

Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay

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Objective: To investigate how moderate and/or high levels of DNA fragmentation (DFI), as measured by the sperm chromatin structure assay (SCSA), affect either IVF or IVF with intracytoplasmic sperm injection (ICSI) fertilization, cleavage, blastulation, implantation, and pregnancy.

Design: Retrospective clinical study.

Setting: Academic human reproduction laboratory.

Patient(s): Eighty-nine couples undergoing IVF with conventional fertilization or ICSI.

Intervention(s): Sperm chromatin structure assay testing (SCSA) of semen aliquot taken from ejaculate used for assisted reproductive technology (ART).

Main Outcome Measure(s): Related DFI to conventional semen parameters and cycle-specific outcomes after ART.

Result(s): No patients achieved clinical pregnancy if SCSA values exceeded the DFI (27%, $P < .01$), moderate DFI (15%, $P < .01$), or high DFI (15%, $P < .05$) thresholds. Dividing the DFI sperm population into moderate-fragmentation and high-fragmentation categories did not improve the prognostic value of the SCSA. No coefficient of determination (r^2) between SCSA parameters and conventional parameters exceeded 0.29.

Conclusion(s): Sperm chromatin structure assay identified thresholds for negative pregnancy outcome after ART not identified using conventional semen parameters. This is the first study analyzing the clinical value of sperm DFI to [1] include a large number of ART patients ($n = 89$), [2] perform SCSA analysis on a semen aliquot from the ejaculate used for ART, and [3] examine how the extent (moderate and high DFI) of DFI influenced ART outcomes. (Fertil Steril® 2003;80:895–902. ©2003 by American Society for Reproductive Medicine.)

Key Words: Sperm chromatin structure assay, SCSA, sperm DNA fragmentation, IVF, ICSI, assisted reproductive techniques, ART, fertilization, pregnancy, infertility

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Male factor is the single most common cause of infertility, with sperm defects representing 30% to 50% of clinical infertility cases (1–3). Abnormal sperm parameters including extremely low sperm concentration, poor sperm motility, and abnormal morphology contribute to natural fertility problems. Before 1992, severe sperm abnormalities were not completely overcome by contemporary infertility treatments like IUI and conventional IVF. The advent of intracytoplasmic sperm injection (ICSI) in 1992 revolutionized the treatment of male factors, allowing men with just a single

living sperm of any quality to become candidates for infertility treatment (4). Yet, many clinics using ICSI were not finding the level of success anticipated (5, 6). These failed ICSI cycles have become a source of scientific debate for infertility specialists and personal anguish for infertility patients.

In clinical practice, the etiology of most failed ICSI cycles is typically attributed to female factors, usually female age (7), regardless of sperm quality. Therefore, many couples with failed assisted reproductive technology (ART) cycles consider oocyte donation to overcome

the influence of putatively poor oocyte quality and to improve their success with ART. This decision is often based on the assumption that injecting a sperm into the oocyte assures the necessary contribution of the paternal genome and ignores the possibility that ART, specifically ICSI, risks forcing fertilization with spermatozoa that contain hidden DNA abnormalities (8). This potential risk is pronounced, as a significant proportion of infertile men have elevated levels of DNA damage (9–15). Furthermore, recent evidence from a study using single-cell gel electrophoresis (comet assay) suggests that “selection of sperm for ICSI in terms of DNA damage was random” (13).

Abnormal embryo (13) and blastocyst development have been linked to poor sperm quality (16). Specifically, sperm DNA fragmentation (DFI), as assessed by the sperm chromatin structure assay (SCSA), has been shown to influence pregnancy that is initiated naturally (11, 17) and via ART. Pilot studies showed that high levels of DNA fragmentation (DFI; >27% DFI) decreased fertility in patients using ART (12), even in men with completely normal standard semen parameters (18). Consequently, the identified relationship between conventional semen parameters and sperm DNA fragmentation (5, 9, 14, 19, 20) is not strong enough to eliminate DNA fragmentation as a potential source of infertility in normozoospermic men and requires a distinct assessment of sperm DNA fragmentation in male infertility evaluations.

