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ORIGINAL RESEARCH

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Molecular analysis of sperm DNA fragmentation and apoptotic effects for unexplained infertility leading to recurrent pregnancy loss: a case-control study

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Abstract

Background: Conventional semen analysis often fails to uncover subtle paternal factors contributing to unexplained infertility (UI) and recurrent pregnancy loss (RPL), conditions traditionally attributed to maternal causes. Emerging evidence highlights the significance of sperm DNA integrity, particularly sperm DNA fragmentation (SDF) and apoptosis, as potential underlying factors. This study aimed to evaluate sperm molecular quality in men with UI or RPL despite normal semen parameters. **Methods**: A hospital-based prospective case-control study was conducted at an *in vitro* fertilization (IVF) centre in Delhi, India, involving 106 male participants: fertile controls (n = 52), RPL cases (n = 20), and UI cases (n = 34). All participants met the World Health Organization (WHO) 2021 semen reference standards and had no evidence of male factor infertility. Sperm DNA fragmentation was measured using the Sperm Chromatin Dispersion (SCD) test and Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay, while apoptosis was analyzed by Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (Annexin V-FITC/PI) staining with flow cytometry. Statistical analyses were performed using Analysis of Variance (ANOVA), Kruskal-Wallis H-test, and Chi-square tests, with statistical significance set at $p < 0.05$. **Results**: Despite normal semen profiles, DNA Fragmentation Index (DFI) was significantly higher in RPL ($31.79 \pm 24.23\%$) and UI ($41.38 \pm 16.32\%$) groups compared to controls ($15.62 \pm 5.18\%$) ($p < 0.01$). DFI correlated positively with paternal age ($r = 0.277$, $p = 0.004$) and negatively with progressive motility ($r = -0.203$, $p = 0.043$). Apoptosis levels were elevated in both patient groups, with RPL cases showing the most severe damage. Lifestyle factors, including higher alcohol use in UI and greater smoking prevalence in RPL, also showed associations. **Conclusions**: Sperm DNA fragmentation and apoptosis are overlooked contributors to male infertility in UI and RPL, underscoring the need for molecular sperm function tests beyond routine semen analysis to enhance diagnosis and management.

Keywords

Sperm DNA fragmentation; Unexplained infertility; Recurrent pregnancy loss; Apoptosis; Sperm chromatin dispersion; TUNEL assay; Male factor infertility

1. Introduction

Recurrent pregnancy loss (RPL) refers to the loss of two or more pregnancies occurring from conception up to 20–24 weeks of gestation, before the fetus reaches viability. In contrast, the loss of a single intrauterine pregnancy, confirmed by ultrasonography or histology within the same gestational period, is generally termed a miscarriage [1]. Unexplained infertility (UI), sometimes referred to as subfertility, is diagnosed when couples fail to conceive despite having regular, unprotected intercourse, and no clear cause can be identified. It is considered a diagnosis of exclusion, made only after ruling out known factors such as ovulatory disorders, male factor infertility, and anatomical conditions like endometriosis, cervical or vaginal obstruction, uterine abnormalities, or blocked fallopian tubes [2]. Around 15% of reproductive-age couples are reported to be infertile, with either the male or female partner or both contributing to the condition [3]. In recent years, pregnancy loss has become increasingly common, accounting for approximately 15–25% of clinically recognized pregnancies [4].

The pathophysiology of RPL varies depending on maternal and gestational age; however, different underlying mechanisms often converge on a final common pathway leading to pregnancy loss. Chromosomal abnormalities in the concep-

tus, as well as disruptions in the fetal-maternal interface, are frequently implicated and manifest as clinical symptoms like vaginal bleeding, uterine cramping, and miscarriage [5]. Yet, over 50% of women who experience pregnancy loss have no identifiable risk factors [6, 7]. While maternal factors have received the most research attention, growing evidence suggests that paternal contributions may also be significant. One area of focus is the sperm DNA fragmentation index (DFI), which quantifies the proportion of sperm with DNA damage [8]. A strong association exists between elevated sperm DNA fragmentation and sporadic miscarriage, with a relative risk of 2.16 in a cohort of 2969 couples (95% confidence interval (CI): 1.54–3.03) [9]. In addition to DNA integrity, endocrine markers such as serum 17α-hydroxy-progesterone (17αOH-P) have also been implicated in predicting spermatogenic response to Follicle-Stimulating Hormone (FSH), underscoring the role of steroidogenesis in male infertility [10]. More recent investigations have reinforced this association, highlighting the diagnostic and prognostic role of SDF testing in unexplained infertility and recurrent miscarriage.

Sperm DNA is densely packed to safeguard the paternal genome from external stressors. Although the majority of DNA is tightly condensed with protamines, certain regions remain associated with histones, making them more susceptible to oxidative damage. Fragmentation in these vulnerable regions can compromise embryonic development after fertilization [11, 12].

To address the limitations of conventional semen analysis, the sixth edition of the World Health Organization (WHO) laboratory manual now includes SDF testing in the "Extended Semen Examination" section [13]. However, the manual lacks standardized methodology or defined cut-off values, prompting laboratories to establish their own reference thresholds [14]. Several techniques are available to examine SDF, including sperm chromatin structure assay (SCSA), TUNEL assay, Comet assay, acridine orange test, and sperm chromatin dispersion (SCD) test. Among these, SCD is widely used due to its simplicity, speed, and cost-effectiveness [15]. Studies have shown a strong correlation between SCSA and SCD results [16]. Importantly, elevated SDF is observed in 5–8% of infertile men who otherwise have normal semen parameters and is recognized as a potential cause of infertility. One of the studies also reported increased SDF in 9 of 17 RPL patients [17].

