according to WHO guidelines 2010 [42].

In the present study, our finding showed a positive relationship between morphologically abnormal and DNA damage using the SCD test, which reflects the quality of the processes controlling the differentiation and maturation of the spermatozoa. Extensive data exist on the relationship between sperm DNA damage and sperm morphology; all of these studies have shown a significant negative association between sperm morphology and sperm DNA fragmentation [43–48]. Our data are...
The existence of high levels of DNA fragmentation is associated with male infertility. Thus, DNA fragmenta-
tion may be considered a complementary parameter to predict sperm function and diagnose the cause of spermatozoal dysfunction. Our data also conclusively showed a negative correlation between degree of DNA fragmentation and morphology of sperm in native semen samples.

Acknowledgements
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Conflicts of interest
None declared.

References

Table 1 Data for semen parameters in two groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A (n = 65) (mean ± SD)</th>
<th>Group B (n = 69) (mean ± SD)</th>
<th>t-Test</th>
<th>P value</th>
</tr>
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<tr>
<td>Age</td>
<td>33.92 ± 8.09</td>
<td>32.7 ± 7.39</td>
<td>0.918</td>
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<tr>
<td>BMI</td>
<td>24.46 ± 0.61</td>
<td>24.62 ± 0.83</td>
<td>1.47</td>
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<td>Volume (ml)</td>
<td>2.63 ± 0.96</td>
<td>2.99 ± 1.43</td>
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<td>Concentration (x 10^9/ml)</td>
<td>46.68 ± 18.12</td>
<td>44.89 ± 18.53</td>
<td>0.565</td>
<td>0.573</td>
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<tr>
<td>Total motility (%)</td>
<td>61.54 ± 4.14</td>
<td>59.61 ± 8.92</td>
<td>1.59</td>
<td>0.114</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>36.09 ± 3.96</td>
<td>34.96 ± 3.47</td>
<td>1.77</td>
<td>0.079</td>
</tr>
<tr>
<td>Abnormal forms (%)</td>
<td>95.54 ± 5.1</td>
<td>99.02 ± 1.3</td>
<td>8.55</td>
<td>0.001**</td>
</tr>
<tr>
<td>Pus cells (x 10^5)</td>
<td>0.2 ± 0.36</td>
<td>0.3 ± 0.55</td>
<td>1.18</td>
<td>0.246</td>
</tr>
<tr>
<td>DNA fragmentation (%)</td>
<td>10.55 ± 4.85</td>
<td>30.53 ± 6.62</td>
<td>19.84</td>
<td>0.001**</td>
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Table 2 Correlation analysis between sperm morphology and sperm DNA fragmentation

<table>
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<th>Correlation coefficient (r)</th>
<th>P value</th>
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<td>Abnormal sperm morphology</td>
<td>0.568</td>
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The study by Sun et al [25] reported a significant inverse correlation between the presence of DNA breaks and the other basic semen parameters including sperm concentration, motility, and morphology.

Morphologically abnormal sperm cannot penetrate the oocyte successfully and are considered the best predictor in ICSI outcome. Many reports have shown that abnormal sperm negatively affect fertilization rates and clinical outcomes in both in-vitro fertilization [50,51] and ICSI. However, normal DNA fragmentation level is required for successful fertilization, embryo development, and improved clinical ICSI outcomes. Thus, it is important to select morphologically normal sperm by an operator (ICSI) to significantly reduce the risk of injecting sperm with fragmented DNA into the oocyte.

The results of the present study also point to significantly lower DNA damage in fertile patients than in infertile patients. This result is corroborated by data from other studies [43,52,53,54] that proved higher DNA fragmentation in infertile men compared with fertile men. Irving et al. concluded that male infertility is strongly correlated with the existence of high levels of DNA fragmentation in their ejaculated spermatozoa [55] using the comet assay. Data collected by Sheikh et al [43] found significantly higher DNA damage, higher morphologically abnormal forms, and lower motility in infertile men compared with fertile men. Because of the evidence of correlations between sperm DNA fragmentation and infertility, sperm DNA fragmentation should be considered during the assessment of semen quality. Also, high levels of sperm DNA fragmentation are correlated positively with lower pregnancy rates and higher pregnancy loss rates after ICSI [52]. Thus, use of a sperm DNA fragmentation test can be effective in cases of male infertility before considering ART procedures.
18 Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal 2011; 14:367–381.


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<td>References [54, 55] are cited in the text; however they are not included in the reference list. Please provide complete publication details so that it can be included in the reference list.</td>
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