To determine the diagnostic and prognostic role of sperm nuclear DNA fragmentation analysis, it is critical to understand the effect that DNA fragmentation has on fertilization, embryo quality, implantation, and pregnancy in a large group of ART patients. The SCSA is the most statistically robust tool used to measure sperm nuclear DNA fragmentation because of instrument-defined criteria that evaluate 5,000 cells per sample. Light-microscopic measurements are typically based on 100–200 observations and are classified by human interpretations. The technical soundness and demonstrated in vivo fertility thresholds of the SCSA test make it an excellent tool for identifying DNA fragmentation thresholds for fertility potential in couples choosing to endeavor highly invasive and complex ART (21). Furthermore, because the SCSA is a quantitative (on a continuous scale), as opposed to a qualitative (damaged or undamaged), measurement, fertility thresholds may be refined to identify how the extent of DNA fragmentation interferes with the normal expression of the paternal genome.

There is a wealth of evidence supporting the impact of DNA fragmentation in ejaculated sperm on preimplantation and postimplantation embryonic development. Yet, the absence of a published, statistically validated clinical threshold for infertility in a large group of ART patients makes the clinical application of these data extremely difficult. Therefore, the objective of our research was to examine the relationship of sperm nuclear DNA fragmentation (as assessed

by the SCSA test) with fertilization, embryo development, implantation rates, and pregnancy after conventional IVF and IVF with ICSI.

MATERIALS AND METHODS

Patients and Conventional Semen Analysis

Institutional review board approval was granted for this study, including 89 men (mean age $\pm$ SD, 34.4 ± 5.4 years) undergoing IVF ($n = 55$), ICSI ($n = 26$), or both IVF and ICSI ($n = 8$) cycles at an academic ART laboratory. Semen samples were obtained by masturbation after 36–120 hours of abstinence. Samples were ejaculated into nontoxic specimen containers and placed in a 37°C water bath for 20–30 minutes to liquefy. After liquefaction, conventional semen parameters were evaluated as part of the preparation for insemination and injection, which included sperm concentration ($\times 10^6$ per milliliter) and motility (percentage motile) as determined by World Health Organization (22) procedures and morphology (percentage normal) as determined by Tygerberg strict criteria (23).

Sample Handling

A 200- μ L subsample of raw semen was taken from the ejaculate used for the ART procedure, placed in 1-mL cryovial, and snap-frozen without cryoprotectants in liquid nitrogen. Samples were shipped in liquid nitrogen tanks, in which they were stored until SCSA analysis.

