Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study

Ramadan A. Saleh, M.D., Ashok Agarwal, Ph.D., David R. Nelson, M.S., Essam A. Nada, M.D., Mohammed H. El-Tonsy, M.D., Juan G. Alvarez, M.D., Anthony J. Thomas, Jr., M.D., and Rakesh K. Sharma, Ph.D.

Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio

Objective: To evaluate levels of sperm nuclear DNA damage in infertile men with normal and abnormal standard semen parameters.

Design: Prospective study.

Setting: Male infertility clinic.

Patient(s): Ninety-two men seeking infertility treatment and 16 fertile volunteers.

Intervention(s): Standard semen analysis was performed according to the World Health Organization guidelines.

Main Outcome Measure(s): Sperm DNA damage was assessed by sperm chromatin structure assay and the results expressed as %DFI.

Result(s): Of the 92 patients, 21 (23%) had normal standard sperm parameters (concentration, motility, and normal sperm forms), while 71 (77%) had an abnormality in one or more of these parameters. The %DFI [median (25th and 75th percentiles)] in infertile men with normal sperm parameters [23 (15, 32)] was significantly higher than fertile donors [15 (11, 20)] (P=.02), but not significantly different from infertile men with abnormal sperm parameters [28 (18, 41)] (P=.27).

Conclusion(s): The results of this study indicate that a significant increase in SCSA-defined DNA damage can be found in sperm from infertile men with normal standard sperm parameters. Therefore, sperm DNA damage analysis may reveal a hidden abnormality of sperm DNA in infertile men classified as idiopathic based on apparently normal standard sperm parameters. (Fertil Steril 2002;78:313–8. ©2002 by American Society for Reproductive Medicine.)

Key Words: Male infertility, nuclear DNA, sperm

Male-factor infertility plays a role in approximately 50% of infertile couples (1). The pathophysiology of male infertility is still poorly understood, however, and various diagnostic tests are unable to determine the underlying cause of sperm dysfunction (2). Until the causes of male infertility are better understood, it is unlikely that any given descriptive test of sperm quality or sperm function will predict with absolute certainty that a man will be fertile or infertile in a given time period (3).

Standard semen analysis using a light microscope has been widely used in most laboratories for initial evaluation of male fertility potential (4); however, diagnosing defective sperm function by standard semen analysis is difficult because the spermatozoon is a highly specialized cell that expresses a diverse array of biological properties to achieve fertilization (5). In addition, results of standard semen analyses can be very subjective and prone to intra- and inter-observer variability (6).

An individual’s semen quality can vary widely due to factors such as days of abstinence from ejaculation, febrile illness, stress, and even problems with sample collection. The female partner’s relative fertility is also another confounding factor, which can, of course, vary tremendously (7). Despite its weaknesses as a diagnostic tool, standard semen analysis allows for the detection of absolute causes of infertility such as azoospermia. In addition, with re-
Semen Samples
Following approval of the study by Cleveland Clinic Foundation’s Institutional Review Board, semen samples were obtained from infertile men (n = 92) and from fertile donors (n = 16) during March 2000 through March 2001. All samples were collected by masturbation, after a period of 48 to 72 hours of sexual abstinence.

Standard Semen Analysis
Following liquefaction, semen specimens were evaluated for semen volume, appearance, pH, and viscosity. Manual semen analysis was performed according to World Health Organization (WHO) guidelines (1) to determine sperm concentration and motility. Five microliter aliquot of liquefied semen was loaded on a Microcell counting chamber (Concept Technologies, San Diego, CA) and examined under 200× magnification. Sperm concentration was expressed as ×10^6/mL semen, while motility was expressed as a percentage. Smears of the raw semen were stained using the Diff-Quik kit (Allegiance Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology using the WHO classification (1). Immediately after staining, the smears were rinsed in distilled water, air-dried, and scored. In this study, normal values were: sperm concentration ≥20×10^6/mL semen, motility ≥50% and normal sperm forms ≥30% (1).

