Localization of single-stranded DNA in human sperm nuclei

Xiaoyang Zhang, M.D., Maria San Gabriel, Ph.D., Jamie Libman, M.D., Simon Phillips, M.Sc., Annick Courchesne, B.Sc., and Armand Zini, M.D.

Objective: To localize foci of single-stranded (ss) DNA in sperm nuclei of fertile and infertile men.

Design: Prospective, observational study.

Setting: University infertility clinic.

Patient(s): Semen samples from 12 consecutive asthenoteratospermic men presenting for infertility evaluation and 5 consecutive fertile men presenting for vasectomy.

Intervention(s): Semen analysis, semen processing, and immunocytochemistry using an antibody targeting ssDNA.

Main Outcome Measure(s): Sperm nuclear ssDNA immunostaining pattern in whole and processed semen samples.

Result(s): Immunocytochemistry (using ssDNA antibody) demonstrated one of two sperm nuclear staining patterns: [1] faint punctated staining, and [2] diffuse nuclear staining. Infertile men had a higher proportion of spermatozoa exhibiting diffuse ssDNA staining than did fertile men (52 ± 19 vs. 14 ± 13, respectively). The proportion of spermatozoa exhibiting diffuse ssDNA staining was significantly higher in whole compared with processed semen. Positive (DNAse-treated nuclei) and negative controls (S1 nuclease-treated nuclei) were obtained to validate the specificity of the antibody.

Conclusion(s): Human sperm nuclei generally exhibit discrete (presumably peripheral) foci of ssDNA. The data also show that infertile men have a higher proportion of sperm nuclei with diffuse areas of ssDNA than do fertile men, and suggest that spermatozoa with diffuse nuclear staining are abnormal. (Fertil Steril 2007;88:1334–8. ©2007 by American Society for Reproductive Medicine.)

Key Words: Spermatozoa, sperm DNA damage, immunocytochemistry, male infertility, single-stranded DNA

Mammalian fertilization involves the direct interaction of the sperm and oocyte, fusion of the cell membranes, and subsequent union of male and female gamete genomes (1). Animal studies have shown that there may be a threshold of sperm DNA damage (e.g., DNA fragmentation) beyond which embryo development and pregnancy outcome are impaired (2). Similarly, clinical studies indicate that sperm DNA damage has an adverse impact on reproductive outcomes after in vivo and in vitro fertilization (3–8).

There is now clear evidence that infertile men possess substantially more sperm DNA damage than do fertile men (6, 9–12). We have shown that the mean percentage of spermatozoa with denatured DNA and DNA fragmentation is 25% and 28%, respectively, in infertile men and 10% and 13%, respectively, in fertile men (12). This is relevant given that infertile men (especially those with severe male-factor infertility and with poor sperm DNA integrity) will be seeking treatment with advanced reproductive technologies.

To date, little is known about the specific localization of sperm DNA damage in human spermatozoa (13, 14). As such, we sought to further characterize the nature and localization of human sperm DNA damage by immunocytochemical studies of spermatozoa from fertile and infertile men.

MATERIALS AND METHODS

Materials

Unless specified, all the chemicals in this study are from Fisher Chemical (Elvet Scientific, Bearpark, Durham, UK).

Study Subjects and Semen Handling

Semen samples were obtained from consecutive asthenoteratospermic men (<50% sperm motility and <15% normal forms but with normal sperm concentration [≥20 million sperm/mL]) presenting for infertility evaluation (n = 12). All couples presenting for infertility evaluation had primary infertility (no prior pregnancy), and had been unable to conceive naturally for a period of at least 1 year. Infertile men presented with a varicocele or with idiopathic infertility. None of the men had a recent illness, high fever, complete asthenosperma, semen hyperviscosity, or a primary hormonal abnormality. The asthenoteratospermic infertile men were selected based on the observation that abnormalities in sperm motility and morphology have been associated with sperm DNA damage in transgenic animals (15). Couples with
significant female-factor infertility (tubal obstruction or ovar- 
ian failure) were excluded. It must be noted that because the 
infertile men included in the study were all asthenoterato-
spermic, the conclusions drawn form this study may not be 
applicable to a cohort of unselected infertile men.

Semen samples (n = 5) were also obtained from consecu-
tive fertile controls (men presenting for vasectomy who 
had previously fathered at least two children, with one in the 
past 5 years).

Samples were produced by masturbation after 3 to 5 days of 
sexual abstinence and allowed to liquefy at room temperature. 
After liquefaction of semen, standard semen parameters (vol-
ume, concentration, motility) were obtained according to 
World Health Organization (16) guidelines. All of the semen 
samples had motile sperm and none had significant numbers 
of round cells or leukocytospermia as per World Health Orga-
ization guidelines (<1 million round cells per milliliter).

