Original Article

Association of Sperm DNA Fragmentation and Intracytoplasmic Sperm Injection Outcomes in an Age-adjusted Cohort: A Retrospective Cohort Study

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Abstract

Background: Sperm DNA fragmentation index (DFI) is increasingly recognised as a critical parameter in male infertility assessment. However, its prognostic utility in assisted reproduction, particularly in intracytoplasmic sperm injection (ICSI) cycles, remains uncertain. **Aim:** This study aims to evaluate the association between sperm DFI and clinical outcomes following ICSI and to examine whether this relationship is independent of maternal age. **Settings and Design:** A multicentre retrospective cohort study across private fertility centres in India. **Materials and Methods:** A total of 688 couples undergoing ICSI were analysed. Subjects were categorised based on sperm DFI levels (≤15%, 15%–30% and >30%). Semen characteristics and pregnancy outcomes were compared across all DFI groups. Multivariate logistic regression was performed to adjust for female age. **Statistical Analysis Used:** Kruskal–Wallis test, Chi-square test, univariate and multivariate logistic regression analysis. **Results:** Higher DFI was associated with poorer semen parameters, including lower motility, concentration and morphology. There was no statistically significant association between DFI levels and biochemical pregnancy, clinical pregnancy, ongoing pregnancy or live birth rates in couples who conceived after ICSI. These findings remained consistent after adjusting for maternal and paternal age. **Conclusions:** Sperm DFI was related to semen quality but did not independently predict ICSI outcomes. While it may provide adjunctive information in selected cases, it is not a prognostic marker of ICSI success.

Keywords: *Assisted reproductive technologies outcomes, infertility, maternal age, sperm chromatin dispersion assay, sperm DNA fragmentation*

Introduction

The global burden of infertility has continued to rise over the decades, positioning it as a significant domain for scientific exploration. Male partners are responsible for approximately 20% of infertility cases and contribute to 30%–40% of additional cases.[1] The introduction of intracytoplasmic sperm injection (ICSI) was initially regarded as a breakthrough in addressing male factor infertility by improving clinical and neonatal outcomes. However, suboptimal outcomes in certain ICSI cases have prompted further investigation.[2,3] These inconsistencies suggest that factors beyond conventional semen parameters may influence reproductive success, particularly the integrity of the sperm genome.

With the advancement in technologies to study the sperm genome, there has been a growing interest in assessing sperm DNA integrity as a part of the diagnostic work-up for assisted reproductive technologies (ART).

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Sperm DNA fragmentation (SDF) refers to the presence of single-or double-strand DNA breaks in the sperm nucleus[4] and is commonly assessed using the DNA fragmentation index (DFI), which quantifies the proportion of sperm exhibiting DNA breaks.[5] According to the World Health Organization's (WHO) 6th edition manual, several assays are available to evaluate SDF, including terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), single-cell gel electrophoresis (Comet), sperm chromatin dispersion (SCD) and acridine orange flow cytometry.[6,7] Selection of the assay often depends on the clinician's or embryologist's preference and laboratory accessibility, with SCD being the most widely adopted due to its simplicity and practicality.[8,9]

Animal studies have demonstrated a clear association between elevated SDF and compromised reproductive outcomes;[10-12] however, in humans, the evidence remains equivocal. Some studies and meta-analyses have reported a negative association between DFI and key reproductive metrics such as fertilisation, embryo development, pregnancy rates and miscarriage.[13,14] Conversely, other investigations have found no significant correlation between DFI and embryo aneuploidy, blastocyst quality or outcomes in oocyte donation cycles.[15] The heterogeneity in study design, small sample sizes and lack of standardisation limit the generalisability of these findings. Beyond these, the age of the female partner is thought to be another confounder that may influence the outcomes of assisted reproduction in males with high DFI. Liang X observed that the adverse outcomes associated with high DFI are only observed in couples where the female age is >35 years of age, but not in younger women.[16] Along with same lines, Horta F hypothesised that the aged oocytes have decreased DNA repair capacity and hence lead to an adverse outcome in assisted reproduction.[17]

Akin to maternal age, another variable thought to influence the association between DFI and ART outcomes is the method of fertilisation. Studies utilising conventional IVF have consistently reported a negative association between high DFI and pregnancy or live birth rates (LBRs). However, such associations were not observed in ICSI cycles,[18] while other reports, including more recent work, have demonstrated adverse effects of elevated DFI even in ICSI.[19,20] These discrepancies are unlikely to arise solely from methodological variations or differences in inclusion criteria; maternal age may also act as an influencing factor. Nevertheless, limited evidence exists regarding the impact of SDF on ICSI outcomes, specifically in younger couples. Such information is crucial for counselling and development of personalised approaches in young couples with high DFI.