Sperm Chromatin Structure Assay (SCSA)
Assessment of SCSA-defined sperm nuclear DNA damage was carried out as previously described (17). Sperm chromatin structure assay measures the susceptibility of DNA to acid-induced denaturation in situ. Semen samples, stored at −196°C, were thawed in a 37°C water bath and immediately diluted with a buffer (0.15 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4) to obtain a sperm concentration of 1 to 2×10^6 sperm cells/mL. The sample was sonicated for 30 seconds with a Branson Sonifier 450 (VWR, Scientific Products, West Chester, PA) operating at a power setting of 3 and utilizing 70% of 1-second pulses. Under these conditions, more than 95% of the sperm tails were separated from their heads. A 200-μL aliquot was removed and mixed with 400 μL of a low pH-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.01% Triton X-100, pH 1.2).

After 30 seconds, spermatozoa were stained by adding 1.20 mL acridine orange (AO) staining solution containing 6μg/mL of AO (chromatographically purified; Cat. No. 04539, Polysciences, Warrington, PA) per mL of buffer (0.037 M citric acid, 0.126 M Na₂HPO₄, 0.0011 mM EDTA [disodium], 0.15 M NaCl, pH 6.0). Immediately after staining, the sample was placed into a Ortho Cytofluorograf 30 flow cytometer (Ortho Diagnostic Inc., Westwood, MA) sample chamber, and the sample was run for 2.5 minutes to allow for hydrodynamic and stain equilibrium before data were collected on approximately 7,000 cells per sample. The flow cytometer was equipped with a Lexel 100 mW argon
ion laser operated at 35 mW and interfaced to a Cicero data-handling unit with PC-based Cyclops Software (Cyto- 
mation, Fort Collins, CO). Green (515 nm to 530 nm) and 
red (>630 nm) fluorescence, corresponding to amounts of 
native DNA and denatured DNA, respectively, were col-
lected for each sperm that was measured at a rate of approx-
imately 250 cells/sec. Computer-generated means and stan-
dard deviations (SD) of green and red fluorescence values, 
derived from a population of approximately 7,000 cells, 
were analyzed.

Alpha t (αt), the basis of all SCSA parameters, was 
calculated as the ratio of the red fluorescence to the total of 
red and green fluorescence of an individual sperm cell (αt = 
red/total [red + green] fluorescence). The parameter Xαt 
represents the mean population of αt, while SDαt represents 
the variability of chromatin structure abnormalities within 
the sperm population. DNA fragmentation index (%DFI) 
represents the percentage of cells outside the main popula-
tion of αt, which represents the population of cells with 
DNA damage. Normal, native chromatin structure remains 
structurally intact and produces a narrow αt distribution. Spermatozoa with abnormal chromatin structure are suscep-
tible to acid-induced DNA denaturation, and have a higher 
%DFI, Xαt, and SDαt. The SCSA measurements were per-
formed twice for each sample, and the results were averaged.

Statistical Analysis
Pairwise comparisons of donors, infertile men with nor-
mal semen parameters, and infertile men with at least one 
abnormal semen parameter were performed using Wilcox-
on’s rank sum tests for continuous variables. The sample size 
of infertility patients provided a 90% power to detect a 10% 
difference in %DFI between normal and abnormal semen 
parameter patients. Comparisons of percentages between 
groups were performed with chi-squared or Fisher’s exact 
tests, as appropriate. Spearman correlation coefficients were 
used to evaluate relationships between continuous variables. 
All tests were two-tailed with a significance level of P<.05. 
Summary statistics were presented as median and interquar-
tile values (25th and 75th percentiles). Calculations were 
performed with SAS version 8.2 software (SAS Institute 
Inc., Cary, NC).