All patients signed an informed consent, and the informa-
tion for this study remained confidential and within the insti-
tution. This study was approved by the ethics review board at 
McGill University.

Semen Processing

Seven of the 12 semen samples from infertile men were 
loaded onto four-layer (20–40–65–95%) Percoll density gra-
dients and centrifuged at 2500 × g for 30 minutes, as previ-
ously described (17). Spermatozoa at the 65% to 95% Percoll 
interface and in the 95% Percoll layer were collected and 
pooled (highly motile fraction, designated as the high den-
sity-gradient fraction or HDGF). Sperm motility and round 
cell contamination was assessed in HDGF. Only those 
 washed samples with less than one round cells/100 spermato-
za were used (to eliminate the possible interference by 
leukocyte-derived oxidants).

Immunocytochemistry

Whole semen and percoll-washed spermatozoa (HDGF) 
were washed with phosphate-buffered saline (PBS, pH = 
7.4) and smears were prepared on Fisher Superfrost Plus 
slides (Elvet Scientific, Bearpark, Durham, UK). The smears 
were fixed in 100% methanol for 2 hours, air dried, and then 
stored at −70°C. Before immunostaining, smears were 
brought to room temperature, rehydrated with PBS for 30 
minutes, and decondensed in 5 mM dithiothreitol and 0.3 
µg/mL heparin for 30 to 60 minutes (to ensure full deconden-
sation of >90% of the spermatozoa).

Immunostaining for single-stranded (ss) DNA was per-
formed using mouse anti-ssDNA (Chemicon, Charlottesville, 
VA). Smears were blocked with 5% goat serum in PBS con-
taining 0.1% Triton X-100 (PBS-T) for 30 minutes, then 
washed with PBS-T and incubated with the ssDNA antibody 
(1:300 dilution) for 1 hour at 20°C. Smears were then washed 
with PBS-T and incubated with biotinylated rabbit antimouse 
IgM (Jackson ImmunoResearch Laboratories, West Grove, 
PA). After washing, the smears were then incubated with 
streptavidin-conjugated Alexa Fluor 555 (Invitrogen, Carls-
bad, CA) for 45 minutes. The smears were mounted with Pro-
long Antifade and observed under a Carl Zeiss Axioshot 
microscope (exciter filter BP 450 and emission filter BP 
590) at 1,000× magnification. All immunostaining experi-
ments were performed on the same run and the data were re-
corded by two separate and blinded observers (interobserver 
variability was 9.9%). At least 200 spermatozoa were as-
essed per slide. The mean percentage (±SD) of spermatozoa 
exhibiting sparse, focal punctated staining and those exhibit-
ing diffuse staining was recorded.

Negative controls were prepared as above, but in the ab-
scence of primary antibody. An additional negative control 
was obtained by treating decondensed spermatozoa with 
S1 nuclease (2,000 U/mL, Fermentas, Burlington, ON, 
Canada) for 60 minutes at 37°C before immunostaining 
(S1 nucleases are enzymes that degrade ss DNA). Positive 
controls were obtained by treating decondensed spermato-
zoa with DNase (0.1 µg/mL) for 60 minutes at room tem-
perature before immunostaining (DNases are enzymes that 
troduce DNA fragmentation). Immunocytochemistry ex-
periments conducted with a lower antibody dilution 
(1:100 dilution) demonstrated similar sperm nuclear immu-
nostaining patterns to that obtained with a 1:300 antibody 
dilution.

Data Analysis

Results are expressed as mean ± SD. Intergroup (fertile and 
infertile men) differences in standard semen parameters (and 
sperm nuclear staining patterns) were assessed by parametric 
and nonparametric tests as appropriate. Differences in the 
sperm nuclear staining between whole and washed spermato-
zoa (HDGF) were assessed by the Wilcoxon signed-ranks test. 
All hypothesis testing was two sided, with a probability value 
of 0.05 deemed as significant. Statistical analysis was per-
formed using Sigma Stat software (SPSS Inc., Chicago, IL).

RESULTS

Sperm Parameters

As expected, the mean (±SD) sperm concentration and per-
cent motility were higher in fertile compared with infertile 
men, although the difference in sperm concentration did 
not reach statistical significance (114 ± 51 vs. 70 ± 40 
sperm/mL and 82 ± 8 vs. 45 ± 24%, respectively). Mean se-
men volume was not significantly different in fertile com-
pared with infertile men (4.6 ± 1.5 vs. 3.1 ± 1.8 mL, 
respectively). All semen samples from infertile men demon-
strated poor morphology (mean [±SD] percent normal 
forms: 2.5 ± 1.8, range 1–6).