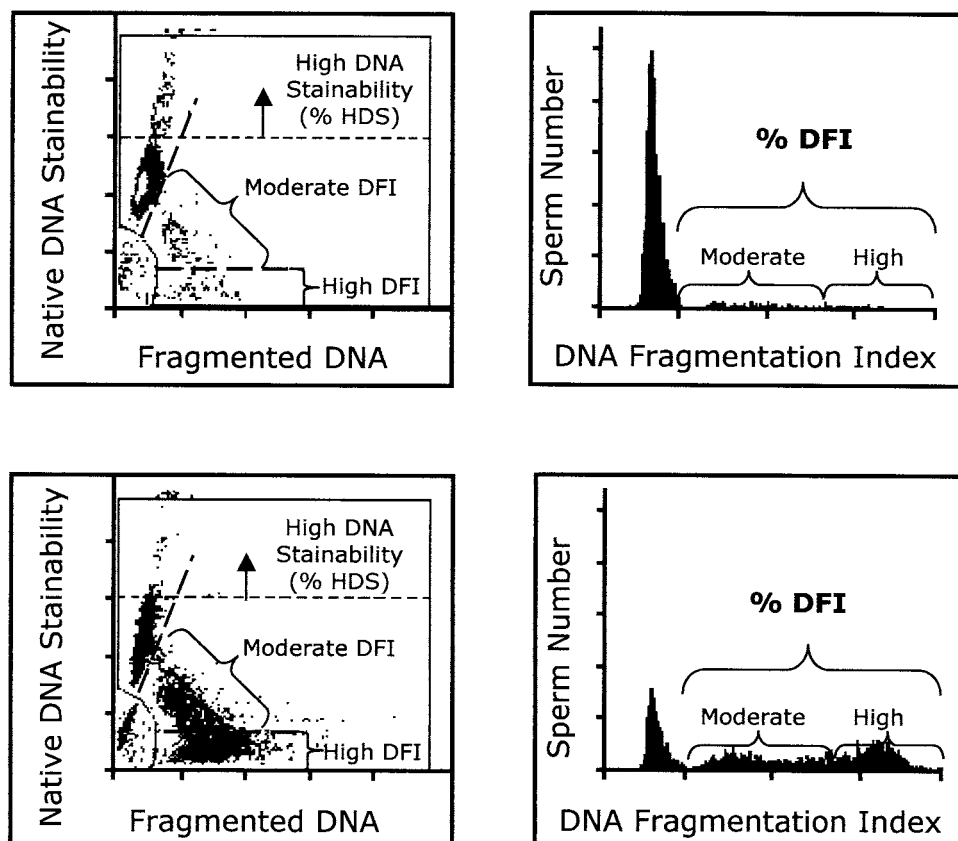
Assisted Reproductive Techniques

Oocytes were retrieved from women (mean age $\pm$ SD, 32.4 ± 4.5 years; age range, 23–41 years) after ovarian stimulation for 7–11 days with hMG (Repronex; Ferring Pharmaceuticals, Tarrytown, NY) and/or recombinant FSH (Gonal-F; Serono, Randolph, MA), followed by a single injection of hCG (Profasi, Serono) 35 hours before oocyte retrieval. Patients were suppressed with luteal phase GnRH agonist (Lupron; TAP Pharmaceuticals, Abbott Park, IL) before stimulation.

Sperm were prepared for insemination by centrifugation over a two-step density gradient (Enhance-S Plus; Conception Technologies, San Diego, CA). For conventional IVF, oocytes were inseminated 40–42 hours after hCG with 10^5 motile sperm per 0.9 mL of insemination medium (IVC-Two medium; InVivoCare, San Diego, CA; supplemented with 10% synthetic serum substitute; Irvine Scientific, Santa Ana, CA). Intracytoplasmic sperm injection was performed 40–44 hours after hCG was administered. Oocytes were assessed for fertilization 18 hours after insemination. Fertilized zygotes were cultured in IVC-Two medium + 10% synthetic serum substitute from days 1 to 3 after retrieval. Embryos cultured beyond day 3 were transferred to IVC-Three medium (InVivoCare) with 10% synthetic serum substitute on day 4. Embryos (3.1 ± 0.2) were transferred, usually on day 3 after retrieval, using an Edwards Wallace catheter (Cooper Surgical, Shelton, CT).

FIGURE 1

Sperm chromatin structure assay–derived cytograms (*left*) showing green fluorescence (double-stranded DNA, *y-axis*) vs. red (fragmented DNA, *x-axis*) fluorescence of 5,000 sperm from semen obtained from highly fertile (*top*) and infertile (*bottom*) men. Dots on the cytograms represent single spermatozoa with dual-parameter green and red fluorescence values acquired from 1,000 channels on the flow cytometer. The *horizontal short dashed line* indicates the threshold for HDS, and the *long dashed line* indicates the threshold for elevated DNA fragmentation (DFI). The frequency histogram (*right*) of DFI with brackets indicating where the total percentage of sperm with elevated levels of DNA fragmentation (DFI, channels 250 to 1,000) was divided into two groups: the percentage of spermatozoa with moderately elevated levels of DNA fragmentation (moderate DFI, channels 250 to 649) and the percentage of spermatozoa with high levels of DNA fragmentation (high DFI channels 650 to 1,000).



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In Vitro Fertilization–ICSI Outcomes

The percentage of zygotes that underwent at least one cleavage (cleavage rate) and the percentage of embryos kept in culture beyond day 3 that reached the blastocyst stage by the end of day 6 (blastulation rate) were recorded. Pregnancy was initially determined 14 days after embryo transfer by a positive serum hCG. Ultrasound detection for fetal sac was used to confirm a positive chemical pregnancy. Implantation rate was defined as the number of gestational sacs on ultrasound as a percentage of embryos transferred.

Sperm Chromatin Structure Assay

The SCSA, a flow-cytometric test that measures the susceptibility of sperm nuclear DNA to acid-induced DNA denaturation in situ, was used to determine the percentage of

sperm with elevated levels of DNA fragmentation in raw semen. Sperm chromatin structure assay parameters are based on the red/(red + green) fluorescence intensity of 5×10^3 sperm/sample after a 30-second pH 1.2 treatment and acridine orange staining (24). Acridine orange is a metachromatic dye that fluoresces red when associated with denatured (fragmented) DNA and green when bound to double-stranded (normal) DNA. Therefore, an increase in the percentage of cells with a high ratio of red to green fluorescence indicates an overall increase in DNA fragmentation in the sperm from that ejaculate.