In the present study, we aimed to explore the association between sperm DFI and clinical outcomes in couples undergoing ICSI. As female age is a well-recognised confounder in ART outcomes, this factor was also considered and adjusted for in our analysis to ensure a more accurate assessment of the independent impact of sperm DFI.

Materials and Methods

Ethical approval

The retrospective cohort study was reviewed and approved by the Institutional Review Board and Ethics Committee (IEC Approval No. IIVF-UA00081). All procedures were conducted in accordance with the ethical standards of the responsible committee and with the principles of the Declaration of Helsinki (1975) and its later amendments.

Study design and participants

Retrospective data from 44 centres of our institute between January 2023 and September 2023. This limited time frame was chosen to minimise any unknown variables such as minor changes in protocols over time, varying patient populations and the inability to control for all lifestyle and exposome factors that affect outcomes. Couples undergoing ICSI treatment were eligible if the male partner had undergone SDF testing as part of their clinical work-up following counselling. Eligible participants included females aged 21–40 years and males with normal karyotypes, 2–3 days of sexual abstinence and no prior antioxidant therapy. Only cycles involving transfer of day 5 grade A blastocysts (AA, AB, and BA) were considered. Couples experiencing recurrent pregnancy loss (RPL), recurrent implantation failure (RIF), secondary infertility or advanced paternal age (APA; >40 years) were included if they were undergoing their first ICSI cycle after DFI testing. Exclusion criteria were couples where the male partner had abnormal semen parameters such as severe oligozoospermia, azoospermia, asthenozoospermia or cryptozoospermia

Semen collection, analysis and Sperm DNA fragmentation testing

Semen samples were collected via masturbation after 2–3 days of abstinence in sterile containers. Patients completed a structured questionnaire capturing clinical history, lifestyle habits (alcohol, smoking, recreational drug use and caffeine intake) and occupational exposures. Certified andrologists performed semen analyses in accordance with WHO 2021 guidelines.[21]

SDF testing was performed on raw semen samples before ICSI, on average of 44 days before the treatment cycle, using the SCD assay with the Sperm Chroma kit (Cryolab, India) as per the manufacturer's protocol. The assay differentiates sperm with intact DNA, which exhibit large halos, from sperm with fragmented DNA, which exhibit small or no halos. To minimise inter-observer variability, all andrologists underwent standardisation training against a reference laboratory. Throughout the study period, at each centre, the DFI analysis was done by the same andrologist for all the patients included herein. Representative photomicrographs of normal and high DFI samples are shown in Figure 1, with a 20 μm scale bar applied.

Controlled ovarian stimulation and endometrial preparation

Controlled ovarian stimulation was carried out using recombinant follicle-stimulating hormone (Intas Pharmaceuticals, India) in combination with a GnRH antagonist protocol (BDR Pharmaceuticals, India). Ovulation was triggered with 250 μg recombinant human chorionic gonadotropin (hCG, Bharat Serums and Vaccines, India) or a GnRH agonist once at least three follicles reached 18–20 mm in diameter, and oocyte retrieval was performed 36 h post-trigger.

Endometrial preparation was initiated with oral estradiol valerate (Abbott, USA), with development monitored by transvaginal ultrasonography. Progesterone support was provided using an 8% vaginal gel (Akums Drugs, India) administered twice daily and oral dydrogesterone 10 mg twice daily until the pregnancy test. In positive cases, luteal support was continued until the end of the first trimester. Embryo transfer (ET) was performed when endometrial thickness reached ≥7 mm, with a maximum of two embryos transferred per cycle. Throughout the study period, at each centre, the same clinician performed the ET procedure.

Intracytoplasmic sperm injection procedure and embryo culture

Retrieved oocytes were incubated in one-step culture medium (Vitromed, Germany) at 37°C in a 6% CO_2 environment. ICSI was performed 2-4 h after retrieval using an ICSI workstation (Shivani Scientific, India). Sperm preparation was performed using either a discontinuous gradient (PureSperm, Prime Healthcare, India) or a swim-up method (Vitromed, Germany)

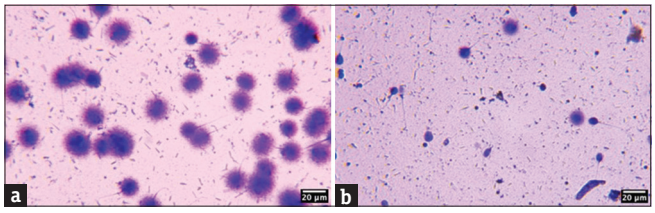


Figure 1: DNA fragmentation index (DFI) detection using sperm chromatin dispersion assay, representative photomicrograph of a sample with (a) normal DFI (b) high DFI. Bar is 20μm

Fertilisation was confirmed by the presence of two pronuclei 17–18 h post-injection. Embryos were cultured to day 5 or day 6; only day 5, grade A[22] embryos were included for analysis. Serum β-hCG testing was performed 14 days post-transfer to confirm biochemical pregnancy. Clinical pregnancy was defined by the presence of a gestational sac with cardiac activity at 6–8 weeks, and pregnancies were followed until live birth.