RESULTS
Among the fertile donor group, the highest observed 
%DFI was 24%; this cut-off value was used to classify 
inertile men into high (>24%), and low (≤24%) DNA 
damage groups. The distribution of 16 fertile donors and 92 
inertile men, according to the results of standard semen 
analyses (1) and SCSA-defined sperm DNA damage 
(%DFI), is illustrated in Figure 1. Of the 16 fertile donors, 
only 1 (6%) had abnormal sperm concentration (10×106/ 
ML) and motility (≤50%), while in the infertile group, 71 of 92 
patients (77%) had abnormalities in one or more of the 
standard sperm parameters. The remaining 21 of 92 patients 
(23%) were normal according to the WHO standards; how-
ever, levels of sperm DNA damage (%DFI) higher than 24% 
were observed in 9 of 21 (43%) infertile men with normal 
standard sperm parameters compared with 44 of 71 (62%)
infertile men with abnormal sperm parameters—a difference that was not statistically significant ($P=.12$). The distribution of %DFI in fertile donors and in infertile men with normal as well as abnormal standard semen parameters is illustrated in Figure 2.

A comparison of standard semen parameters and SCSA parameters in fertile donors and in infertile men with normal and abnormal standard semen parameters is shown in Table 1. The only significant difference between fertile donors and infertile men with normal standard semen parameters was observed in levels of DNA damage (%DFI) ($P=.02$). On the other hand, no significant difference was observed between infertile men with normal and abnormal semen parameters in %DFI ($P=.27$). Levels of $X_{at}$ and $SD_{at}$ in infertile men with abnormal standard semen parameters, however, were significantly higher as compared with the levels in infertile men with normal standard semen parameters ($P=.02$ and $<.0001$, respectively), as well as in the fertile donor ($P=.0003$ and $<.001$, respectively). The strong positive correlation of the SCSA parameters $X_{at}$ and $SD_{at}$ with the extent of sperm DNA damage, as expressed by %DFI ($r = .97$, $P<.0001$ and $r = .79$, $P<.0001$, respectively), indicates that the severity of DNA damage in sperm from infertile men with abnormal semen parameters was even greater.

The median age as well as the 25th and 75th percentile values of infertile men with abnormal semen parameters [33 (31, 36), respectively] were not significantly different from infertile men with normal semen parameters [33 (30, 36)] ($P=.72$) or fertile men [32 (29, 34)] ($P=.76$). The duration of infertility, in years, in the group with abnormal semen parameters [2 (1, 4)] was significantly longer than in the group with normal semen parameters [1 (1, 2)] ($P=.02$).

**DISCUSSION**

The molecular basis of many forms of male infertility is still poorly defined (18). Research on the integrity of the sperm nuclear DNA has recently been the subject of intense study. Since the initial reports of pregnancies using intracytoplasmic sperm injection (ICSI), tremendous progress has occurred in the treatment of male factor infertility, apparently regardless of the sperm defect (19–20). Because a spermatozoon is injected directly into the cytoplasm of the mature oocyte during ICSI, classical sperm parameters or
sperm–oocyte interactions are no longer relevant, which places increased emphasis on the quality of sperm chromatin. It is unclear if assisted reproductive techniques (ARTs), including ICSI, are effective in compensating for poor chromatin packaging and/or DNA damage, or if suboptimal chromatin integrity is responsible for poor implantation rate (>20%) in the majority of ART patients (21).

The results from this study indicate that levels of SCSA-defined DNA damage in sperm from the infertile men with abnormal standard sperm parameters (i.e., concentration, motility, and normal forms) were significantly higher than the levels of the fertile men. This observation is in agreement with the reports of several recent studies, which indicated a negative correlation between the percentage of spermatozoa with DNA damage and the standard sperm parameters (14–16). In view of the association between poor semen quality with increased sperm DNA damage, it is probable that spermatozoa selected for ARTs may originate from samples with high percentages of sperm with damaged DNA (22). A recent study by Zini et al. (23) indicated that improvement in sperm motility following semen processing by density gradient technique is not associated with a similar improvement in sperm DNA integrity. As a result, there is a substantial risk that spermatozoa carrying damaged DNA are being used for ART (24).

