Sperm deoxyribonucleic acid fragmentation: predictors, fertility outcomes, and assays among infertile males

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Objective: To examine the factors associated with increased deoxyribonucleic acid fragmentation index (DFI), evaluate the pregnancy outcomes of men with increased DFI, and compare three independent DFI assays.
Design: Secondary analysis.
Setting: Nine US-based fertility centers.
Patient(s): Infertile men (N = 147) with sperm concentration $\leq 15 \times 10^6$/mL, motility $\leq 40\%$, or normal morphology $\leq 4\%$ were enrolled. The female partners were ovulatory, ≤ 40 years old, and had documented tubal patency.
Intervention(s): At a baseline visit, the men provided a semen sample. The couples attempted conception without assistance for 3 months and with ovarian stimulation and intrauterine insemination in the subsequent 3 months.
Main Outcome Measure(s): The DFI was analyzed using the sperm chromatin structure assay (SCSA) with increased DFI defined as $>30\%$. The predictors of increased DFI were determined by a multivariable linear regression model. The pregnancy outcomes were compared using the χ^2 test. The independent DFI assays (SCSA, deoxynucleotidyl transferase-mediated dUTP nick end labeling, and Comet) were compared with Pearson and Spearman correlations.

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Result(s): The 19% of men with increased DFI were older (36.0 vs. 33.0 years) and had lower total sperm motility (38.2% ± 20.5% vs. 45.2% ± 15.6%). Increased male age was found to be a significant predictor of DFI (0.75, 95% confidence interval [0.06, 1.45]). Increased DFI was not associated with conception or live birth. There was a modest correlation of the deoxynucleotidyl transferase-mediated dUTP nick end labeling assay with the SCSA (r = 0.34) and Comet assay (r = 0.19).
Conclusion(s): Older age was associated with increased DFI among infertile men. The DFI assays were only weakly correlated, indicating a standard definition of DFI is needed to truly interrogate how sperm deoxyribonucleic acid fragmentation impacts male fertility.
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Key Words: DNA sperm fragmentation, male factor infertility, DNA fragmentation indexes

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Traditionally, male infertility was diagnosed on the basis of the results of a semen analysis, including sperm concentration, motility, and morphology. The predictive value of a conventional semen analysis for male fertility potential is poor, and therefore new markers for determining male infertility are needed (1, 2). In theory, abnormalities in sperm deoxyribonucleic acid (DNA) integrity would be expected to be associated with reproductive outcomes (3). Reactive oxygen species and oxidative stress are responsible for DNA fragmentation (4), which could introduce DNA damage into the embryo and, without adequate self-repair, could lead to the disruption of embryo development in both the preimplantation and postimplantation stages of early human development (5). Understanding the factors associated with increased sperm DNA damage can potentially lead to improved counseling and treatments for couples with male factor infertility.

The effect of increased DNA fragmentation on reproductive outcomes was evaluated in both intrauterine insemination (IUI) cycles and in vitro fertilization (IVF) cycles; the findings were conflicting, because some studies indicated a detrimental effect of increased DNA fragmentation on outcomes, whereas others found no effect (6–10). Furthermore, if an increased DNA fragmentation index (DFI) was noted, some studies demonstrated that using intracytoplasmic sperm injection with IVF cycles could improve outcomes (11). In addition, an increased DFI was reported to be associated with higher rates of pregnancy loss (12–16). A meta-analysis of 10 randomized trials found that an increased DFI was associated with poorer reproductive outcomes in IUI cycles (6), but a more recent meta-analysis reported no difference in IUI outcomes (9). Both of these meta-analyses included studies that used differing assays and definitions for measuring DNA fragmentation, making the interpretation of the findings difficult.

Multiple assays exist for measuring sperm DNA damage, including the sperm chromatin structure assay (SCSA), deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and the Comet assay. Most studies that examined sperm DNA fragmentation used a single method, most frequently either SCSA or TUNEL (17). However, a consensus does not exist as to which method is preferred, nor are there common threshold definitions for increased DFI for each assay, thereby complicating the interpretation of the data and the implementation of DNA fragmentation testing for infertility.