Study outcomes

The primary outcome was LBR. Secondary outcomes included biochemical pregnancy rate (BPR), clinical pregnancy rate (CPR) and ongoing pregnancy rate (OPR) at 12 weeks. All outcomes were defined as per standard clinical criteria. LBR was defined as the delivery of at least one infant beyond 20–24 weeks of gestation. BPR was defined as the presence of serum β-hCG after 14 days of ET. CPR was confirmed with the presence of a gestational sac detected by transvaginal ultrasound after 6 weeks of ET. Pregnancy that continued beyond 12 weeks of gestation, confirmed by ultrasonography, was determined as OPR at 12 weeks.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 28.0 (IBM Corp., Armonk, NY, USA). Continuous variables were assessed for normality through visual inspection (histograms, Q–Q plots) and numerical methods (skewness and kurtosis). Owing to non-normal distributions in several variables, continuous data are presented as mean ± standard deviation, median with interquartile range and range (minimum–maximum). Categorical variables are expressed as counts and percentages.

Between-group comparisons for continuous variables across DFI categories (≤15%, 16%–30%, >30%) were performed using the Kruskal–Wallis test, with *post hoc* pairwise comparisons where applicable. Categorical variables were compared using Pearson's Chi-square test.

Univariate logistic regression was employed to assess the effect of DFI (as a continuous predictor) on clinical pregnancy, ongoing pregnancy at 12 weeks and live birth outcomes. Results are reported as odds ratios (ORs) with 95% confidence intervals (CIs). Model fit was evaluated using −2 log-likelihood, Cox and Snell R^2 and Nagelkerke R^2 values.

Multivariable logistic regression analyses were further conducted to adjust for potential confounders, incorporating DFI, male age, female age and interaction terms (DFI × female age, DFI × male age) to explore both main and moderating effects on ART outcomes; $P < 0.05$ was considered statistically significant for all the tests.

Results

A total of 1124 patient records were screened from the institute's electronic database. After applying the eligibility criteria and data completeness, 688 couples were included in the final analysis. Patients were stratified into three groups according to sperm DFI levels: ≤15% (n = 179), 16%–30% (n = 380) and >30% (n = 129). A threshold of 30% was used to define abnormal DFI, in line with the manufacturer's protocol, in-house standard operating procedures (SOPs) and published literature.[23]

Baseline demographic and clinical characteristics

The mean male age of the patients involved in this study was 36.72 ± 5.38 years. Comprehensive semen analyses, including volume, pH, concentration, motility and morphology, are presented in Table 1. Semen liquefaction and viscosity were normal in 88.1% and 86.4% of cases, respectively [Supplementary Table 1].

Corresponding female characteristics are detailed in Supplementary Table 2. The mean female age was 33.12 ± 5.20 years. The cohort included 56.1% (n = 386) of cycles using autologous oocytes and 43.9% (n = 302) with donor oocytes [Supplementary Table 1]. The majority of transfers were frozen (74.1%), with double ETs performed in 83.1% and single ETs in 16.9% [Supplementary Table 1]. Embryo development metrics, including blastulation rate and quality, are summarised in Supplementary Table 3.

Analysis of patient characteristics based on DNA fragmentation index

Participants were categorised into three DFI groups: ≤15% (n = 179), 16%–30% (n = 380) and >30% (n = 129). Male characteristics across DFI groups are summarised in Supplementary Table 4. While these were classified as normozoospermic as per the latest WHO standards, patients with DFI >30% were significantly associated with poorer semen quality as compared to men with DFI ≤15%. They had reduced sperm concentration, total motility (especially progressive motility and rapid motility) and fewer proportion of sperm with normal morphology ($P < 0.05$). Head defects and immotility were also more prevalent in higher DFI groups (DFI >30%).

Among female partners [Supplementary Table 4], age, body mass index, antral follicle count and oocyte maturity did not differ significantly across DFI categories. However, AMH levels and endometrial thickness were marginally lower in the >30% DFI group (P = 0.025 and P = 0.024, respectively). Embryo development parameters, including total blastocysts, good-quality blastocysts and blastulation rates, were comparable across all DFI groups [Supplementary Table 4].