In this study, all male partners underwent clinical and laboratory evaluation, including semen analysis according to the WHO 2021 guidelines, hormonal profiling, and detailed medical history. Men with identifiable causes of infertility such as varicocele, urogenital infections, abnormal semen parameters, chromosomal abnormalities, systemic illness, or history of gonadotoxic therapy were excluded. As no apparent male factor could be identified despite standard diagnostic evaluation, these individuals were categorized as having idiopathic or UI.

2. Materials and methods

2.1 Study population

The study design was a hospital-based analytical prospective case-control study, conducted at an IVF Centre, located in Delhi, India. The recruitment of participants took place between November 2018 to March 2024. For the purpose of this study, RPL and UI patients were selected. There was no indication of risk factors for UI and RPL in the female partners, and all couples (UI and RPL) had normal karyotypes. These included negative antiphospholipid antibody, no endocrine disorders, no uterine structural abnormalities, lupus antibody tests, and normal coagulation. There was no history of chronic illness, varicocele, orchitis, toxic exposure, testicular torsion, testicular trauma or previous gonadotoxic therapy among the male partners. Patients had normal fertility parameters meaning their semen profiles met the WHO 2021 criteria: semen volume of at least 1.4 mL, sperm concentration of 16 million/mL or higher, total motility of 42% or more, progressive motility of at least 30%, sperm morphology of 4% or more (strict Kruger criteria), and vitality above 54%. These values reflect the updated WHO 2021 reference limits for semen analysis, which have been recently re-evaluated for their impact on male infertility diagnostics [7]. These values were confirmed through initial semen screening, and all controls also had normal hormonal levels and no signs of infection, systemic illness, or reproductive dysfunction. Importantly, they were the partners of women who had conceived successfully and were at least 20 weeks into pregnancy, with fertility treatment required only for female-related issues. All participants, including those in the control group, were non-smokers. Semen samples were obtained through masturbation following 3–5 days of abstinence and were allowed to liquefy for 20 minutes at 37 °C before being analyzed.

2.2 Sperm DNA integrity was assessed using two methods

2.2.1 Sperm chromatin dispersion (SCD) test

All SDF measurements were performed in triplicate to ensure precision. Only those with intra-assay variability below 5% were considered valid for analysis [3]. This test is based on the halo test and our findings that sperm nuclei with fragmented DNA either produce very little or no DNA dispersion halos, which are seen in sperm nuclei with non-fragmented DNA following the removal of nuclear proteins, when treated with an acid solution before the lysis buffer [16]. For the assay, sperm suspensions were first adjusted in modified Human Tubal Fluid (mHTF) medium to reach a final concentration of approximately 5–10 million cells per milliliter. The samples were then combined with 1% low-melting point agarose (resulting in a working concentration of 0.7%) at 37 °C. About 50 μL of this mixture was carefully spread onto microscope slides previously coated with 0.65% standard agarose and heat-fixed at 80 °C. After placing a coverslip, the slides were allowed to solidify at 4 °C for 5 minutes. This process, similar in principle to halo or comet assays, provided a gel matrix in which unfixed sperm could be evaluated in a suspension-like environment. Following solidification, coverslips were gently lifted, and the slides were immersed in freshly prepared 0.08 N Hydrochloric

Acid (HCl) for 7 minutes at room temperature (22 °C) in the dark, enabling the formation of limited single-stranded DNA regions at sites of strand breaks. The acid treatment was then neutralized, and nuclear proteins were removed by immersing slides in two consecutive lysis buffers: the first containing 0.4 M Tris, 0.8 M Dithiothreitol (DTT), 1% Sodium Dodecyl Sulfate (SDS), and 50 mM Ethylenediaminetetraacetic Acid (EDTA) (pH 7.5) for 10 minutes, followed by a second solution of 0.4 M Tris, 2 M Sodium Chloride (NaCl), and 1% SDS (pH 7.5) for 5 minutes. After lysis, slides were rinsed with Tris-borate-EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 minutes, dehydrated through a graded ethanol series (70%, 90%, and 100% for 2 minutes each), and finally air-dried before further analysis [18]. This approach has also been supported by more recent studies comparing different methodologies for SDF detection, which confirmed the reliability of SCD and TUNEL assays as practical clinical tools [14]. All SCD slides were independently evaluated by two experienced observers who were blinded to group allocation. Inter-observer discrepancies greater than 10% were resolved by consensus, as manual scoring of halos is known to be operator-sensitive [19].

2.2.2 TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay utilizes terminal deoxynucleotidyl transferase (TdT) to catalyze incorporation of fluorescein-dUTP at 3′-OH ends of single and double-stranded DNA breaks in sperm. After semen liquefaction, samples are washed by centrifugation to isolate sperm from seminal plasma. Cells are then fixed in paraformaldehyde and permeabilized with cold ethanol before labeling. Samples are incubated with TdT enzyme and fluorescein-dUTP at 37 °C for 1 hour to label damaged sites. Flow cytometry then quantified the sperm apoptosis.

