

Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility

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Objective: To examine the effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility.

Design: Prospective study.

Setting: Infertility clinic at a tertiary care academic institution.

Patient(s): Ninety-two infertile men with normal female partners. Sixteen fertile donors served as the control group.

Intervention(s): Standard semen analysis and assessment of levels of seminal oxidative stress. Assisted reproductive techniques in 33 of the 92 patients (IUI [n = 19], IVF [n = 10], and intracytoplasmic sperm injection [n = 4]).

Main Outcome Measure(s): Sperm DNA damage by sperm chromatin structure assay. Results were expressed as DNA fragmentation index.

Result(s): Patients were classified as having either idiopathic (n = 23) or male factor infertility (n = 69). Patients with idiopathic and male factor infertility had significantly higher DNA fragmentation index and oxidative stress compared with the case of fertile donors. A clinical pregnancy was achieved in 9 (27%) of 33 patients with assisted reproductive techniques. Significantly higher DNA fragmentation index and oxidative stress were found in men who failed to initiate a pregnancy after assisted reproductive techniques (n = 24), compared with the cases of those who succeeded and of the fertile donors. DNA fragmentation index was correlated positively with oxidative stress ($r = 0.27$), and negatively with fertilization ($r = -0.70$) and embryo quality ($r = -0.70$).

Conclusion(s): Sperm DNA damage is significantly increased in men with idiopathic and male factor infertility and in men who failed to initiate a pregnancy after assisted reproductive techniques. Such an increase may be related to high levels of seminal oxidative stress. (Fertil Steril® 2003;79(Suppl 3):1597–1605. ©2003 by American Society for Reproductive Medicine.)

Key Words: Male infertility, nuclear DNA, oxidative stress, spermatozoa

Data accumulated over the past few decades indicate that male factor infertility plays a role in approximately 50% of infertile couples (1). In some cases, standard investigations of the infertile couple reveal no detectable abnormalities, and therefore the couple is diagnosed with unexplained or idiopathic infertility (2). Despite the presence of numerous tests of sperm quality and function, no single laboratory test can determine with accuracy and precision whether a man is fertile (3). Standard semen

analysis using light microscope is widely used in most laboratories for the initial evaluation of the male partner of an infertile couple. However, diagnosing defective sperm function by standard semen analysis is difficult because spermatozoa are highly specialized cells that express a diverse array of biological properties to achieve fertilization (4). In addition, results of standard semen analysis can be very subjective and prone to intraobserver and interobserver variability (3).

There is growing evidence to suggest that seminal oxidative stress is involved in many aspects of male infertility (5). Seminal oxidative stress is caused by an imbalance between reactive oxygen species (ROS) production and antioxidant scavenging activities in semen (6). Increased levels of seminal oxidative stress have been correlated with sperm dysfunction through different mechanisms that include lipid peroxidation of sperm plasma membrane and impairment of sperm metabolism, motility, and fertilizing capacity (7). In addition, oxidative stress has been shown to affect the integrity of the sperm chromatin and to cause high frequencies of single and double DNA strand breaks (8). Exposing spermatozoa to artificially produced ROS significantly increases DNA damage by modifying all bases and producing base-free sites, deletions, frameshifts, and DNA cross-links (9).

The biological impact of an abnormal sperm chromatin structure depends on the combined effects of the extent of DNA or chromatin damage in the spermatozoa and the capacity of the oocyte to repair that damage (10). Therefore, if spermatozoa are selected from samples with extensively damaged DNA and used for IVF, the repair capacity of oocytes may be inadequate, leading to a low rate of embryonic development and high early pregnancy loss (11). Haaf and Ward (12) have suggested that sperm chromatin abnormalities may influence the initiation and regulation of paternal gene activity during early stages of embryo development. Defects in the male genome have been shown to lead to postfertilization failure (13). Recent studies have suggested that sperm chromatin or DNA is an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters (concentration, motility, and morphology) (14, 15).

The objectives of our study were the following: to [1] examine levels of sperm DNA damage by sperm chromatin structure assay (SCSA) in infertile men with idiopathic and male factor infertility and [2] determine the correlation of the SCSA-defined DNA damage with seminal oxidative stress indices; standard sperm characteristics; and fertilization, embryo quality, and clinical pregnancy in a cohort of patients who underwent assisted reproductive techniques.