Association of sperm DNA fragmentation index and pregnancy outcomes following intracytoplasmic sperm injection

Clinical outcomes stratified by DFI group are illustrated in Figure 2. The BPR was highest in the DFI ≤15% group at 125/179 (68.80%), followed by 241/380 (63.40%) in the DFI 16%–30% group and 80/129 (62.00%) in the DFI >30% group. Similarly, CPR was 108/179 (60.30%)

Table 1: Characteristics of the male partners included in the study

Characteristics	Mean±SD	Median (IQR)	Range (minimum–maximum)
Age (years)	36.72±5.38	36.00 (33.00–40.00)	34.00 (21.00–55.00)
Abstinence (days)	4.21±1.17	5.00 (3.00–5.00)	5.00 (2.00–7.00)
Semen volume (mL)	2.36±1.04	2.10 (1.80–3.00)	12.20 (0.30–12.50)
Semen pH	7.60±0.60	8.00 (7.00–8.00)	9.00 (1.00–10.00)
Sperm concentration (million/mL)	51.66±28.13	50.00 (30.00–70.00)	158.00 (2.00–160.00)
Total sperm motility PR + NPR (%)	43.02±21.03	45.00 (30.00–60.00)	90.00 (0.00–90.00)
Progressive (%)	34.36±18.58	36.00 (21.75–47.00)	85.00 (0.00–85.00)
Rapid progressive (%)	20.60±15.28	19.50 (9.00–30.00)	82.00 (0.00–82.00)
Slow progressive (%)	13.75±10.48	10.00 (6.00–20.00)	64.00 (0.00–64.00)
Non-progressive (%)	8.66±6.58	7.00 (5.00–10.00)	52.00 (0.00–52.00)
Immotile (%)	51.75±22.09	51.00 (40.00–68.00)	99.00 (0.00–99.00)
Morphologically normal (%)	1.70±1.14	2.00 (1.00–2.00)	5.00 (0.00–5.00)
Head defects (%)	50.24±18.61	52.00 (39.00–67.00)	88.00 (0.00–88.00)
Mid piece defects (%)	24.07±9.98	24.00 (18.00–30.00)	75.00 (0.00–75.00)
Tail defects (%)	18.75±11.13	16.00 (11.00–25.00)	80.00 (0.00–80.00)

IQR=Interquartile range, PR=Progressive motility, NPR=Non-PR, SD=Standard deviation

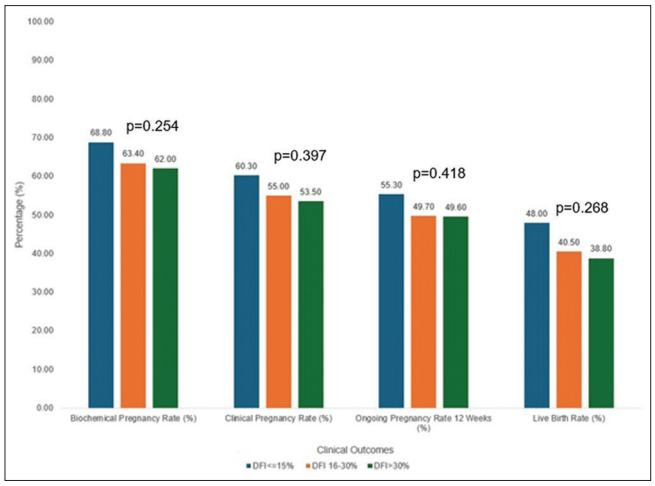


Figure 2: Impact of sperm DNA fragmentation on pregnancy outcomes; $P < 0.05$ was considered significant

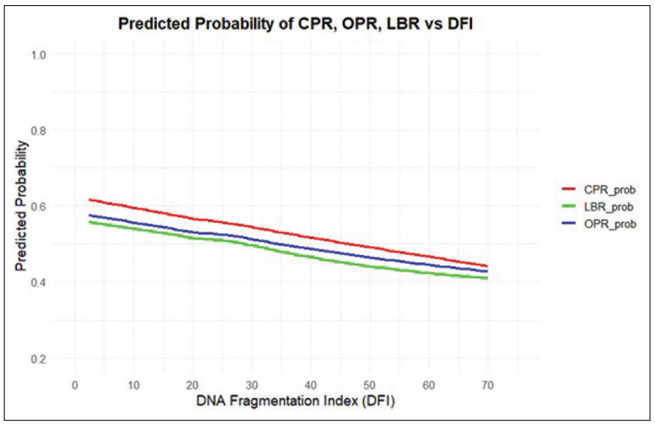


Figure 3: Predictive probability plot. CPR = Clinical pregnancy rate, OPR = Ongoing pregnancy rate, LBR = Live birth rate, DFI = DNA fragmentation index

in the DFI ≤15% group, 209/380 (55.00%) in the DFI 16%–30% group and 69/129 (53.50%) in the DFI >30% group. The OPR also declined across DFI strata, with 99/179 (55.30%) in DFI ≤15%, 189/380 (49.70%) in DFI 16%–30% and 64/129 (49.60%) in DFI >30%. The LBR was 86/179 (48.00%) in the DFI ≤15% group, 154/380 (40.50%) in the DFI 16%–30% group and 50/129 (38.80%) in the DFI >30% group. These differences failed to reach statistical significance ($P > 0.05$).