To validate the SCD assay results, findings were cross-referenced with sperm morphology and apoptotic markers such as Annexin V/PI. Prior studies have shown strong concordance between SCD, TUNEL, and SCSA methods, with apoptosis as an orthogonal marker of DNA integrity [20, 21].

2.2.3 Annexin V-FITC/PI staining to detect sperm apoptosis

To analyze sperm apoptosis, 1 million sperm cells were isolated from semen samples by centrifugation at 300×g for 7 minutes and washing with cold phosphate buffered saline (PBS). This removed seminal fluid while maintaining live cell membrane integrity. Washed sperm were resuspended in 200 μL of binding buffer. Apoptotic cells were stained by adding 2 μL each of Annexin V conjugated to fluorescein isothiocyanate (FITC: 100 ng/μL) and propidium iodide (PI; 50 μg/mL). Annexin V binds to externalized phosphatidylserine on the membrane surface of apoptotic cells, while PI permeates the membranes of necrotic cells. The stained sample was incubated for 15 minutes at room temperature in the dark. Cells were then immediately analyzed by flow cytometry, measuring Annexin V-FITC fluorescence in the Fluorescence channel 1 (FL 1) channel and PI fluorescence in the FL2 channel [18]. This dual staining paired with standardized protocols enabled differentiation of viable, early apoptotic, late apoptotic, and necrotic sperm subpopulations for each semen sample tested as shown in Fig. 1. Apoptosis samples were run on an Accuri C6 measuring FITC in FL1 and PI in FL2. Flow cytometric analysis was performed on sperm cells stained with Annexin V-FITC and Propidium Iodide (PI) to evaluate apoptosis and necrosis. The scatter plots generated by flow cytometry divided the sperm populations into four quadrants: the lower left (LL) quadrant represented viable sperm cells (Annexin V-/PI-), the lower right (LR) quadrant indicated early apoptotic cells (Annexin V+/PI-), the upper right (UR) quadrant corresponded to late apoptotic or necrotic cells (Annexin V+/PI+), and the upper left (UL) quadrant denoted necrotic cells (Annexin V-/PI+). Panel (A) depicts the negative control (unstained sample), whereas Panel (B) shows a representative patient sample with an increased proportion of apoptotic sperm cells. This dual-staining approach enabled clear discrimination between viable, early apoptotic, and necrotic sperm populations, thereby providing insights into the extent of cellular damage across study groups. Fluorescence channels (FL1 and FL2) were used for detecting Annexin V-FITC and PI, respectively.

2.3 Statistical analysis

Statistical analysis was performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA), and measures' mean and standard deviations were reported for each group. Analysis of Variance (ANOVA) or its non-parametric equivalent was been used to compare means across demographics and clinical parameters, and Pearson's Chi squared test was used to compare three groups for Lifestyle factors. In addition, Kruskal Wallish H-test was used to compare DNA fragmentation indices, where differences were assessed for statistical significance. $p < 0.05$ was considered as statitsically significant and $p < 0.01$ was considered as significant in the Kruskal Wallish H-test.

3. Results

3.1 Study population and group allocation

A total of 106 men were enrolled based on the inclusion and exclusion criteria. Participants were categorized into three groups: 52 men with normal fertility parameters and ongoing pregnancies at 20 weeks (control group), 20 men whose partners experienced RPL (RPL group), and 34 men whose partners were diagnosed with UI (UI group).

3.2 Baseline clinical and lifestyle characteristics

Table 1 presents the demographic, lifestyle, and hormonal profiles of the study participants. No significant differences were found in age, Body Mass Index (BMI), duration of infertility (in the control group. it is based on duration of treatment in purely female factor cases), testosterone, FSH, or Luteinizing Hormone (LH) levels among the groups. However, lifestyle factors revealed notable group differences. The UI group showed a significantly higher prevalence of alcohol

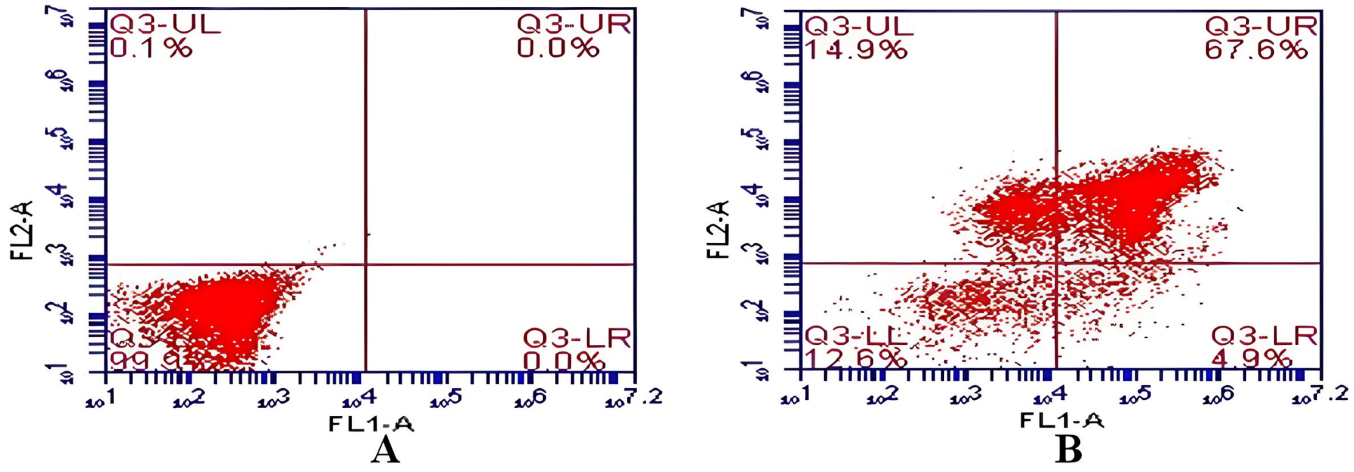

FIGURE 1. Representative flow cytometry diagrams of sperm apoptosis using Annexin V-FITC/PI dual staining. UL: upper left; FL: Fluorescence channel; UR: upper right; LR: lower right; LL: lower left.