The total percentage of cells with elevated levels of DNA fragmentation (DFI, channels 250 to 1,000) was divided into two groups: the percentage of spermatozoa with moderately

TABLE 1

Relationship between conventional sperm and SCSA parameters (r^2).

Parameter	Moderate DFI	High DFI	DFI ^a	HDS
Concentration, $\times 10^6/\text{mL}$	NS	0.11 ($P=.002$)	0.08 ($P=.006$)	0.05 ($P=.037$)
Motility, % motile	0.12 ($P<.001$)	0.19 ($P<.0001$)	0.18 ($P<.0001$)	NS
Strict morphology, % normal (P)	0.14 ($P=.0004$)	0.29 ($P<.0001$)	0.25 ($P<.0001$)	NS

Note: NS = not significant; $P>.05$.^aTotal percentage of sperm with elevated levels of DNA fragmentation.Larson-Cook. Assisted reproduction and sperm DNA damage. *Fertil Steril* 2003.

elevated levels of DNA fragmentation (moderate DFI, channels 250 to 649) and the percentage of spermatozoa with high levels of DNA fragmentation (high DFI, channels 650 to 1,000; Fig. 1). In addition, the SCSA identifies ejaculates with an increased percentage of sperm with high DNA stainability (HDS). These sperm are thought to be immature with incomplete chromatin condensation (24).

Statistical Analysis

The likelihood ratio χ^2 test was used to determine whether the SCSA parameters (DFI, moderate DFI, high DFI, and HDS) provided statistically significant thresholds for pregnancy. Regression analysis was used to determine the relationship between SCSA parameters and ART outcomes including fertilization rates, embryo grade, and implantation rates. Regression analysis was also used to determine the strength of the relationship between SCSA and conventional semen parameters (Table 1). Analysis of variance was used to test for differences in the conventional semen parameters, male age and female age between couples with and without a pregnancy (Table 2). To identify the clinical utility of the SCSA test, the positive predictive value, negative predictive value, sensitivity, and specificity were calculated for each SCSA parameter (Table 3).

TABLE 2

Fertility values between couples with and without a clinical pregnancy.

Parameter	Pregnant (n = 28)		Not pregnant (n = 61)	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
Female age	32.0 $\pm$ 3.7	27–39	32.6 $\pm$ 4.8	23–41
Male age	34.3 $\pm$ 5.1	27–47	34.4 $\pm$ 5.6	22–48
Density (M/mL)	92.6 $\pm$ 61.5	4–235	84.4 $\pm$ 54.0	2–281
Motility (%)	60.9 $\pm$ 7.4	40–70	60.0 $\pm$ 10.4	19–76
Strict morphology (%)	6.9 $\pm$ 2.7	2–13	7.3 $\pm$ 2.5	2–13

Note: Fertility values did not vary significantly between pregnant and nonpregnant groups ($P>.05$).Larson-Cook. Assisted reproduction and sperm DNA damage. *Fertil Steril* 2003.

RESULTS

Fertilization rate ($72.5\% \pm 0.2\%$ overall) was not related to DFI, moderate DFI, high DFI, or HDS (Fig. 2). All but 12 of the 89 patients had 100% cleavage rates, and cleavage was not related to SCSA parameters. Forty-six couples had embryos maintained in culture beyond day 3. In all but 8 cases, these were spare embryos remaining after day 3 embryo transfers. Blastulation rate ($36.5\% \pm 5.2\%$ overall) was not significantly related to SCSA parameters.

Sperm chromatin structure assay parameters predicted a 0 implantation rate and no pregnancy (confirmed by ultrasound) after ART. One patient achieved a chemical pregnancy with $>27\%$ DFI but subsequently lost the pregnancy before ultrasound. All 28 patients who achieved a pregnancy had SCSA values lower than the DFI (27%, $P<.01$), moderate-DFI (15%, $P<.01$), and high-DFI (15%, $P<.05$) thresholds (Fig. 3A–C). Total DFI, moderate-DFI, and high-DFI thresholds had 100% specificity and positive predictive value for failure to initiate an ongoing pregnancy (Table 3). Because of the many factors that lead to infertility, the sensitivities and negative predictive value of DFI thresholds were low (Table 3). High DNA stainability also appeared to have a threshold (15%) for pregnancy; however, the confidence level was not statistically significant ($P>.05$; Fig. 3D), and the sensitivity and positive predictive value were lower than DFI thresholds (Table 3).