Using the data from participants in the Males, Antioxidants, and Infertility (MOXI) trial, a multicenter, randomized, placebo-controlled trial conducted by the Reproductive Medicine Network of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (18), we sought to examine the factors associated with increased sperm DNA fragmentation from a baseline sperm sample in infertile men. Secondarily, we aimed to determine whether an increased DFI was associated with subsequent pregnancy and live birth outcomes in non-IVF cycles. Furthermore, we sought to compare the results obtained using the most common three assays for sperm DNA fragmentation in a single population with known male factor infertility.

MATERIALS AND METHODS

Study Design

This secondary analysis included 147 participants enrolled in the MOXI randomized, controlled trial (clinicaltrials.gov NCT02421887). The trial, analysis, design, baseline characteristics, and trial outcomes of the participating couples were previously published (18). All participants provided written informed consent, and institutional review board approval was obtained at each participating study site. Participating men were ≥18 years of age and had at least one abnormal semen parameter according to the World Health Organization criteria (sperm concentration $\leq 15 \times 10^6$/mL, total motility ≤40%, normal morphology [Kruger] ≤4%) on semen analysis within the past 6 months. Female partners were ≥18 years of age and ≤40 years of age with regular menstrual cycles, showed evidence of ovulation, and had a normal uterine cavity with at least one patent fallopian tube (18). The men were randomized to treatment with a commercially available combination antioxidant formulation or placebo. The couples attempted conception for 3 months using ovulation predictor kits and timed intercourse. The couples who did not conceive after three months of timed intercourse underwent ovarian stimulation (OS)-IUI for three cycles or until pregnancy was achieved.

Methods

The baseline demographics, complete medical and fertility history, and lifestyle and environmental questionnaires for all participants were obtained using standardized forms at enrollment (18). At visit 1 before randomization, a semen

analysis was performed, and 3 aliquots of neat semen were stored at −80°C and shipped to the Utah Andrology Laboratory for DNA fragmentation analysis by SCSA (19), TUNEL, and Comet assays (20). To assure the quality of the DFI testing, all samples were analyzed at the same time. Control samples for each assay with known low, moderate, and high sperm DNA damage were analyzed after the reagents were prepared.

Sperm chromatin structure assay. The SCSA assay was performed as previously described by Simon et al. (20). Briefly, a small aliquot of semen was diluted, treated with an acid detergent solution, and stained with purified acridine orange in a phosphate-citrate buffer, pH 6.0. The cells were analyzed using a flow cytometer (Accuri C6; Accuri Cytometers, Inc., Ann Arbor, MI) that was equipped with an air-cooled argon ion laser.

TUNEL assay. The TUNEL assay was performed using the In-situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) as previously described by Chohan et al. (21). A total of 200 sperm per individual slide were evaluated by the same examiner using fluorescence microscopy (20).

Alkaline Comet assay. The alkaline single-cell gel electrophoresis or Comet assay was performed as previously described by Hughes et al. (22) and Donnelly et al. (23). The sperm was considered damaged or normal on the basis of the presence or absence of a visible "comet tail" with 50–100 Comets scored per sample. If the variation in DNA fragmentation was low among the 50–100 comets scored for an individual, the sample was reanalyzed to confirm accuracy.

Data Analysis

This analysis included all subjects for whom a DFI result was available, which included 147 of the 171 total subjects enrolled in the MOXI trial. The subjects were assigned to a cohort of either increased or normal DFI on the basis of the visit 1 SCSA analysis results before the initiation of the study medication for the MOXI trial. The entire cohort was used for the analysis of pregnancy outcomes. In addition, a subgroup analysis was conducted for those subjects receiving placebo to assess the impact of the study medication on pregnancy outcomes.

The SCSA assay is one of the most commonly used assays for both clinical and research purposes, with elevated DFI defined by a range of >25% to 40%. For the data analysis, the investigators defined >30% as elevated DFI for SCSA. On the basis of data from Simon et al. (20), an elevated DFI was defined as >10% for the TUNEL and >82% for the Comet assays. Conception was defined by a rising human chorionic gonadotropin serum level on two consecutive tests 48 hours apart. Pregnancy loss was defined as a nonviable pregnancy before 20 weeks gestational age. Live birth was defined as the delivery of a viable infant after 20 weeks gestation.