TABLE 1. Baseline characteristics of the control and study groups.

Parameter	Control (Mean ± SD)	RPL (Mean ± SD)	UI (Mean ± SD)	*p* value
N	52	20	34	
Demographics				
Age (yr)	34.69 ± 5.58	37.7 ± 4.82	36.71 ± 4.81	0.143[a]
BMI (kg/m^2)	25.53 ± 3.8	26.02 ± 3.69	26.1 ± 3.52	0.613[a]
Duration of Infertility (yr)	2.6 ± 2.5	2.3 ± 3.3	2.7 ± 2.5	0.854[a]
Lifestyle Factors				
Smoking (Yes, %)	47.0%	51.9%	35.0%	0.351[b]
Alcohol (Yes, %)	34.6%	23.5%	45.0%	0.006[b]
Exercise (Yes, %)	15.0%	18.5%	30.0%	0.025[b]
Clinical Factors				
Testosterone (ng/dL)	15.0 ± 3.4	16.0 ± 2.4	16.5 ± 2.3	0.621[a]
FSH (mUL/mL)	7.0 ± 3.7	8.0 ± 4.1	8.5 ± 2.5	0.421[a]
LH (mUL/mL)	5.1 ± 2.1	5.8 ± 3.4	6.1 ± 2.9	0.451[a]

All values are presented as mean ± SD, unless lifestyle factors.
BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; RPL: recurrent pregnancy loss; UI: unexplained infertility; SD: Standard deviation.
[a]*One-way ANOVA (not significant, $p \geq 0.05$).*
[b]*Pearson's Chi Squared test (not significant, $p \geq 0.05$).*

consumption (45%) compared to the RPL group ($p = 0.006$), and a significantly higher frequency of exercise compared to the control group ($p = 0.025$). Additionally, smoking was most prevalent in the RPL group (51.9%), with a significant association observed between smoking and RPL ($p = 0.013$), suggesting a potential male factor in reproductive outcomes [22].

3.3 Conventional semen parameters do not distinguish between groups

Despite these lifestyle differences, semen parameters including volume, sperm concentration, motility, progressive motility, and morphology did not show significant variation between the groups (Table 2). All participants met WHO criteria for normal semen characteristics. This suggests that traditional semen analysis may be insufficient for identifying underlying sperm abnormalities in cases of RPL and UI.

3.4 Elevated DNA fragmentation reveals hidden sperm defects

A striking difference emerged at the molecular level. The DFI was significantly elevated in both patient groups compared to controls: control group (15.62 ± 5.18%), RPL (31.79 ± 24.23%), and UI (41.38 ± 16.32%) ($p < 0.001$). *Post-hoc* analysis confirmed that both RPL and UI groups had significantly higher DFI than controls, despite normal semen

TABLE 2. Baseline semen parameters of controls and cases.

Sperm Parameter	Control (Mean ± SD)	RPL (Mean ± SD)	UI (Mean ± SD)	*p* value
n	52	20	34	
Volume (mL)	2.23 ± 0.84	2.42 ± 0.8	2.35 ± 1.04	0.867[a]
Sperm Concentration (million/mL)	61.46 ± 23.95	53.25 ± 37.87	60.62 ± 38.32	0.593[a]
Total Motility (%)	59.56 ± 9.7	54.9 ± 17.96	56.97 ± 14.52	0.449[a]
Progressive (%)	42.25 ± 8.11	38.25 ± 13.76	38.82 ± 12.08	0.673[a]
Normal Morphology (%)	8.58 ± 3.23	7.1 ± 2.86	7.88 ± 4.04	0.513[a]
DFI (%)	15.62 ± 5.18	31.79 ± 24.23	41.38 ± 16.32	<0.001[b]

All values are presented as mean ± SD, unless indicated otherwise.
[a]*One-way ANOVA (not significant, $p \geq 0.05$).*
[b]*Kruskal Wallish H-test (not significant, $p \geq 0.001$).*
RPL: recurrent pregnancy loss; UI: unexplained infertility; SD: Standard deviation; DFI: DNA Fragmentation Index.

profiles. This finding highlights a disconnect between standard semen parameters and sperm DNA integrity.

3.5 Correlation analysis between DFI, age, and motility

To further explore the factors influencing DNA fragmentation, correlation analysis was conducted. DFI showed a statistically significant positive correlation with paternal age ($r = 0.277$, $p = 0.004$) (Fig. 2), and a significant negative correlation with progressive motility ($r = -0.203$, $p = 0.043$) (Fig. 3). No significant correlations were found with semen volume, sperm concentration, or morphology. These results suggest that age and motility may influence sperm DNA quality, even when overall semen parameters appear normal.