TABLE 3

Performance characteristics table for SCSA and pregnancy.

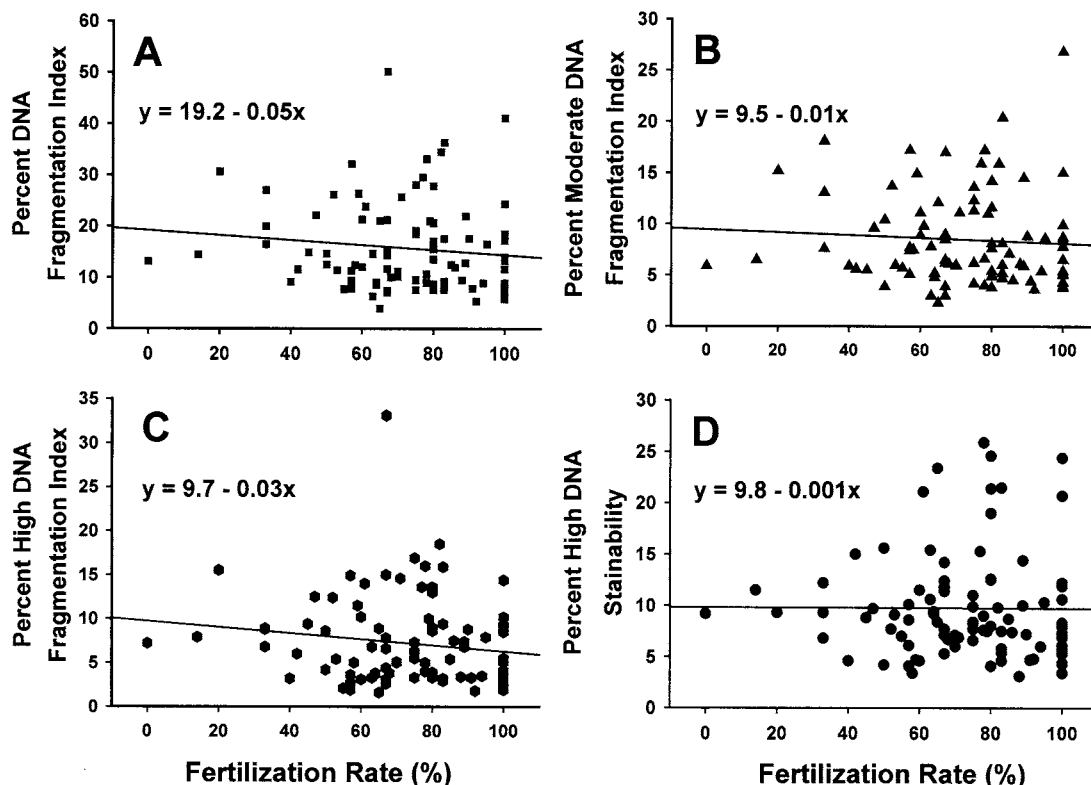
Characteristic	Moderate DFI	High DFI	DFI	HDS
Cutoff value	$\geq 15\%$	$\geq 15\%$	$\geq 27\%$	$\geq 15\%$
Specificity (%)	100.0	100.0	100.0	89.3
Sensitivity (%)	14.8	9.8	16.4	11.5
PPV (%)	100.0	100.0	100.0	70.0
NPV (%)	35.0	33.7	35.4	31.7

Note: PPV = positive predictive values; NPV = negative predictive values.

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FIGURE 2

Regression analysis of fertilization rates and SCSA parameters. Fertilization rate was not significantly ($P > .05$) associated with sperm DNA fragmentation (A–C) or high DNA stainability (D). Moderate and high DNA stainability did not significantly strengthen the relationship between DNA fragmentation and fertilization rates (B, C).



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Dividing the total percentage of cells with elevated levels of DFI into moderate and high categories (moderate DFI and high DFI) did not improve the specificity, positive predictive value, or statistical significance of the likelihood ratio χ^2 test. This indicates that the percentage of spermatozoa with moderate and high DFI increased in proportion to the total percentage of cells with elevated levels of DFI.