MATERIALS AND METHODS

Study Groups

The study was approved by the Cleveland Clinic Foundation's institutional review board. The participants were selected from couples attending the infertility clinic who had a history of infertility of ≥ 12 months ($n = 92$). A detailed medical history was obtained from all participants, including reproductive history and infertility evaluation of the female partner. A male-infertility specialist (A.J.T.) took the medical history and performed the genital examination of the male partner. Patients with normal female partners, that is, normal reproductive history, normal ovulation (by follicular

ultrasound scan, luteal phase progesterone levels, and endometrial biopsy), and tubal patency (hysterosalpingogram) were eligible for the study.

A group of healthy volunteers of proven fertility (initiated a successful pregnancy within the last 12 months before participation in the study) served as the control group ($n = 16$). Patients with azoospermia and those with female factor infertility were not eligible for the study. Of the 92 couples in this study, 33 underwent assisted reproductive techniques (IUI [$n = 19$], IVF [$n = 10$], and intracytoplasmic sperm injection [ICSI; $n = 4$]). Information was obtained on the outcome of the assisted reproductive techniques, including fertilization, embryo quality, and clinical pregnancy.

Standard Semen Analysis

Semen specimens were produced by masturbation at the clinical andrology laboratory after a period of 48 to 72 hours of sexual abstinence. After liquefaction, semen specimens were evaluated for sperm concentration and motility according to the guidelines of the World Health Organization (WHO) (1). Morphology smears were scored using the WHO classification (1) and Kruger's strict criteria (16). Sperm concentration was expressed as 10^6 per milliliter of semen, whereas motility and morphology were expressed as percentage. Sperm parameters were considered normal when sperm concentration was $\geq 20 \times 10^6$ per milliliter of semen, motility was $\geq 50\%$, and normal sperm forms were $\geq 30\%$ by WHO criteria (1) and $> 14\%$ by Kruger's strict criteria (16). Seminal leukocytes were quantified by a myeloperoxidase-staining test, and values were considered to be normal at concentrations of $\leq 1 \times 10^6$ peroxidase-positive leukocytes per milliliter of semen (1).

Measurement of ROS and Total Antioxidant Capacity

Levels of ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as a probe (17). Levels of total antioxidant capacity (TAC) in the seminal plasma were assessed by an enhanced chemiluminescence assay, as described elsewhere (6).

Reactive Oxygen Species-TAC Score

The composite ROS-TAC score was calculated for each sample as a measure of seminal oxidative stress using principal component analysis, as described in an earlier study (6). The ROS-TAC score is a statistical formula that minimizes the variability of the individual parameters of oxidative stress (ROS alone or TAC alone). The ROS-TAC score is an accurate measure of seminal oxidative stress; low ROS-TAC scores indicate high seminal oxidative stress (6).

Sperm Chromatin Structure Assay

Assessment of SCSA-defined DNA damage was performed using a flow cytometer (Ortho Diagnostic Inc., Westwood, MA), as described elsewhere (18). Computer-generated means and SDs of green (515 nm to 530 nm) and red

(>630 nm) fluorescence, corresponding to the amounts of native and denatured DNA, respectively, were derived from a population of approximately 5,000 sperm cells. Alpha_t (α_t), was calculated as the ratio of the red fluorescence to the total of red and green fluorescence of an individual sperm cell ($\alpha_t = \text{red}/\text{total} [\text{red} + \text{green}]$ fluorescence). The parameter X_{α_t} represents the mean population of α_t . Standard deviation of α_t (SD_{α_t}) represents the variability of chromatin structure abnormalities within the sperm.

Cells with abnormal chromatin structure or DNA damage were represented by DNA fragmentation index (DFI), formally called $COMP_{\alpha_t}$ (the percentage of cells outside the main population of α_t) (19). The fourth parameter, percentage of high DNA-stainable sperm, formally called %HGRN (the percentage of high green), identifies the cells with immature nuclei by the characteristic pattern of increased green fluorescence (19). Statistical thresholds were established for DFI as follows: excellent = DFI <15%, for high fertility status; good = DFI between 15% and 24%, for reasonable fertility status; fair = DFI between 25% and 30%; and poor = DFI >30%, for a significant lack of fertility potential (19).