Initially, baseline patient demographics and semen analysis parameters were compared among men with and without increased DFI using the Wilcoxon rank-sum test for the continuous variables and the $\chi 2$ or Fisher's exact test for categorical variables, as appropriate. Subsequently, a prediction modeling was conducted using a multivariable logistic regression model with the dependent variable of DFI, modeled as a bivariate variable. A similar linear regression model was created using DFI as a continuous variable. All models included the sperm concentration at visit 1 (million/milliliter), normal morphology at visit 1 (percentage), total motility at visit 1 (percentage), male age, body mass index (BMI), race/ethnicity, duration of infertility, smoking status, alcohol use, presence of a self-reported varicocele, income, education level, insurance type, occupation, fertility-related quality of life (FertiQOL) score, androgen deficiency (Androgen Deficiency in Aging Males) score, sleep survey score (Epworth Sleepiness Scale), sexual function survey score (International Index of Erectile Function), depression (Patient Health Questionnaire-9) score, and sleep apnea questionnaire (STOP-BANG—snoring, tiredness, observed apnea, blood pressure, body mass index, age, neck size, gender) score as potential predictors. Age, BMI, semen parameters, duration of infertility, and questionnaire response scores were analyzed as continuous variables, and the remaining variables were analyzed as categorical variables. All variables were introduced into a multivariable regression analysis in a stepwise fashion, using a P value of $<.10$ to enter and a P value of $<.05$ to remain. The pregnancy outcomes were compared using Fisher's exact test or $\chi 2$ test. Comparisons of the pregnancy outcomes were analyzed in all subjects irregardless of the study arm and then repeated in only subjects in the placebo arm. Pearson and Spearman correlations were used to compare DNA fragmentation among the SCSA, TUNEL, and Comet assays. Analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC). A $P<.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

One hundred forty-seven MOXI participants had DFI testing completed. The mean ±SD DFI, as measured by SCSA, was 22.86% ± 11.98%. Nineteen percent (n = 28/147) had an increased DFI (SCSA >30%) at baseline. Participants with an increased DFI were older and had lower sperm motility but otherwise had similar BMI, racial/ethnic background, sperm concentration, alcohol use, tobacco use, infertility duration, and self-reported presence of a varicocele (Table 1). The groups had similar socioeconomic characteristics as well as similar responses on intake questionnaires (Supplemental Table 1, available online). Forty-five percent (66 of 146) of males had an increased DFI with the TUNEL assay. No differences were noted between participants with normal DFI or increased DFI on the basis of the TUNEL assay (data not shown). Only six males (4.1%) had an increased DFI using the Comet assay. Because of the small number, no additional predictive analyses were performed.

Predictors of Increased DFI

In the multivariable logistic regression model, male age (odds ratio [OR] [95%confidence interval {CI}]: 1.16 [1.02, 1.32]) and sperm concentration (OR [95% CI]: 1.02 [1.017, 1.03]) were predictors of increased DFI (defined as DFI >30% on SCSA). A sensitivity analysis was performed, varying the

TABLE 1

Baseline characteristics for all enrolled male subjects categorized by the SCSA result.