Univariate logistic regression analysis for predicting the effect of sperm DNA fragmentation index on pregnancy outcomes following intracytoplasmic sperm injection

To evaluate the impact of DFI on treatment outcomes, univariate logistic regression was performed using DFI as a continuous predictor for clinical pregnancy, ongoing pregnancy and LBRs [Supplementary Table 5]. For clinical pregnancy, the regression coefficient (B) was −0.010 (SE = 0.007), with an OR of 0.990 (95% CI: 0.976–1.005), and the association was not statistically significant ($P = 0.188$). For ongoing pregnancy, the regression coefficient was −0.008 (SE = 0.007) OR = 0.992 (95%CI: 0.978–1.006; $P = 0.274$). The live birth outcome yielded a regression coefficient of −0.012 (SE = 0.008) OR = 0.988 (95% CI: 0.974–1.004; $P = 0.133$). The Nagelkerke R^2 values for all models were low (0.003 for CPR, 0.002 for OPR and 0.005 for LBR). The predicted probability plot [Figure 3] illustrates a modest decline in the likelihood of clinical pregnancy, ongoing pregnancy and live birth with increasing DFI values, in alignment with the logistic regression results

Multivariable logistic regression analysis after adjusting for maternal and paternal age

Multivariate logistic regression models were constructed to assess the impact of DFI on reproductive outcomes after adjusting for both male and female age, as well as their interactions with DFI [Supplementary Table 6]. In the clinical pregnancy model, DFI was not significantly associated with outcome (B = −0.047, SE = 0.052; OR = 0.954; 95%CI: 0.861–1.057; $P = 0.367$). Similarly, female age ($P = 0.137$), male age ($P = 0.558$) and the interaction terms (DFI female age, $P = 0.397$; DFI male age, $P = 0.771$) did not demonstrate significant effects. The models for ongoing pregnancy and live birth outcomes showed comparable patterns, with no significant predictors. For live birth, DFI had a regression coefficient of −0.031 (SE = 0.054) OR = 0.970 (95%CI: 0.872–1.079; $P = 0.573$). Across all three models, the Nagelkerke R^2 values remained low (0.009–0.014)

Predictive impact of DNA fragmentation index on live birth rate

A logistic regression analysis was conducted to evaluate the association between elevated DFI levels and live birth outcomes, using the DFI ≤15% group as the reference category. As shown in Table 2, couples with DFI >30% demonstrated 28.9% lower odds of achieving live birth compared to those with DFI ≤15% (OR = 0.70; 95%CI: 0.43–1.13; $P = 0.146$). Similarly, patients in the intermediate DFI group (16%–30%) had 22.8% lower odds of live birth (OR = 0.77; 95%CI: 0.53–1.12; $P = 0.170$). The model demonstrated an adequate fit, as indicated by a null deviance of 864.44 (df = 625), a residual deviance of 861.81 (df = 623) and an AIC value of 867.81. The small difference between the null and residual deviance suggests that the model fits the data reasonably well.

Discussion

This large multicentric cohort evaluated the association between sperm DFI and ICSI outcomes. No statistically significant association was observed between DFI levels and biochemical pregnancy, clinical pregnancy, ongoing

Table 2: Logistic regression predicting live birth from DNA Fragmentation Index categories

Predictors	*B*	SE	OR	95% CI for OR	*P*
Intercept	0.059	0.154	1.06	0.78–1.44	0.699
DFI 15%–30%	−0.259	0.189	0.77	0.53–1.12	0.170
DFI >30%	−0.352	0.242	0.70	0.43–1.13	0.146
Null deviance=864.44 (df=625)					
Residual deviance=861.81 (df=623)					
AIC=867.81					

SE=Standard error, OR=Odds ratio, CI=Confidence interval, DFI=DNA Fragmentation Index, AIC=Akaike Information Criterion

pregnancy or LBRs, even after adjusting for maternal and paternal age.

Routine semen analysis is considered as gold standard to find the cause of infertility and diagnosis of male infertility. However, we observed that although classified as normozoospermic as per WHO standards demonstrated impaired semen quality when DFI was elevated. They were associated with reduced sperm concentration, increased immotility and reduction in morphology as compared to their counterparts that had background rates of DFI (<15%). These findings are consistent with prior reports linking DNA damage to abnormal semen characteristics, likely reflecting defective spermatogenesis and impaired chromatin remodelling.[24,25] Thus, DNA integrity may influence reproductive potential beyond what is captured by routine semen analysis.[26]