3.6 Sperm chromatin dispersion (SCD) assay links morphology to fragmentation

Using the SCD test, we assessed the relationship between sperm morphology and DNA fragmentation at the single-cell level. Among spermatozoa with normal morphology, 29% showed no fragmentation (Fig. 4A), while 3% had fragmented DNA (Fig. 4B). In contrast, 54% of spermatozoa with abnormal morphology were non-fragmented (Fig. 4C), and 14% were fragmented (Fig. 4D). These results reinforce the importance of examining DNA integrity alongside morphology to gain a complete picture of sperm health.

3.7 Flow cytometry reveals increased apoptosis in RPL and UI groups

Annexin V-FITC and PI dual staining revealed significant differences in sperm apoptosis between groups (Fig. 5). Our study reveals fascinating insights into how sperm cells behave differently across various regions when comparing healthy men with those facing fertility challenges. In the lower left region of sperm cells, we observed a clear hierarchy: healthy controls showed the highest cell viability (30.46%), followed by men with UI (28.78%), and those with RPL showed the lowest levels (15.41%). This pattern suggests that as reproductive problems become more severe, sperm cells in this region become increasingly compromised.

Recent research supports our findings, showing that men from couples experiencing unexplained pregnancy losses have significantly higher levels of sperm damage compared to fertile men. This cellular deterioration appears to manifest differently across sperm regions, with the lower left area being particularly vulnerable.

While all groups showed relatively low percentages in the lower right region, the pattern remained consistent with our overall findings. RPL patients showed the most dramatic reduction (3.43%), suggesting this region might serve as an early indicator of severe sperm dysfunction. Interestingly, this area seems more resistant to damage, possibly representing a cellular compartment with different protective mechanisms. The upper right region proved most intriguing, showing the highest levels of cellular activity across all groups. Here, RPL patients demonstrated dramatically elevated levels (64.6%) compared to both controls (51.4%) and UI patients (47.67%). This finding aligns with established research showing that men with RPL often have significantly increased sperm abnormalities and cellular damage.

This region appears to bear the brunt of cellular stress, potentially serving as a biomarker for identifying men at highest risk of causing pregnancy complications. Studies have consistently shown that elevated sperm DNA damage correlates strongly with increased miscarriage rates, making this finding clinically relevant for counseling couples. Perhaps most-telling was the progressive increase we observed in the upper left region: controls (5.62%) → UI patients (12.36%) → RPL patients (16.54%). This stepwise pattern suggests we may be witnessing the progression of cellular dysfunction as fertility problems become more severe.

4. Discussion

The aim of this study was to evaluate underlying molecular defects in male partners of couples experiencing unexplained infertility (UI) and recurrent pregnancy loss (RPL), conditions where conventional semen parameters often fail to reveal ab-