Immunocytochemistry

Immunocytochemistry experiments using ssDNA antibody 
demonstrated one of two sperm nuclear staining patterns: [1]
faint punctated staining (Fig. 1A), and [2] diffuse nuclear staining (Fig. 1B). Infertile men had a significantly higher proportion of spermatozoa exhibiting diffuse ssDNA staining than did fertile men (Table 1). Negative controls (absence of primary antibody and S1 nuclease treatment) demonstrated no detectable sperm nuclear staining (Fig. 1C). Positive controls (DNase-treated spermatozoa) demonstrated diffuse and intense nuclear staining for ssDNA (Fig. 1D).

The percentage of spermatozoa (±SD) exhibiting diffuse immunostaining was significantly lower in the HDGF (obtained by Percoll density-gradient centrifugation) compared with raw semen (9.9 ± 3.6 vs. 65.7 ± 12.6%, respectively, P < .001). The percentage of motile spermatozoa was higher in the HDGF compared with raw semen, although the difference was not statistically significant (39 ± 22 vs. 32 ± 18%, respectively, P > .05).

**DISCUSSION**

This study is the first to examine the localization of sperm DNA damage by immunocytochemistry. Our data show that in samples from fertile and infertile men a subpopulation of spermatozoa possess low (and presumably normal) levels of ssDNA. The punctated nuclear immunostaining pattern suggests that the ssDNA is located at the nuclear periphery. The nuclear localization results reported in this study are representative of the native (nondecondensed) state of the sperm head because nuclear architecture is maintained following sperm decondensation, as performed in this study (18). As such, our data suggest that the discrete foci of ssDNA (as observed in most spermatozoa) may be associated with telomeres and histones (specifically, histone H2B), as these structures have also been localized to the nuclear periphery of human spermatozoa (19, 20). The nature (physiologic or pathologic) of the observed peripheral foci of ssDNA is unknown.

Our data demonstrate that infertile men have a significantly higher percentage of spermatozoa with diffuse (and presumably abnormal) nuclear staining than fertile controls. These data are in keeping with clinical studies showing that infertile men possess substantially more sperm DNA damage than do fertile men (6, 9–12). The etiology of the observed diffuse nuclear staining (presumably indicative of extensive DNA damage) is likely multifactorial. Sperm protamine deficiency and semen oxidative stress have been strongly linked to sperm DNA damage (21, 22).

The data also support the notion that the population of spermatozoa in the ejaculate is heterogeneous with respect...
to DNA integrity (6, 12, 23). The significantly lower percentage of spermatozoa exhibiting diffuse immunostaining in Percoll-washed (HDGF) compared with whole semen samples further supports the concept that spermatozoa with diffuse ssDNA immunostaining pattern are abnormal.

Studies have shown that measures of sperm DNA denaturation correlate with measures of sperm DNA fragmentation and DNA oxidation, suggesting that the ssDNA detected in this study may be indicative of more substantial DNA damage (12, 24, 25). Embryos can repair sperm DNA damage; however, there may be a threshold beyond which sperm DNA damage cannot be repaired (2, 26). The discrete, focal areas of ssDNA at the nuclear periphery (possibly within telomeres) would likely be repaired early as telomeric regions are among the first to be exposed after nuclear decondensation at fertilization (27). On the other hand, extensive sperm DNA damage (as observed in that subset of spermatozoa with diffuse areas of ssDNA) is likely beyond the repair capacity of the embryo. Moreover, spermatozoa with extensive sperm DNA damage would likely not participate in fertilization because of associated plasma membrane lipid peroxidation (28).

Sperm DNA damage is clinically relevant, as it has been associated with reduced fertility potential in vivo and in vitro (3–8). Infertile couples in whom the man has high levels of sperm DNA damage experience lower pregnancy rates and a higher rate of spontaneous abortions (6, 28, 29). Couples undergoing insemination also tend to have poorer pregnancy outcome when the man’s spermatozoa possess high levels of DNA damage (4, 5). Sperm DNA damage has been associated with poor pregnancy outcome after IVF; however, with intracytoplasmic sperm injection, the impact of sperm DNA damage on pregnancy outcome does not appear to be significant (3, 4, 7).

In summary, we have demonstrated that human sperm nuclei exhibit either sparse, discrete foci of ssDNA (presumably peripheral) or extensive and diffuse areas of ssDNA. Our data also show that infertile men have a higher proportion of spermatozoa with diffuse nuclear staining for ssDNA than fertile men and suggest that spermatozoa with a diffuse nuclear staining pattern are abnormal.

**TABLE 1**

<table>
<thead>
<tr>
<th>Spermatozoa with diffuse ssDNA staining (%)</th>
<th>Fertile</th>
<th>Infertile</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Mean (±SD) percentage of sperm nuclei with diffuse single-stranded (ss) DNA staining pattern in fertile and infertile men.</td>
<td>14 ± 13</td>
<td>52 ± 19</td>
<td>.0030</td>
</tr>
</tbody>
</table>

*Comparison between fertile and infertile men by Mann-Whitney rank sum test.

**REFERENCES**