The relationship between DFI and ICSI outcomes remains debated. Simon *et al*. in a single-centre study reported that high DFI did not significantly affect live birth after ICSI, though IVF outcomes were negatively influenced.[27] Subsequently, multiple studies with different sample sizes and using either IVF or ICSI reported contradictory findings.[28-30] Therefore, Osman *et al*. conducted a meta-analysis, where they included the data from six studies, which had information from 652 men with low DFI and 169 with high DFI. They observed a very high heterogeneity across the results in different studies and concluded that DFI was not consistently associated with fertilisation failure or adverse pregnancy outcomes after ICSI.[31] In contrast, Agarwal *et al*. noted that elevated DFI is linked with abnormal semen quality and adverse reproductive outcomes.[4] Differences in study design, sample size, patient inclusion and exclusion criteria, heterogeneity in assays used and different thresholds used to classify the patients could be possible reasons for such inconsistent results. This also makes the interpretation of the association of DFI and pregnancy outcomes in ICSI cycles difficult and makes informed decisions in a clinical setup harder. To circumvent some of these variables, we analysed the data of our real-world Indian cohort where DFI testing was performed using uniform methods and the same cutoffs were used to classify the patients. In this dataset, we did not find any significant association between DFI and ICSI outcomes. Based on our observations and those observed previously, we conclude that in patients with high DFI, ICSI would yield a favourable outcomes.

Female age is thought to be a major confounder that has an influence on ART outcomes, where men above 35 years in general have poorer outcomes. Further, it is reported that in couples with advanced maternal age and male partner with high DFI, the reproductive outcomes are poorer.[32,33] Notwithstanding this notion, we failed to detect any association of sperm DFI and ART outcomes after adjustments for female age. These observations imply that in a larger real-world context, female age may not be a major confounder in couples where the male partner has elevated DFI and ICSI is sufficient intervention, and these men need not be subjected to expensive therapeutics.

In the present study, we observed that couples where male partners had higher DFI did not have lower LBRs as compared to their normal DFI counterparts. However, despite the use of ICSI, it is imperative to note that couples with DFI >30% had ~10% lower LBRs than those with DFI ≤15%. Furthermore, even in couples where the DFI was within the normal range but towards the higher side (16%–30%) had LBRs ~7% lower compared to the normal DFI group. This reduction, though not statistically significant, is clinically noteworthy. In real-world practice, even modest differences in live birth probability can impact emotional and financial consequences for patients, and therefore, such findings should not be disregarded. From a clinical perspective, this suggests that while high DFI should not alter the choice of treatment, clinicians may consider DFI as an adjunctive parameter during counselling. In this context, it is also important to note that high DFI is associated with high miscarriage rates and reduced birth weight in the offspring.[23] Therefore, healthcare providers must be aware of these trends for counselling, cycle planning and expectation management.

The 2023 ESHRE guidelines do not recommend the routine use of DFI testing for all infertile men but acknowledge its utility in specific contexts.[34] In the present study, we could not conduct a subgroup analysis of our data due to the limited sample size. Future studies must focus on the impact of sperm DFI and its association with ART outcomes in cohorts of couples with unexplained infertility, RIF, RPL, varicocele and other such conditions. This should allow stratification

and triaging of couples for personalised approaches and help them for better ART outcomes.

The strengths of our study include its multicentric design, large sample size and standardised clinical and laboratory protocols. Adjustments for maternal and paternal age further strengthen the analysis. The main limitation lies in the use of the SCD assay. While the SCD assay is simple, cost-effective and feasible for routine clinical use without the need for expensive equipment, it is prone to high inter-observer variation in halo interpretation.[35] It is also assumed to be less sensitive than assays such as TUNEL or Comet.[36,37] However, comparative studies have shown that these methods, in general, correlate very well and have similar predictive values for DFI.[38,39] Thus, the use of SCD may not be a major limitation in our study. Although intra-observer variations in SCD is a well-recognised problem, the relatively short timeframe of data collection in our study minimised such potential confounders. Nevertheless, these methodological considerations should be kept in mind when interpreting our findings.

DNA breaks can occur either on one or the two strands or on both the strands. Unfortunately, SCD lacks the ability to distinguish between single- and double-strand DNA breaks. Recent studies have suggested that single-stranded and double-stranded DNA breaks have differential impacts on ART outcomes,[40] where it is shown that double-stranded breaks in sperm have more adverse outcomes in ART than single-stranded breaks.[41,42] Future studies must be planned not only to validate this association but also to determine if differentiating single- and double-stranded breaks have any clinical utility in real-world scenarios.

Conclusions

This multicentric cohort study found no statistically significant association between sperm DFI and ICSI outcomes, even after adjusting for maternal and paternal age. Elevated DFI was linked with poorer semen quality but did not independently predict pregnancy or LBRs. These findings suggest that while DFI reflects male gamete quality, it is not a prognostic marker for ICSI success.

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Authors contribution

SN = Concept, Design, Literature search, Manuscript review and editing; TD = Concept, Literature search, Statistical analysis, Manuscript writing; MD = Intellectual content, Statistical analysis, Manuscript review and editing; RS = Data acquisition; CV = Concept, Design, Intellectual content, Manuscript review and editing; MK = Concept; MN = Concept".

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Conflict of interest

There are no conflicts of interest.