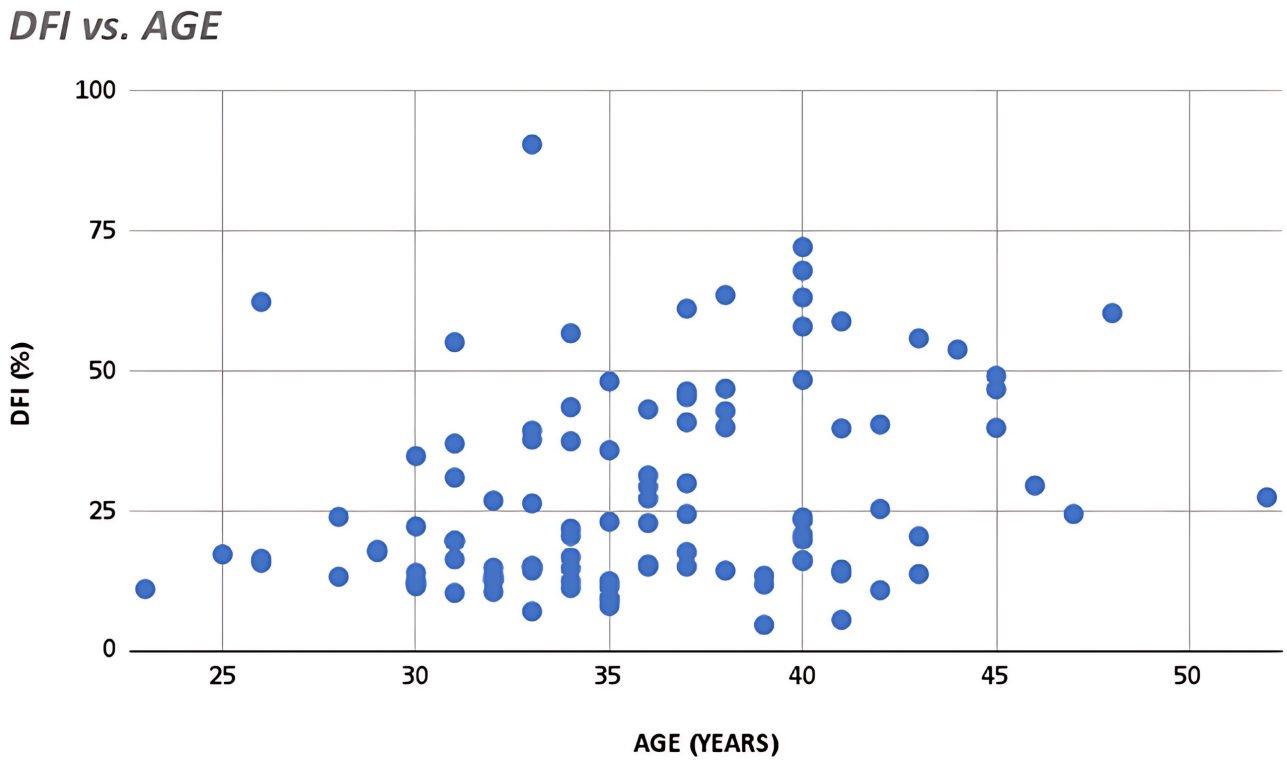

FIGURE 2. Correlation between paternal age and DNA Fragmentation Index (DFI): Scatter plot illustrating the positive correlation between paternal age and sperm DFI across all study participants ($r = 0.277$, $p = 0.004$). Each dot represents an individual subject. The trend line indicates that increasing paternal age is associated with higher levels of sperm DNA damage, supporting age as a contributing factor in idiopathic male infertility and RPL. DFI: DNA Fragmentation Index.

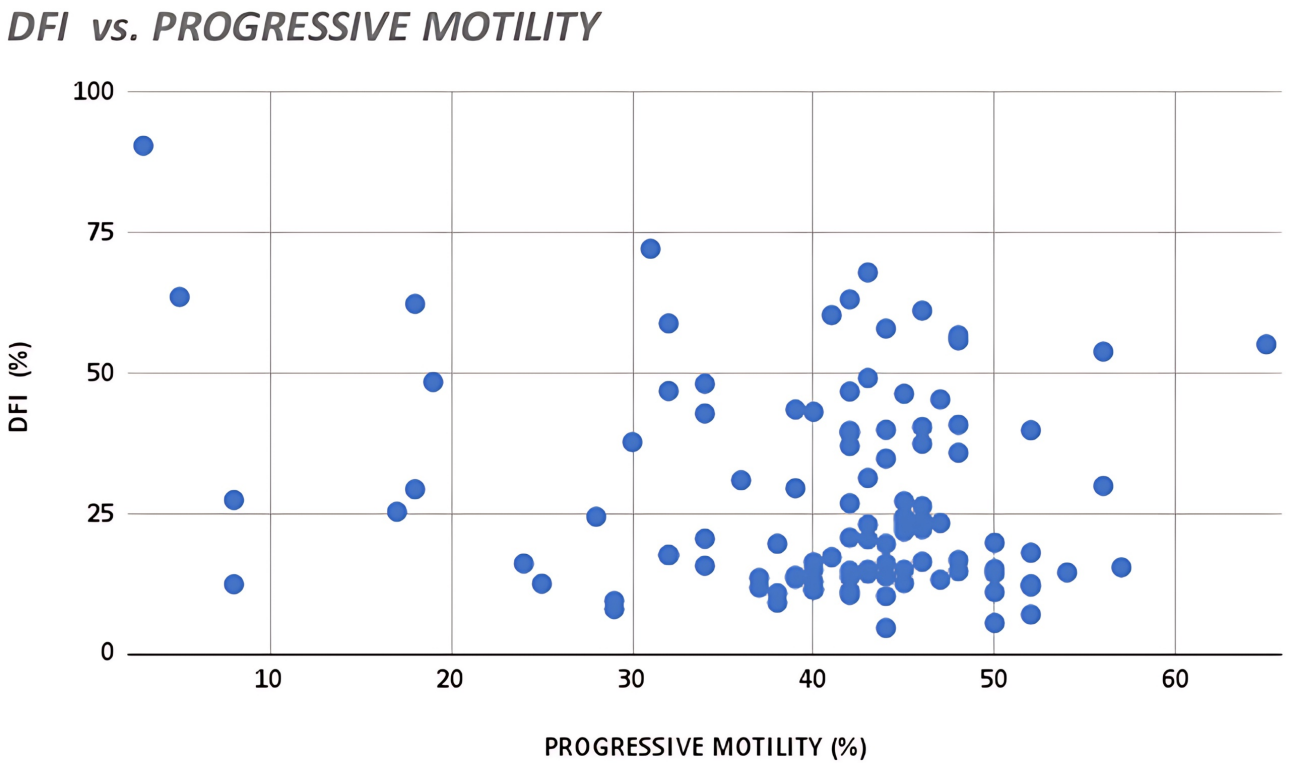

FIGURE 3. The relationship between progressive motility and DNA Fragmentation Index (DFI): scatter plot demonstrating a statistically significant negative correlation between sperm progressive motility and DFI ($r = -0.203$, $p = 0.043$). Each point represents one semen sample. This figure highlights that even in normozoospermic individuals, reduced progressive motility is associated with increased DNA fragmentation. DFI: DNA Fragmentation Index.