Characteristic	0 < DFI ≤ 30% (n = 119)	DFI > 30% (n = 28)	*P* value
Sperm concentration at visit 1 (10^6/mL)	20.0 (11.0, 40.0)	18.0 (12.0, 51.5)	.62
Normal morphology at visit 1 (%)	5.0 (3.0, 8.5)	5.0 (2.0, 11.0)	.67
Total motility at visit 1 (%)	45.2 ± 15.6	38.2 ± 20.5	.04
DFI by SCSA at visit 1	17.4 (13.0, 23.3)	39.4 (34.8, 46.1)	<.001
Male age (y)	33.0 (30.0, 36.0)	36.0 (32.5, 40.0)	.009
Male BMI	27.7 (24.2, 31.3)	28.0 (24.3, 30.9)	.99
Obesity			.64
No obesity	81/119 (68.1)	20/28 (71.4)	
Class I	23/119 (19.3)	7/28 (25.0)	
Class II	7/119 (5.9)	1/28 (3.6)	
Class III	8/119 (6.7)	0/28 (0.0)	
Ethnicity			.15
Hispanic or Latino	9/119 (7.6)	0/28 (0.0)	
Non-Hispanic	105/119 (19.3)	25/28 (89.3)	
Unknown	5/119 (4.2)	3/28 (10.7)	
Race			.62
White	93/119 (78.2)	19/28 (67.9)	
Black	8/119 (6.7)	3/28 (10.7)	
Asian	6/119 (5.0)	3/28 (10.7)	
American Indian or Alaska Native	1/119 (0.8)	0/28 (0.0)	
Unknown	9/119 (7.6)	3/28 (10.7)	
Mixed Race	2/119 (1.7)	0/28 (0.0)	
History of smoking			.74
Never	71/119 (59.7)	17/28 (60.7)	
Current	15/119 (12.6)	2/28 (7.1)	
Former	33/119 (27.7)	9/28 (32.1)	
History of alcohol use			.48
Never	8/119 (6.7)	0/28 (0.0)	
Current (in the past year)	106/119 (89.1)	27/28 (96.4)	
Former (not in the past year)	5/119 (4.2)	1/28 (3.6)	
Presence of varicocele (self-report)			1.00
Yes	11/119 (9.2)	2/28 (7.1)	
No	108/119 (90.8)	26/28 (92.9)	
Duration of Infertility (months)	24.0 (16.0, 36.0), n = 116	24.0 (13.0, 36.0), n = 26	.95

Note: Categorical variables are presented as number/total (percentage); continuous variables by median (interquartile range). Wilcoxon's rank-sum test was used for the continuous variables, and χ^2 or Fisher's exact test was used for categorical variables. BMI = body mass index; DFI = deoxyribonucleic acid fragmentation; SCSA = sperm chromatin structure assay.

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definition of increased DFI for SCSA at 25% and 40%. At DFI >25%, sperm concentration (OR [95% CI]: 1.01 [1.00, 1.20], P = .04) and male age (OR [95% CI]: 1.15 [1.04, 1.27], P = .009) remained significant; however, at DFI >40%, sperm concentration (OR [95% CI]: 1.011 [1.001, 1.02], P = .026) but not male age remained as a predictor of increased DFI. Only male age was found to be a significant predictor of DFI in the multivariable linear regression model. For every 1-year increase in age, the DFI increased by 0.75% (95% CI [0.06, 1.45]).