Data availability statement

The data set used in the study can be made available on request.

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Supplementary Table 1: Categorical variables	
Characteristics	***n* (%)**
Male characteristics	
Male age (years)	
≤40	499 (72.5)
>40	189 (27.5)
Semen liquefaction	
Abnormal	80 (11.9)
Normal	590 (88.1)
Semen viscosity	
High	91 (13.6)
Normal	579 (86.4)
Female characteristics	
Plan	
Autologous oocytes	386 (56.1)
Donor oocytes	302 (43.9)
Female age (years)	
≤35	442 (64.2)
>35	246 (35.8)
Embryo characteristics	
ET type	
Fresh	178 (25.9)
Frozen	510 (74.1)
ET count	
SET	116 (16.9)
DETs	572 (83.1)

ET=Embryo transfer, SET=Single embryo transfer, DET=Double embryo transfers

Supplementary Table 2: Female patient characteristics			
Characteristics	**Mean±SD**	**Median (IQR)**	**Range (minimum–maximum)**
Female age (years)	33.12±5.20	33.00 (29.00–37.00)	28.00 (21.00–49.00)
Female AMH	2.33±2.22	1.83 (0.80–3.11)	13.95 (0.05–14.00)
Female BMI	25.85±3.27	25.80 (23.92–27.70)	16.20 (18.50–34.70)
AFC	14.67±7.26	14.00 (10.00–18.00)	48.00 (0.00–48.00)
Endo thickness (mm)	8.72±1.06	8.60 (8.00–9.40)	6.00 (7.00–13.00)
Oocyte retrieved	11.68±7.16	10.00 (7.00–15.00)	49.00 (1.00–50.00)
MII	8.37±4.35	8.00 (5.00–11.00)	29.00 (1.00–30.00)
MII rate (%)	67.90±15.90	68.75 (58.03–77.98)	89.00 (11.00–100.00)

IQR=Interquartile range, SD=Standard deviation, BMI=Body mass index, MII=Metaphase II, AFC=Antral follicle count, AMH=Anti-Müllerian hormone

Supplementary Table 3: Embryo characteristics			
Characteristics	**Mean±SD**	**Median (IQR)**	**Range (minimum–maximum)**
Blast	5.29±3.20	5.00 (3.00–7.00)	20.00 (0.00–20.00)
Good blast	2.90±2.18	2.00 (1.00–4.00)	13.00 (0.00–13.00)
Blast rate (%)	64.50±25.49	64.29 (50.00–85.71)	100.00 (0.00–100.00)
Good blast rate (%)	36.90±24.63	33.33 (20.00–50.00)	100.00 (0.00–100.00)

SD=Standard deviation, IQR=Interquartile range

Supplementary Table 4: Patients' characteristics in specific DNA fragmentation index groups

Male characteristics				
Characteristics	**DFI ≤15% (*n*=179), mean±SD**	**DFI 16%–30% (*n*=380), mean±SD**	**DFI >30% (*n*=129), mean±SD**	***P***
Male age (years)	36.04±5.02	36.75±5.45	37.55±5.61	0.091
Abstinence (days)	4.17±1.17	4.27±1.19	4.11±1.15	0.352
Semen volume (mL)	2.50±1.30	2.31±0.92	2.34±0.99	0.524
Semen pH	7.60±0.58	7.66±0.53	7.43±0.79	0.003
Sperm concentration (million/mL)	56.84±26.61	50.62±29.67	47.67±24.72	0.013
Total motility (PR+NPR, %)	47.98±21.04	41.87±20.95	39.54±20.25	<0.001
PR (%)	39.88±18.80	33.19±18.38	30.17±17.27	<0.001
Rapid progressive (%)	24.11±15.92	20.65±15.43	15.66±12.48	<0.001
Slow progressive (%)	15.77±12.34	12.54±9.39	14.50±10.30	0.026
Non-progressive (%)	8.10±6.63	8.68±6.66	9.37±6.29	0.071
Immotile (%)	46.30±20.81	52.42±22.34	57.31±21.60	<0.001
Morphologically normal (%)	1.96±1.17	1.69±1.16	1.43±1.00	<0.001
Head defects (%)	47.35±18.66	51.01±18.72	52.04±17.92	0.019
Midpiece defects (%)	24.81±10.99	23.66±9.78	24.24±9.06	0.555
Tail defects (%)	20.17±11.85	17.94±10.71	19.14±11.19	0.093
Female characteristics				
Characteristics	**DFI (≤15%) (*n*=184), mean±SD**	**DFI (16%–30%) (*n*=389), mean±SD**	**DFI (>30%) (*n*=132), mean±SD**	***P***
Female patient age (years)	32.72±4.95	33.00±5.32	34.04±5.13	0.048
AMH	2.42±2.22	2.35±2.12	2.18±2.51	0.025
BMI	25.67±3.10	25.99±3.41	25.69±3.11	0.574
AFC	15.59±7.98	14.60±6.87	13.66±7.24	0.068
Endo thickness (mm)	8.90±1.14	8.69±1.04	8.57±0.99	0.024
Total number of MII	12.36±6.50	11.64±7.45	10.97±7.16	0.155
Total number of oocytes retrieved	8.77±4.20	8.26±4.41	8.18±4.42	0.270
MII rate (%)	69.44±14.96	67.54±17.08	67.02±13.58	0.534
Embryo characteristics				
Characteristics	**DFI (≤15%) (*n*=184), mean±SD**	**DFI (16%–30%) (*n*=389), mean±SD**	**DFI (>30%) (*n*=132), mean±SD**	***P***
Total number of blast	5.39±3.05	5.31±3.34	5.12±3.00	0.719
Total number of good blast	2.96±2.21	2.84±2.17	3.02±2.22	0.649
Blast rate (%)	62.41±24.90	65.22±25.26	65.32±27.02	0.285
Good blast rate (%)	34.94±22.57	36.94±24.54	39.53±27.44	0.492