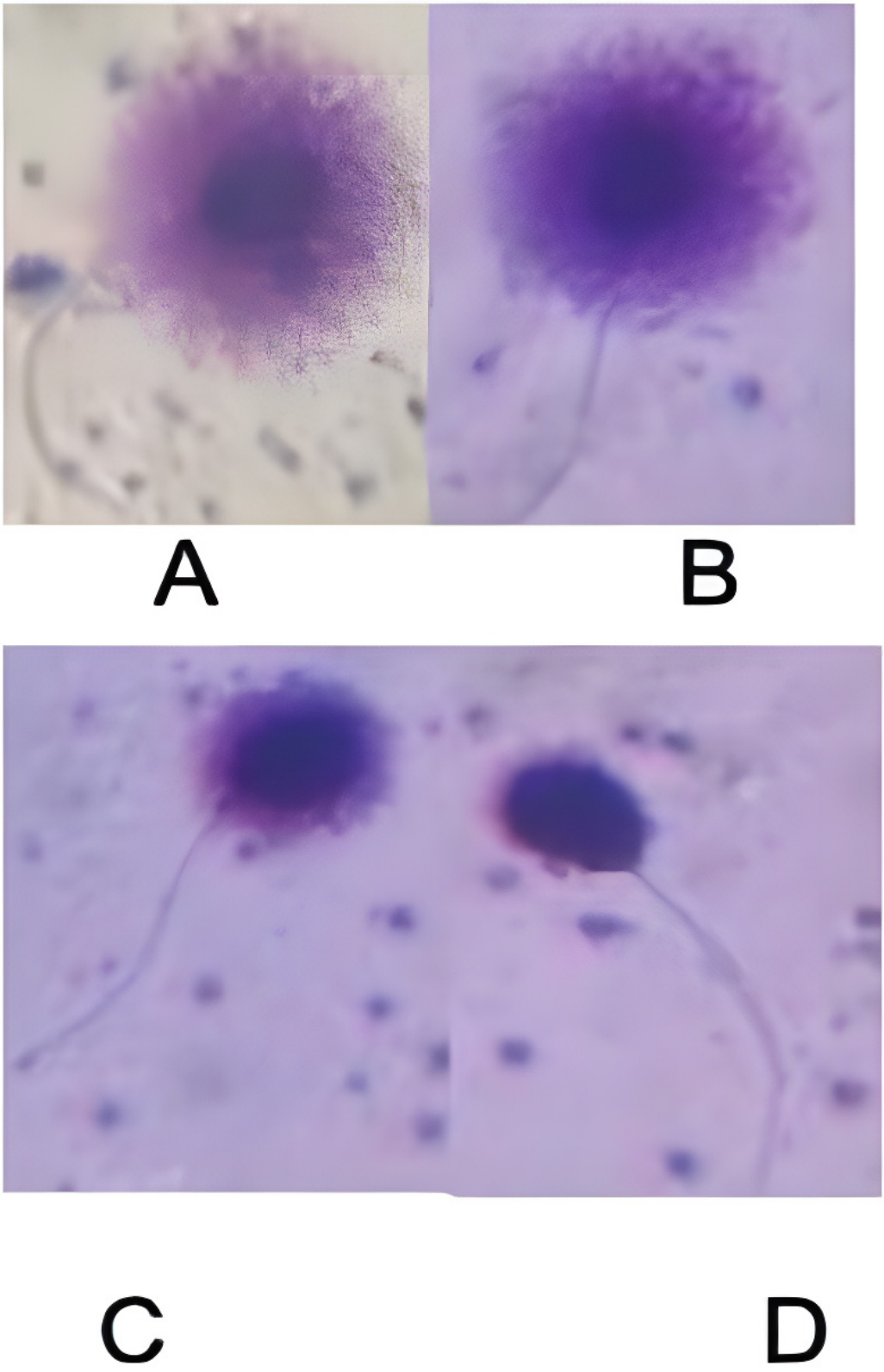

FIGURE 4. Sperm Chromatin Dispersion (SCD) assay showing DNA halo patterns in sperm nuclei: representative micrographs showing four types of halo patterns observed in the SCD test, which reflects the extent of DNA fragmentation in sperm cells. (A) Large halo: Indicates non-fragmented DNA (intact chromatin structure). (B) Medium halo: Mild DNA fragmentation. (C) Small halo: Moderate fragmentation. (D) No halo: Severe DNA fragmentation. These images, taken at 100× magnification, illustrate varying degrees of DNA integrity, with smaller or absent halos suggesting higher fragmentation. The SCD test provides a visual assessment of sperm nuclear DNA integrity.