Assisted Reproductive Techniques

Thirty-three of the 92 couples in this study underwent assisted reproductive techniques (IUI [n = 19], IVF [n = 10], and ICSI [n = 4]). Spermatozoa were prepared for assisted reproductive techniques by density gradient centrifugation technique using ISolate media (Irvine Scientific, Santa Ana, CA). The protocols for ovulation induction for standard IVF and ICSI were the same. A baseline blood estradiol level and vaginal ultrasound were performed on day 1–5 of the cycle. The prerequisites for initiating an induction cycle included a blood estradiol level of <50 pg/mL and absence of ovarian cysts and a thin endometrium (<5 mm) on ultrasound examination.

Oocyte retrieval was performed by ultrasound-guided transvaginal follicular aspiration with the patient under general anesthesia. Mature oocytes were incubated in a humidified CO₂ (5%) incubator for 16–18 hours in the presence of washed spermatozoa (50,000–100,000 motile spermatozoa per milliliter) or after being subjected to ICSI. Fertilization was considered normal if two pronuclei and two polar bodies were identified. Good-quality embryos had blastomeres of equal sizes with no cytoplasmic fragments or blebs. Ultrasound-guided tubal transfer was performed using day 3 embryos.

Patients were scheduled for ultrasound 4–6 weeks after transfer to determine presence of fetal sac. A clinical pregnancy was defined as the occurrence of at least one ultrasound-confirmed gestational sac in the uterus at 4–6 weeks after the assisted reproductive technique procedure.

Statistical Analysis

Continuous variables among the groups were compared using Kruskal-Wallis tests. Pairwise comparisons between

the groups were performed with Wilcoxon rank-sum tests. Fisher's exact test was used for the categorical variables. Correlation between variables was calculated using Spearman's nonparametric method. Logistic regression was used to determine the relationship of several variables with fertility status and assisted reproductive technique outcome measures. All hypothesis testing was two-tailed, and the results were considered statistically significant at $P < .05$. The overall sample size of both patients and donors was sufficient to determine whether a 10-point change in ROS-TAC scores and/or a 10% change in DFI was associated with an increased odds of pregnancy of 2.0 or decreased pregnancy odds by 0.5 with a power of 90%. The sample size solely of patients had 90% power to determine whether a 10-point change in ROS-TAC scores and/or a 10% change in DFI was associated with increased odds of pregnancy of 4.8 or decreased pregnancy odds by 2.0. Summary statistics are presented as median and (interquartile [IQR] values: 25th percentile and 75th percentile). All analyses were calculated using SAS statistical software package (version 8.1; SAS Institute Inc., Cary, NC).

RESULTS

Infertile men participating in this study were classified into one of two groups: [1] patients with idiopathic infertility (with normal standard semen parameters on repeated analyses, normal genital exam and normal female partner; n = 23) or [2] patients with male factor infertility (with abnormality in one or more of the standard semen parameters on repeated analyses, with/without abnormal genital exam, and with a normal female partner; n = 69). No significant differences were found in age between the patients with idiopathic infertility (mean, 33 [30, 36] years) or male factor infertility (33 [31, 37] years) and the fertile donors (33 [29, 34] years). Also, no significant difference was found in the age of the female partners of men with idiopathic fertility (30 [29, 36] years) and male factor infertility (33 [30, 35] years).

The difference in the length of infertility between the idiopathic (1 [1, 2] years) and male factor infertility groups (2 [1, 4] years) was statistically significant ($P = .02$). A clinical pregnancy was achieved in 27% (9/33) of couples who underwent assisted reproductive technique procedures (26% [5/19] with IUI, 30% [3/10] with IVF, and 25% [1/4] with ICSI), whereas pregnancy did not occur in the remaining 73% (24/33).

No significant difference was found in the age of the infertile men who achieved a clinical pregnancy by assisted reproductive techniques (33 [32, 34] years) compared with those who did not (32 [31, 38] years; $P = .97$). Also, the difference in age of the female partners among couples who achieved a clinical pregnancy by assisted reproductive techniques (31 [29, 33] years) and those who did not (34 [30, 36] years) was not statistically significant ($P = .17$).

TABLE 1

Comparison of oxidative stress indices (ROS, TAC, and ROS-TAC scores) between fertile donors and patients with idiopathic and male factor infertility.