Because ICSI is the technique used primarily for the treatment of infertile men with very poor sperm quality, a major concern would be the use of DNA-damaged spermatozoa to fertilize the oocyte, which may have adverse consequences such as fertilization failure, early embryo death, spontaneous abortion, childhood cancer, and infertility in the offspring (15, 25–27). Lopes et al. (14) have shown that men with sperm DNA damage of more than 25% are more likely to experience a fertilization rate less than 20% after ICSI; however, other studies have demonstrated that spermatozoa with significantly damaged DNA still retain a residual capacity for fertilization following ICSI (24, 28).

Interestingly, the results of this study indicate that levels of sperm DNA damage (%DFI) in infertile men with normal standard sperm parameters were significantly higher compared with the levels in the fertile group and were not significantly different from the levels in infertile men with abnormal standard sperm parameters. Levels of Xtot and SDtot, however, were significantly higher in infertile men with abnormal semen parameters than in infertile men with normal semen parameters, which suggests that the severity of DNA damage in the first group was even worse. These findings have important diagnostic and prognostic value in the management of male infertility. Increased DNA damage may be, at least in part, responsible for the low fertility in men who otherwise have normal standard semen parameters on repeated analyses and, as a result, are diagnosed as unexplained or idiopathic.

The mechanism(s) underlying increased nuclear DNA damage in sperm from normozoospermic infertile men is unclear. Under normal circumstances, the sperm undergo a complex process of nuclear remodeling during spermiogenesis, which is characterized by very tight packaging and condensation of sperm chromatin (29). A potential explanation for sperm DNA damage in normozoospermic men could be an inherent defect of sperm chromatin packaging, which is characteristic of this particular group of patients and is not associated with defects in other stages of sperm development or maturation. Another potential explanation is that DNA damage occurs after spermiation and, therefore, will not be related to sperm maturation during the process of spermio-

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fertile donors (n = 16)</th>
<th>Infertile men with normal semen parameters (n = 21)</th>
<th>Infertile men with abnormal semen parameters (n = 71)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (×10^6/mL)</td>
<td>71 (35, 120)</td>
<td>58 (47, 74)</td>
<td>23 (13, 47)</td>
<td>.68</td>
<td>.001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>65 (58, 77)</td>
<td>62 (54, 70)</td>
<td>42 (30, 52)</td>
<td>.39</td>
<td>.0002</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>40 (33, 45)</td>
<td>34 (32, 37)</td>
<td>18 (12, 25)</td>
<td>.22</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>DFI (%)</td>
<td>15 (11, 20)</td>
<td>23 (15, 32)</td>
<td>28 (18, 41)</td>
<td>.02</td>
<td>&lt;.0001</td>
<td>.27</td>
</tr>
<tr>
<td>Xtot</td>
<td>233 (213, 257)</td>
<td>260 (233, 274)</td>
<td>290 (251, 344)</td>
<td>.04</td>
<td>.0003</td>
<td>.02</td>
</tr>
<tr>
<td>SDtot</td>
<td>160 (145, 176)</td>
<td>175 (145, 193)</td>
<td>213 (174, 251)</td>
<td>.44</td>
<td>.001</td>
<td>.001</td>
</tr>
</tbody>
</table>

Note: Values are median (25th and 75th percentiles). DNA fragmentation index (%DFI) = cells outside the main population of sperm with abnormal chromatin structure; Xtot = the mean population of α; SDtot = the variability of chromatin structure abnormalities within the sperm population; A = P value of fertile donors vs. infertile men with normal semen parameters; B = P value of fertile donors vs. infertile men with abnormal semen parameters; C = P value of infertile men with normal semen parameters vs. infertile men with abnormal semen parameters. Wilcoxon’s rank sum test was used for comparison and statistical significance was assessed at P<.05.

Sperm DNA damage in infertile men

Acknowledgments: The authors thank Donald Evenson, Ph.D., and Kjersten Larson, Ph.D., Department of Chemistry and Biochemistry, South Dakota State University, Brookings, SD, for their help with the SCSA.

References