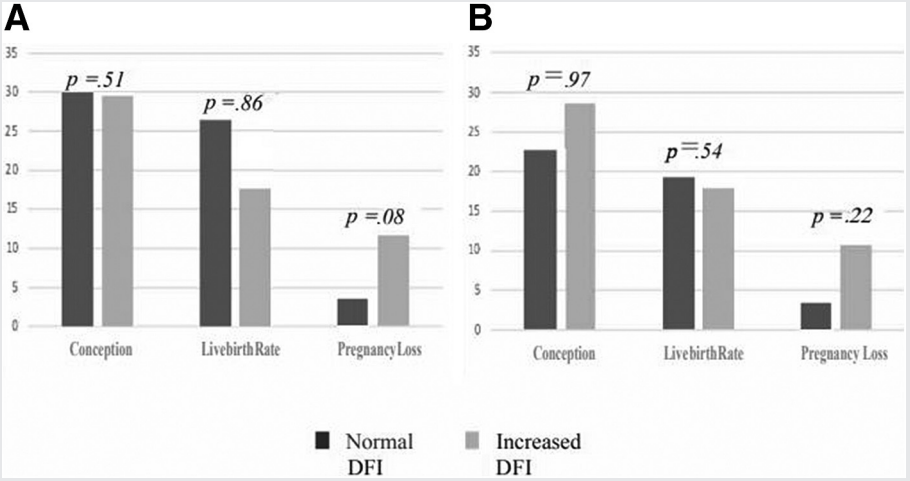
Pregnancy Outcomes

Clinical pregnancy, live birth, and pregnancy loss rates from natural and OS-IUI cycles for couples in which the male partners had normal and increased DFI are presented in Figure 1. There were no statistically significant differences in pregnancy or live birth rates between couples in which the male partner had increased or normal sperm DNA fragmentation by the SCSA assay. More couples with increased DFI achieved pregnancy with natural intercourse (7 of28, 25%) than couples with normal DFI did (5/119, 4.2%, P = .002). There was no difference in pregnancy rate after OS-IUI in couples with normal DFI (22 of 114, 19.3%) compared with that in couples with increased DFI (1/21, 3.6%, P = .08). The results should be interpreted with caution, given the small number of pregnancies in each phase. Pregnancy loss occurred in 37.5% (3 of/8) of couples with increased DNA fragmentation who achieved pregnancy, compared with 11.1% (3/27) of couples with normal DNA fragmentation (P = .083). In addition, there were no significant differences between couples with men with normal DFI or increased DFI by TUNEL in regards to conception (21.2% vs. 25.8%, P = .52), live birth (17.5 vs. 19.7%, P = .73), or pregnancy loss (2.5% vs. 6.1%, P = .41).

Correlation among DFI Assays

The TUNEL assay correlated with both the SCSA (r = 0.34, P<.001) and Comet (r = 0.19, P = .02) assays as continuous variables with Pearson's correlation; however, no correlation was observed between the SCSA and Comet assays (r = −0.004; P = .96) (Table 2). When analyzed using binary definitions of normal and increased DFI with cutoffs for

FIGURE 1



(**A**) The cumulative pregnancy outcomes from timed intercourse and ovarian stimulation-intrauterine insemination cycles in the entire Males, Antioxidants, and Infertility trial cohort who completed DFI testing. (**B**) The cumulative pregnancy outcomes from timed intercourse and ovarian stimulation-intrauterine insemination cycles in couples in which the male partner was in the placebo arm of the Males, Antioxidants, and Infertility trial. The DFI was determined by the sperm chromatin structure assay method. Normal DFI was defined as 0 < sperm chromatin structure assay ≤ 30%. Increased DFI was defined as sperm chromatin structure assay >30%. DFI = deoxyribonucleic acid fragmentation index.

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increased DFI (SCSA >30%, TUNEL >10%, and Comet >82%), no relationship was noted among any of the three assays (Table 2) with Spearman's correlation.

DISCUSSION

We found that male age was predictive of increased sperm DNA fragmentation using a commonly used definition and assay for the DFI. In addition, we reported lower sperm motility in men with an increased DFI. Contrary to our hypothesis, smoking, obesity, or environmental or lifestyle exposures were not associated with increased DFI among infertile males, nor did we find a significant difference in pregnancy outcomes between men with and without an increased DFI. Finally, only a weak correlation was noted among the three most commonly used DFI assays.

Several studies showed that sperm DNA fragmentation was increased in men with male factor infertility (24, 25), and this may have a potential role in the etiology of male infertility (6). Our study found that older male age was associated with an increased DFI. Similar to our study, Das et al. (26) reported an increased DFI in men with increased paternal age in both men with normospermia and men with abnormal sperm parameters. In addition, Belloc et al. (27) found that increasing paternal age was a significant predictor of increased DNA sperm fragmentation in 1,974 men with normospermia. However, not all studies demonstrated the association between DFI and paternal age. Komiya et al. (28) did not find a relationship between paternal age and increased DFI, although this finding was most likely accounted for by an overall younger paternal age in the population studied. Another study reported that men >40 years of age had over twice the odds of increased DFI as men <40 years of age (29). Interestingly, the present investigation assessed predominantly young men (34.2 ± 5.9 years), and despite this younger and more narrowed age range compared with those of other studies, age remained a predictive factor for increased DFI. This finding demonstrates that in men with abnormal sperm parameters, increased age is related to sperm DNA fragmentation.

In addition, we found lower sperm motility in men with increased DFI. Similar to our findings, Belloc et al. (27) and Komiya et al. (28) in addition reported an association between low sperm motility and increased DFI, but the latter in addition reported an association between lower sperm concentration and increased DFI, which was not demonstrated in our study. Our study differs from that of Komiya et al. in that their

TABLE 2

Correlation among the three DFI assays. Data are presented as the correlation statistic (*P* value).