Variable	Fertile donors (n = 16)	Idiopathic infertility (n = 23)	Male factor infertility (n = 69)	P values		
				A ^a	B ^b	C ^c
ROS ($\times 10^6$ cpm)	0.4 (0.1, 2)	1.8 (0.1, 3.1)	10 (1, 48)	.56	.0004	.003
TAC (Trolox equivalent)	895 (724, 1,146)	797 (619, 1,093)	707 (413, 1,010)	.13	.02	.21
ROS-TAC score	53 (50, 58)	47 (45, 51)	43 (32, 49)	.009	.0001	.007

Note: Values are median and IQR (25th and 75th percentiles) unless otherwise indicated. Wilcoxon rank-sum test was used for the analysis, and $P < .05$ was considered significant.

^a P values between fertile donors and idiopathic infertility patients.

^b P values between fertile donors and male factor infertility patients.

^c P values between idiopathic and male factor infertility patients.

Saleh. Increased DNA damage in sperm. *Fertil Steril* 2003.

Standard Semen Parameters

Median (and IQR: 25th and 75th percentiles) for sperm concentration, percentage of motility, and normal sperm forms by WHO and Kruger's methods, in the fertile donors (71 [36, 120], 62 [54, 70], 40 [33, 45], and 9 [8, 11], respectively), were significantly higher than those in patients with male factor infertility (23 [13, 48], 42 [30, 52], 18 [12, 25], and 6 [3, 8], respectively; $P = .0008, 0.0002, 0.0001, \text{ and } 0.0001$, respectively) and were not significantly different from those in patients with idiopathic infertility (58 [47, 74], 65 [58, 77], 34 [32, 37], and 8 [4, 10], respectively; $P = .68, 0.39, 0.22, \text{ and } 0.18$, respectively).

Sperm concentration, percentage of motility, and normal sperm forms, by WHO and Kruger's methods, were, in the fertile donors (71 [36, 120], 62 [54, 70], 40 [33, 45], and 9 [8, 11], respectively), significantly higher than in patients who failed to initiate a clinical pregnancy after assisted reproductive techniques (18 [12, 41], 38 [23, 50], 16 [12, 25], and 7 [4, 8], respectively; $P = .003, 0.001, 0.0001, \text{ and } 0.001$, respectively).

0.001, respectively). Sperm motility was significantly higher in the patients who succeeded in initiating a clinical pregnancy after assisted reproductive techniques than in those who failed ($P = .009$) and was not significantly different from that in the fertile donors ($P = .91$).

Oxidative Stress Indices (ROS, TAC, and ROS-TAC Scores)

Comparisons of ROS, TAC, and ROS-TAC scores between fertile donors and patients with idiopathic and male factor infertility are shown in Table 1. The ROS-TAC scores were positively correlated with sperm concentration ($r = 0.37$; $P = .001$), motility ($r = 0.39$; $P = .0007$), and normal sperm forms by WHO criteria ($r = 0.44$; $P = .0001$) and by Kruger's strict criteria ($r = 0.37$; $P = .001$). The ROS-TAC scores were negatively correlated with seminal leukocyte concentrations ($r = -0.57$; $P < .0001$).

Comparisons of ROS, TAC, and ROS-TAC score between fertile donors and patients who initiated a clinical

TABLE 2

Comparison of oxidative stress indices (ROS, TAC, and ROS-TAC scores) between fertile donors and infertile men who succeeded to initiate a clinical pregnancy after assisted reproductive techniques and those who failed.

Variable	Fertile donors (n = 16)	Pregnancy with assisted reproductive techniques (n = 9)	No pregnancy with assisted reproductive techniques (n = 24)	P values		
				A ^a	B ^b	C ^c
ROS ($\times 10^6$ cpm)	0.4 (0.1, 2)	0.9 (0.1, 18)	14 (1, 178)	.36	.005	.04
TAC (Trolox equivalent)	895 (724, 1,146)	816 (406, 886)	707 (436, 811)	.19	.02	.71
ROS-TAC score	53 (50, 58)	50 (40, 52)	36 (29, 49)	.06	.0006	.04

Note: Values are median and IQR (25th and 75th percentiles) unless, otherwise indicated. Wilcoxon rank-sum test was used for the analysis, and $P < .05$ was considered significant.