Sperm chromatin structure assay and conventional semen parameters were only weakly correlated (Table 1). Specifically, only 3 of the 10 men with poor SCSA test results ($>27\%$ DFI) had asthenozoospermia ($<50\%$ motility) and/or oligozoospermia (<20 million per milliliter), and 2 men had abnormalities in both sperm density and motility (oligoasthenospermia). Three of the men with poor SCSA results had no abnormalities in their conventional semen analysis. Male age was not significantly related to SCSA parameters; however, this patient population did not include any men aged >48 years. The mean female or male age was not significantly different in the 10 patients with SCSA values above vs. below the total-DFI threshold. Furthermore,

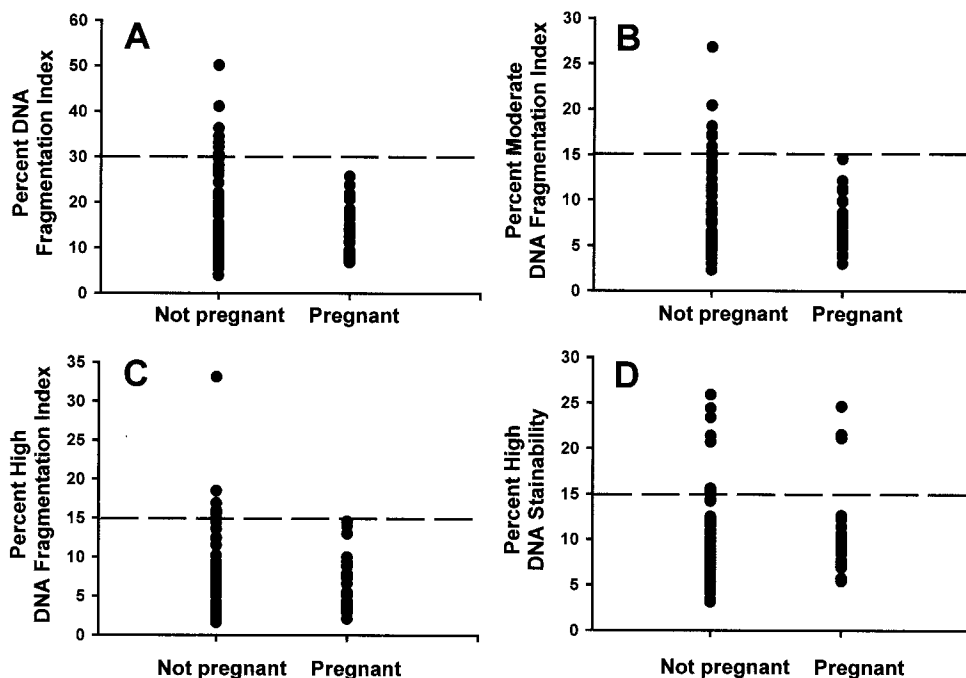
conventional semen analysis as well as male and female age were not significantly different between couples who did and did not initiate a clinical pregnancy (Table 2).

DISCUSSION

Sperm nuclear DNA fragmentation thresholds significantly predicted negative pregnancy outcomes in couples ($n = 89$) attempting pregnancy via ART. Sperm chromatin structure assay analysis was completed on a semen aliquot taken from the ejaculate used for ART, which is important because of the small but consistent percentage of men with fluctuating SCSA results (Evenson D, unpublished observations). These data confirm previous publications reporting that $\geq 30\%$ (11) and $>40\%$ DFI (17) results were significantly related to in vivo male subfertility. More important, these results confirm our previously published pilot studies that found no pregnancies if DFI values were $>27\%$ (12). The current and aforementioned (12) ART studies showed that of 113 IVF and IVF-ICSI cases, there were no clinical

FIGURE 3

No pregnancies when DNA fragmentation is above SCSA-derived clinical thresholds in pre-washed semen. (A), $\geq 27\%$ DFI ($p < .01^*$). (B), $\geq 15\%$ Mod DFI ($P < .01^*$). (C), $\geq 15\%$ High DFI ($P = .03^*$). Asterisk (*) denotes that the logit estimators use a correction of 0.5 for cells that contain a zero. (D), $\geq 15\%$ HDS was not a significant predictor of pregnancy.