PR=Progressive motility, NPR=Non-PR, SD=Standard deviation, DFI=DNA fragmentation index, MII=Metaphase II, AFC=Antral follicle count, BMI=Body mass index, AMH=Anti-Müllerian hormone

Supplementary Table 5: Univariate logistic regression analysis for predicting pregnancy outcomes

Predictors	***B***	**SE**	**Wald**	**df**	***P***	**Exp(*B*)**	**95% CI for Exp(*B*)**
Clinical pregnancy rate							
DFI result	−0.010	0.007	1.73	1	0.188	0.990	0.976–1.005
Constant	0.468	0.186	6.32	1	0.012	1.596	-
−2 log likelihood=941.76, Cox and snell R^2=0.003, Nagelkerke R^2=0.003							
Ongoing pregnancy rate							
DFI result	−0.008	0.007	1.19	1	0.274	0.992	0.978–1.006
Constant	0.265	0.186	2.02	1	0.154	1.303	
−2 Log likelihood=936.25, Cox and Snell R^2=0.002, Nagelkerke R^2=0.002							
Live birth rate							
DFI result	−0.012	0.008	2.260	1	0.133	0.988	0.974–1.004
Constant	0.118	0.194	0.374	1	0.541	1.126	
−2 Log Likelihood=862.15, Cox and Snell R^2=0.004, Nagelkerke R^2=0.005							

DFI=DNA fragmentation index, CI=Confidence interval, SE=Standard error

Supplementary Table 6: Multivariate logistic regression analysis for predicting pregnancy outcomes							
Predictors	***B***	**SE**	**Wald**	**df**	**P**	**Exp(*B*)**	**95% CI for Exp(*B*)**
Clinical pregnancy rate							
DFI	−0.047	0.052	0.815	1	0.367	0.954	0.861–1.057
Female age	−0.079	0.053	2.209	1	0.137	0.924	0.834–1.025
Male age	0.031	0.052	0.344	1	0.558	1.031	0.931–1.143
DFI × female age	0.002	0.002	0.718	1	0.397	1.002	0.998–1.006
DFI × male age	−0.001	0.002	0.084	1	0.771	0.999	0.995–1.003
Constant	1.94	1.34	2.09	1	0.148	6.96	-
−2 Log likelihood=937.15, Cox and Snell R^2=0.009, Nagelkerke R^2=0.012							
Ongoing pregnancy rate							
DFI	−0.035	0.052	0.451	1	0.502	0.966	0.872–1.070
Female age	−0.083	0.053	2.488	1	0.115	0.920	0.830–1.020
Male age	0.039	0.052	0.548	1	0.459	1.039	0.938–1.151
DFI × female age	0.002	0.002	0.790	1	0.374	1.002	0.998–1.006
DFI × male age	−0.001	0.002	0.216	1	0.642	0.999	0.995–1.003
Constant	1.59	1.34	1.403	1	0.236	4.92	-
−2 Log likelihood=931.27, Cox and Snell R^2=0.009, Nagelkerke R^2=0.012							
Live birth rate							
DFI	−0.031	0.054	0.317	1	0.573	0.970	0.872–1.079
Female age	−0.079	0.055	2.063	1	0.151	0.924	0.830–1.029
Male age	0.038	0.054	0.513	1	0.474	1.039	0.936–1.154
DFI × female age	0.002	0.002	0.637	1	0.425	1.002	0.997–1.006
DFI × male age	−0.001	0.002	0.259	1	0.611	0.999	0.995–1.003
Constant	1.31	1.38	0.895	1	0.344	3.70	-
−2 Log Likelihood=857.70, Cox and Snell R^2=0.011, Nagelkerke R^2=0.014							

DFI=DNA Fragmentation Index, CI=Confidence interval, SE=Standard error