^a P values between fertile donors and patients who achieved pregnancy with assisted reproductive technique.

^b P values between fertile donors and patients who did not achieve pregnancy with assisted reproductive technique.

^c P values between patients who achieved pregnancy with assisted reproductive technique and those who failed.

Saleh. Increased DNA damage in sperm. *Fertil Steril* 2003.

TABLE 3

Comparison of SCSA-defined DNA damage parameters between fertile donors and patients with idiopathic and male factor infertility.

Variable	Fertile donors (n = 16)	Idiopathic infertility (n = 23)	Male factor infertility (n = 69)	P values		
				A ^a	B ^b	C ^c
X _{at} (%)	232 (214, 258)	260 (233, 274)	290 (251, 344)	.04	.0003	.02
SD _{at} (%)	175 (145, 193)	160 (145, 274)	213 (174, 251)	.44	.001	.0001
DFI (%)	15 (11, 21)	23 (15, 32)	28 (18, 41)	.02	.0001	.27
HDS (%)	8 (5, 12)	9 (6, 11)	11 (8, 16)	.76	.07	.03

Note: Values are median and IQR (25th and 75th percentiles) unless otherwise indicated. Wilcoxon rank-sum test was used for the analysis, and $P < .05$ was considered significant. X_{at} = the mean population of at; SD_{at} = the variability of chromatin structure abnormalities within the sperm population; HDS = high DNA stainable.

^a P values between fertile donors and idiopathic infertility patients.

^b P values between fertile donors and male factor infertility patients.

^c P values between idiopathic infertility and male-factor infertility patients.

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pregnancy with assisted reproductive techniques and those who failed are shown in Table 2. Levels of seminal ROS were negatively correlated with fertilization ($r = -0.59$; $P = .03$) and embryo quality ($r = -0.89$; $P = .007$) after conventional IVF and ICSI.

Sperm Chromatin Structure Assay–Defined DNA Damage Parameters

Comparisons of SCSA-defined DNA damage parameters between fertile donors and patients with idiopathic and male factor infertility are shown in Table 3. The DFI was negatively correlated with sperm concentration ($r = -0.31$; $P = .001$), percentage motility ($r = -0.47$; $P < .0001$), and normal sperm forms by WHO criteria ($r = -0.40$; $P < .0001$) and by Kruger's strict criteria ($r = -0.31$; $P = .002$). Levels of ROS were positively correlated with X_{at} ($r = 0.23$; $P = .017$) and SD_{at} ($r = 0.41$; $P = .0001$).

The ROS-TAC scores were negatively correlated with the SCSA parameters: DFI ($r = -0.27$; $P = .009$), X_{at} ($r = 0.26$; $P = .01$), SD_{at} ($r = -0.31$; $P = .002$), and high DNA-stainable sperm ($r = -0.31$; $P = .003$). Comparisons of SCSA-defined DNA damage parameters between fertile donors and men who succeeded in initiating a clinical pregnancy by assisted reproductive techniques and those who failed are shown in Table 4.

Levels of DFI were negatively correlated with fertilization ($r = -0.70$; $P = .03$) and embryo quality ($r = -0.70$; $P = .03$) after IVF and ICSI. Levels of DFI were negatively correlated with the overall pregnancy in the patients who underwent assisted reproductive techniques ($P < .0001$). The distribution of DFI percentage in relation to standard sperm parameters (normal or abnormal) and clinical pregnancies (positive or negative) in the patients who underwent IUI

TABLE 4

Comparison of SCSA-defined DNA damage parameters between fertile donors and infertile men who succeeded in initiating a clinical pregnancy after assisted reproductive techniques and those who failed.

Variable	Fertile donors (n = 16)	Pregnancy with assisted reproductive techniques (n = 9)	No pregnancy with assisted reproductive techniques (n = 24)	P values		
				A ^a	B ^b	C ^c
X _{at} (%)	232 (214, 258)	250 (227, 267)	305 (286, 353)	.33	.0001	.007
SD _{at} (%)	175 (145, 193)	176 (169, 185)	219 (180, 253)	.57	.004	.09
DFI (%)	15 (11, 21)	21 (13, 25)	38 (28, 43)	.15	.0001	.001
HDS (%)	8 (5, 12)	11 (10, 14)	10 (8, 22)	.22	.23	.9

Note: Values are median and IQR (25th and 75th percentiles) unless otherwise indicated. Wilcoxon rank-sum test was used for the analysis, and $P < .05$ was considered significant. X_{at} = the mean population of at; SD_{at} = the variability of chromatin structure abnormalities within the sperm population; DFI = DNA fragmentation index; HDS = high DNA stainable.