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pregnancies if the DFI of the sample used for the procedure was $>27\%$.

The level of sperm DNA fragmentation (percentage DFI) was not strongly related to conventional semen parameters. In addition, unlike SCSA results, conventional semen parameters did not have an important influence on the outcome of ART, confirming results of studies of ICSI patients that were reported by Nagy et al. (4, 25). Thus, SCSA results provided significant additional prognostic information with regard to negative pregnancy outcome in men with high levels of sperm DNA fragmentation.

In contrast to the strong predictive value of the SCSA for negative pregnancy outcome, fertilization rate was not associated with sperm DNA fragmentation or high DNA stainability. This indicates that normal fertilization does not ensure high-quality DNA in the paternal genome and supports previous studies that showed no relationship between fertilization rate and DNA fragmentation (8, 13, 26). In contrast to these reports, other investigators have shown that there is a significant, negative correlation between sperm DNA fragmentation and IVF (27) and ICSI (5) fertilization rates. However, the correlation coefficient for ICSI was relatively small ($r = -0.23$; $P = .0117$), indicating that 5.3% of the

variation in fertilization rates could be predicted by sperm DNA fragmentation levels (5). Although statistically significant, this analysis provides little clinically applicable prognostic information.

Our results confirm data of other investigators who showed that DNA-damaged sperm negatively affect preimplantation and postimplantation embryonic development (13, 28). In contrast to animal studies presented by Ahmadi and Ng (28), this study failed to show a relationship between blastulation rate and the level of sperm DNA fragmentation. However, the statistical power of the analysis was far too low to exclude a relationship because only five men with DFI of $>27\%$ had embryos cultured beyond day 3. Consistent with the results of Ahmadi and Ng (28), implantation rates were significantly related to the percentage of sperm with elevated levels of DFI.

These data support those from studies elsewhere showing that ICSI overrides safeguards that typically prevent sperm with damaged DNA to fertilize via spontaneous pregnancy or conception after conventional IVF (13, 26). The significant decrease in implantation and pregnancy rates using sperm with DFI indicated that the damaged paternal genome is selected against during embryonic development. This pro-

vides a possible explanation for the lack of evidence for an increased incidence of major congenital malformations among children born after ICSI (29–31) and suggests that microassisted fertilization does not place children at a significantly greater risk for genetic damage. However, another recent study suggested that ICSI may increase the odds of major and minor birth defects (32). This increased risk is believed to be related to parental background factors that required the use of ICSI, not the technique itself (33, 34). The role that high levels of sperm DNA fragmentation plays in these background factors should be evaluated to better understand whether children conceived via ART have a higher rate of chromosome abnormalities (35), childhood cancers (27, 36, 37), and infertility (36), which may not be expressed until the child reaches puberty or adulthood (9).

This study provides new information regarding how the extent of DNA fragmentation in sperm impacts pregnancy rates. We anticipated that there would be a difference in the relationship between ART end points and moderate DFI and high DFI because of the fact that the SD of sperm DNA fragmentation was the most sensitive SCSA parameter in dose–response toxicology studies (39). Instead, data indicated that when >27% of spermatozoa have DNA fragmentation (DFI), the oocyte DNA repair capacity was exceeded, and pregnancy was prevented regardless of the percentage of sperm with moderate DFI or high DFI. This loss of embryos created with sperm samples containing DFI follows evidence that there is a progressive loss of chromosomally abnormal embryos during preimplantation development (40).

The percentage of spermatozoa with DNA fragmentation (percentage DFI) was composed of nearly equal percentages of sperm with moderate (percentage moderate DFI) and high levels of DNA fragmentation (percentage high DFI). Although this was true for the patients included in this study, the percentage of moderate and high DNA fragmentation is not always equal (Evenson D, unpublished observations). Therefore, a future study including patients with an unequal percentage of moderate and high DFI may be used to determine whether these categories provide predictive value superior to that of DFI alone.

The overall results of this study confirm previous studies indicating that SCSA parameters provide independent prognostic information to infertility patients using ART. Furthermore, the prognostic value of the DFI was not improved by ascertaining the extent of DNA fragmentation in spermatozoa. Therefore, DFI was shown to be the most effective quantitative assessment of sperm nuclear DNA fragmentation. It provides clinicians with valuable information regarding significantly decreased fertility potential in men having elevated levels of sperm DNA fragmentation.

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