As a continuous variable

	SCSA	Comet
TUNEL	0.35 (<.001)	0.19 (.02)
Comet	−0.004 (.96)	1

As a binary variable

	SCSA	Comet
TUNEL	0.15 (0.07)	−0.05 (0.55)
Comet	0.08 (0.37)	1

Note: Pearson correlation was used for analysis as a continuous variable and Spearman correlation was used for analysis as a binary variable. For SCSA, >30% was considered increased. For TUNEL, >10% was considered increased. For Comet, >82% was considered increased. DFI = deoxyribonucleic acid fragmentation index; SCSA = sperm chromatin structure assay; TUNEL = deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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study included men with both normal and abnormal sperm parameters and enrolled a younger male population. In addition, we found that higher sperm concentration was a predictor of increased DFI, which conflicts with the results of other studies. Although a significant correlation, it was not a strong correlation as that seen with paternal age.

Contrary to our hypothesis, we did not find a predictive relationship between substance use and increased DFI. This differs from the study by Komiya et al. (28), who found a relationship between increased sperm DFI and chronic alcohol use. The studies used different definitions for alcohol use, which may account for this difference. In addition, the amount of alcohol or tobacco use was not quantified in our study; therefore, in our population, the level of alcohol use was unknown and may have additionally affected this result. The effect of substance use on DNA fragmentation may be more apparent when comparing infertile males with fertile controls and requires additional research.

There has been sharpened research focus recently on the relationship between sperm DNA fragmentation and pregnancy outcomes after fertility cycles. We did not find any significant associations in pregnancy outcomes during natural or OS-IUI cycles on the basis of the DFI. Similar to our findings, Muriel et al. (30) in addition found no difference in pregnancy outcomes from IUI cycles when the men had increased DNA fragmentation. In contrast, Belloc et al. (31) and Bungum et al. (32) reported decreased pregnancy rates in IUI cycles when the male partners had higher DNA fragmentation. Both of these studies involved larger sample sizes and included men with and without abnormal sperm parameters. In addition, it is possible that men with abnormal sperm parameters already have a lower pregnancy rate, and the effect of DFI is not additive. Larger effects may be seen in men with normal sperm parameters. Our study lacked adequate power to assess definitively pregnancy outcomes and included cumulative pregnancy outcomes from both unassisted and OS-IUI cycles after subject randomization. Our finding of a higher pregnancy loss rate in couples with increased DFI, although not statistically significant given the small numbers, suggested that we were underpowered for this outcome and call for larger studies in men with abnormal sperm parameters to understand the role of DFI in clinical outcomes from IUI treatments in this patient population.

Discrepancies in the literature regarding the association between increased DNA fragmentation and clinical outcomes may result from using different assays to measure sperm DNA damage. In support of this notion, our study did not find any correlations among the three most commonly used assays when compared as binary variables (i.e., normal vs. increased). However, the TUNEL assay did weakly correlate with both the SCSA and Comet assays when compared as continuous variables. Simon et al. (20) reported that increased sperm DNA damage according to both the TUNEL and Comet assays was associated with poorer outcomes with IVF cycles, but they did not report the same difference with results according to the SCSA assay. This demonstrated that a standard definition and assay are needed to define increased DNA fragmentation to investigate its impact on infertility and its treatments.

The present investigation has several strengthsas follows: reporting on DFI in males with abnormal semen parameters seeking fertility treatments; assessing a vast number of baseline clinical characteristics or exposures that may impact DFI; reporting pregnancy outcomes in non-IVF cycles for couples with male factor infertility; comparison of three commonly used assays for DNA fragmentation testing using the same laboratory with standardized procedures for each method; and use of data collected during a multicenter, double-blind, randomized controlled trial. Despite these strengths, the study was limited by its relatively small sample size and the small number of men with increased DFI. In addition, the men in this study did not undergo an evaluation with a physical exam and self-reported many factors that can affect DFI, which may have impacted the findings. Finally, increased DFI was a parameter for enrollment in the study; however, no subjects were enrolled in the study by increased DFI alone.

CONCLUSION

In summary, we evaluated the relationship of increased DFI with patient characteristics and pregnancy outcomes in infertile men. We found that paternal age was a risk factor for increased DFI, and that tests for DNA fragmentation were not interchangeable. A standard definition of DFI is needed to truly determine the role of sperm DNA fragmentation in male infertility.

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