^a P values between fertile donors and patients who achieved pregnancy with assisted reproductive technique.

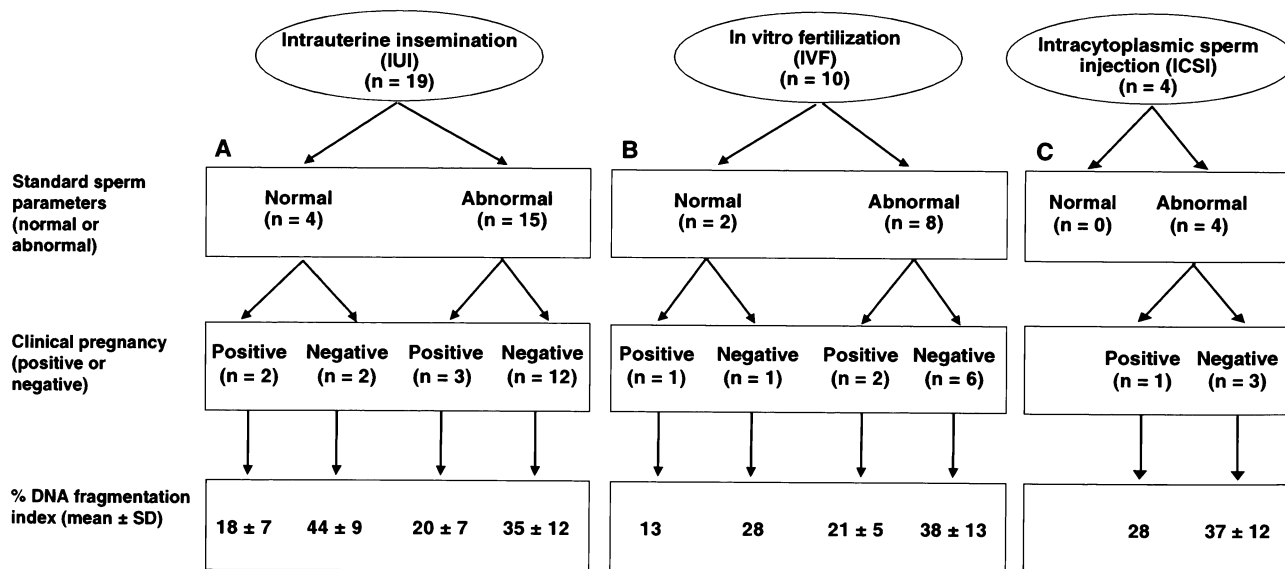
^b P values between fertile donors and patients who did not achieve pregnancy with assisted reproductive technique.

^c P values between patients who achieved pregnancy with assisted reproductive technique and those who failed.

Saleh. Increased DNA damage in sperm. *Fertil Steril* 2003.

FIGURE 1

The distribution of DFI (%) in relation to standard sperm parameters (normal or abnormal) and pregnancies (positive or negative) in patients who underwent (A) IUI (n = 19), (B) IVF (n = 10), and (C) ICSI (n = 4).



Saleh. Increased DNA damage in sperm. Fertil Steril 2003.

(n = 19), IVF (n = 10), and ICSI (n = 4) is shown in Figure 1.

DISCUSSION

Physicians and scientists dealing with practical aspects of male infertility are driven toward identifying why spermatozoa from a particular male have not achieved fertilization and whether spermatozoa from a particular male have the capacity to fertilize an egg (19). In clinical practice, assessment of male fertility still relies on the traditional, manual/visual, light-microscopic methods for standard semen analysis. These do not adequately address all parameters of sperm quality and function that are required for an optimum fertility status, and a definitive diagnosis of male infertility cannot be made as a result of such basic semen analysis (20).

In our study, conventional sperm characteristics (sperm concentration, motility, and normal forms) were completely normal in a subset of infertile men diagnosed as idiopathic and were not significantly different from the fertile donors. Therefore, the finding of normal conventional sperm parameters does not guarantee that the sperm population in an ejaculate will be fertile.

In addition, no differences were found in the conventional sperm parameters, in our study, between the patients who failed to initiate a clinical pregnancy with assisted reproductive techniques and those who succeeded, except for the percentage sperm motility, which was significantly higher in

the later group. However, the fact that assisted reproductive techniques, particularly ICSI, have been successful in treating infertile men with very poor sperm characteristics implies that classical parameters of sperm quality, including motility, are no longer relevant; this places more emphasis on the quality of DNA in the sperm nucleus (19).

It is still unclear whether assisted reproductive techniques can compensate for poor chromatin packaging and/or DNA damage or if suboptimal chromatin integrity is responsible for poor implantation rate (<20%) in the majority of assisted reproductive technique patients (21). Results from our study indicate a significant increase in the SCSA-defined DNA damage (DFI percentage) in the infertile men who failed to initiate a clinical pregnancy with assisted reproductive techniques vs. those who succeeded. In addition, levels of DFI, in our study, were negatively correlated with fertilization and embryo quality after IVF and ICSI. In a recent study, sperm DNA damage measured by the TUNEL assay was negatively correlated with fertilization rates after conventional IVF, but not ICSI, in patients with unexplained infertility and oligozoospermia (22).

These results are significant and indicate that pregnancy failure after assisted reproductive techniques may be related, at least in part, to increased DNA damage in spermatozoa. Pregnancy failure, in this case, may be mediated by impairment of fertilization and/or embryo quality. Increased sperm DNA damage in infertile men who failed to initiate a preg-

nancy via assisted reproductive techniques in our study may be caused by the high levels of seminal oxidative stress found in these patients. Such speculation is supported by the finding of a significant positive correlation between seminal oxidative stress and SCSA parameters (%DFI, X_{ox} , and SD_{ox}) that are indicative of the extent and severity of DNA damage in spermatozoa.

High levels of seminal oxidative stress in the patients who failed to initiate a clinical pregnancy after assisted reproductive techniques in our study are likely caused by increased levels of ROS production in this group. Recent studies have shown a significant positive correlation between sperm DNA fragmentation and levels of ROS in testicular tissue (23) and in semen (24, 25).

Therefore, it is likely that spermatozoa selected for assisted reproductive techniques originate from an environment experiencing high seminal oxidative stress and, consequently, high levels of sperm DNA damage (26). As a result, there is a substantial risk that spermatozoa carrying damaged DNA are being used for assisted reproductive techniques (27). Levels of seminal ROS in our study were negatively correlated with rates of fertilization and embryo development after IVF and ICSI. In this case, impairment of fertilization and embryo development in relation to high ROS may be mediated by ROS-induced damage to the sperm nuclear DNA.

In our study, the median (IQR) of DFI in infertile men who succeeded in initiating a clinical pregnancy with assisted reproductive techniques was 21% (13%, 25%) compared with 15% (11%, 21%) in the fertile donors, a difference that was not statistically significant ($P=.15$). The DNA damage values in the fertile donors and the patients who were able to initiate a clinical pregnancy with assisted reproductive techniques in our study were consistent with the thresholds reported by Evenson et al. (19) as being excellent (DFI <15%), for high fertility status, and good (DFI = 15% to 24%), for reasonable fertility status.

On the other hand, in this study, the median (IQR) DFI in infertile men who failed to initiate a clinical pregnancy with assisted reproductive techniques was 38% (28%, 43%), which was consistent with the thresholds reported by Evenson et al. (19) as being fair (DFI = 25% to 30%) and poor (DFI >30%) for a significant lack of fertility potential. In addition, the maximum level of DFI in infertile men who initiated a clinical pregnancy after assisted reproductive techniques in our study was 28%. This level is close to the DFI threshold of 27% reported in a recent study to be indicative of pregnancy failure after IVF or ICSI (14).

The same group has also reported that a DFI threshold of 30% is incompatible with natural pregnancy (4). The 30% threshold was described as the “tip of the iceberg effect,” that is, chromatin damage identified in 30% of the spermatozoa indicated a level of DNA strand breaks that is simply

incompatible with proper paternal gene contributions to the embryo, causing in vivo infertility (19). The remaining 70% of spermatozoa, the “rest of the iceberg,” may not be sufficient for fertility because they also contain the same kind of damage, albeit to a lesser degree, but to an extent that significantly reduces paternal genome competence.

Spano et al. (28) also reported that in vivo fecundity decreased progressively when >20% of the spermatozoa were identified by the SCSA as being damaged and became essentially zero when $\geq 40\%$ of the sperm population was damaged. Results from our study indicate that the infertile men classified as idiopathic, based on normal standard semen parameters, have significantly higher levels of SCSA-defined DNA damage (percentage DFI) compared with the men in the fertile group. In addition, levels of DNA damage in the patients with normal standard semen parameters were not significantly different from those in the patients with abnormal semen parameters.

Therefore, it may be speculated that increased DNA damage is responsible, at least in part, for the low fertility in a subset of infertile men who have normal standard semen parameters on repeated analysis and as a result are diagnosed as having unexplained or idiopathic fertility. However, the remaining SCSA parameters (X_{ox} , SD_{ox} , and high DNA-stainable sperm) were significantly lower in the patients with normal semen parameters compared with those with abnormal semen parameters, which indicates that the severity of DNA damage in the later group was even worse. A recent study by Sakkas et al. (29), using the comet assay, has indicated that ejaculated sperm from infertile men had single- and double-stranded DNA breaks and that both were more prevalent in men with abnormal semen parameters.

In this study, increased levels of sperm DNA damage in infertile men with idiopathic and male factor infertility may be caused by the high levels of seminal oxidative stress found in these patients. Another explanation for the link between seminal oxidative stress and sperm DNA damage may be related to a defect in spermiogenesis that causes the release of spermatozoa that are immature and have abnormal chromatin structure/high DNA damage (13, 29) and abnormal morphology (30). Spermatozoa with abnormal morphology have been shown to have a capacity to generate high levels of ROS that, on exceeding critical levels, can cause oxidative stress (30).

In addition, levels of SCSA-defined sperm DNA damage in our study were negatively correlated with sperm concentration, motility, and normal sperm forms. Recent studies have shown a similar correlation between the percentage of spermatozoa with DNA damage and conventional sperm parameters (31, 32), whereas other studies showed only a weak correlation (3, 15).

Before a conclusion can be reached from the results of our study, it is important to bear in mind that our study had some

limitations in relation to the potential negative effects of sperm DNA damage on the outcome of assisted reproductive techniques. The total number of patients who underwent assisted reproductive techniques in our study was relatively small, and therefore we were unable to stratify the patients into groups based on the assisted reproductive technique (IUI, IVF, or ICSI). As a result, we were unable to study the effects of sperm DNA damage in each well-defined group.

Large-scale, randomized studies in the future may help determine the effects of sperm DNA damage on the outcome of assisted reproductive techniques in different clinical settings. Despite these limitations, some firm relationships are evident from our study, particularly in relation to the negative effects of increased sperm DNA damage and seminal oxidative stress on the rates of fertilization, embryo development, and pregnancy via assisted reproductive techniques.

In conclusion, our study indicates a significant increase in SCSA-defined DNA damage and levels of seminal oxidative stress in men with idiopathic and male factor infertility and in infertile men who failed to initiate a clinical pregnancy via assisted reproductive techniques. Levels of DNA damage in spermatozoa from infertile men were correlated positively with seminal oxidative stress and negatively with standard sperm parameters (concentration, motility, and normal forms). Levels of DNA damage in spermatozoa from infertile men were negatively correlated with the outcome of assisted reproductive technique (fertilization, embryo quality, and clinical pregnancy).

In addition, our study shows that normal-looking spermatozoa that have increased DNA damage can certainly be infertile, whereas spermatozoa with abnormal standard parameters, such as motility and morphology, but with low DNA damage can still be fertile. Therefore, sperm DNA damage analysis may be a tool for evaluation of semen samples before their use in assisted reproductive techniques.

Testing sperm DNA damage may also help selection of semen samples with the least amount of sperm DNA damage to be used in assisted conception. This may help reduce the risks associated with the use of DNA-damaged sperm for fertilization and avoid financial, social, and emotional problems associated with failed assisted reproductive technique attempts.

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