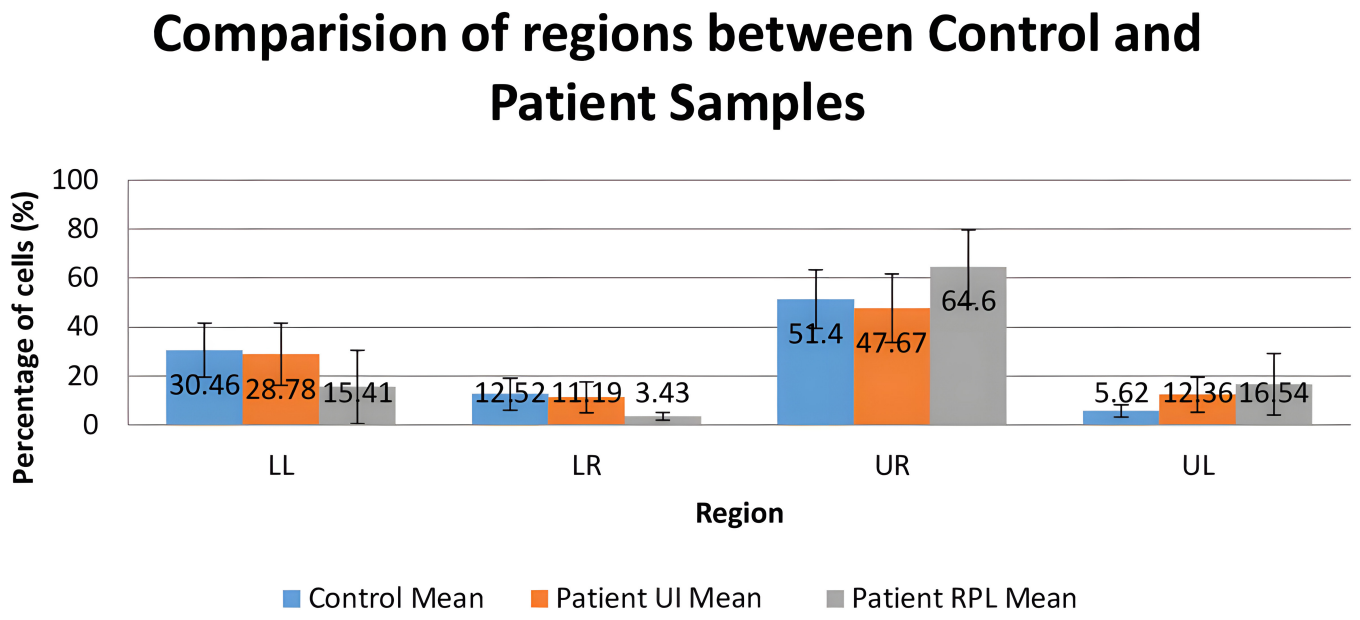

FIGURE 5. Bar chart showing the mean percentage (± standard deviation) of sperm cells in each flow cytometry quadrant: Lower Left (LL, viable cells), Lower Right (LR, early apoptotic cells), Upper Right (UR, late apoptotic/necrotic cells), and Upper Left (UL, necrotic cells with DNA damage but not apoptotic). Blue bars represent the control group, orange bars represent unexplained infertility (UI) patients, and grey bars represent recurrent pregnancy loss (RPL) patients. Statistical analysis was performed using one-way ANOVA, with significant differences observed in the Lower Right quadrant ($p = 0.0019$) and the Upper Right quadrant ($p = 0.0053$). RPL: recurrent pregnancy loss; UI: unexplained infertility.

normalities. While basic semen analysis remains a standard diagnostic approach, our findings reiterate its limitations, as all study groups including RPL and UI exhibited semen parameters within normal WHO reference ranges (WHO, 2021). This reinforces the emerging consensus that traditional parameters alone are insufficient to identify subtle but clinically significant sperm dysfunctions [23].

To address this diagnostic gap, we employed sperm DNA fragmentation (SDF) testing via sperm chromatin dispersion (SCD) and TUNEL-based assays, alongside apoptosis evaluation using Annexin V-FITC/PI flow cytometry. Consistent with previous reports, both UI and RPL groups exhibited significantly elevated SDF indices compared to controls, with the highest DNA damage and apoptosis observed in RPL cases. These findings support growing evidence that compromised sperm DNA integrity is an important contributor to reproductive failure, particularly in the absence of female-factor infertility or detectable abnormalities in routine semen tests.

In our cohort, elevated SDF appeared to occur independently of sperm concentration and morphology, although a modest negative correlation was observed [24] between progressive motility and DFI. This suggests that sperm DNA damage may be influenced by underlying cellular stress mechanisms not captured by basic semen parameters. Oxidative stress (OS), one such mechanism, has been widely implicated in idiopathic male infertility (IMI) and is known to induce both DNA fragmentation and apoptosis in spermatozoa [25, 26]. Ribas-Maynou and Benet have emphasized that different types of SDF such as single-strand and double-strand breaks are linked to distinct clinical consequences and should be analyzed separately where possible [27].

While SCD provided valuable insights into DNA integrity, it did not fully differentiate the RPL group from UI in our study, highlighting the need for orthogonal validation. Flow cytometric analysis of apoptotic markers complemented the SDF data by revealing a higher percentage of early and late apoptotic spermatozoa in RPL cases, indicating more advanced sperm cell damage. These findings underscore the clinical utility of combining molecular markers for a comprehensive assessment of male fertility potential [28].

One challenge in interpreting SDF results is the lack of universally accepted thresholds. Although some studies, suggest a 20% cutoff for infertility risk [29], inter-laboratory variability in assay sensitivity necessitates the use of population- and lab-specific reference values [18]. In our study, we addressed this by establishing baseline thresholds using fertile donor samples, as recommended by the WHO (2021). Additionally, we ensured assay reproducibility through internal quality controls, triplicate testing, and independent blinded slide evaluations [3, 18, 30, 31].

Beyond intrinsic sperm defects, our findings also point to the influence of systemic male health on fertility. Diabetes mellitus, metabolic syndrome, and cardiovascular risk factors have all been linked to increased sperm DNA fragmentation and apoptosis. Chronic hyperglycemia, insulin resistance, dyslipidemia, and vascular dysfunction contribute to oxidative stress and hormonal imbalance, disrupting spermatogenesis even in men with otherwise normal semen parameters [10, 32, 33]. A recent meta-analysis showed diabetic men had significantly higher SDF compared to non-diabetics, while men with metabolic syndrome had nearly 1.85 times greater odds of abnormal DNA fragmentation [34]. Cardiovascular risks

such as hypertension also compromise testicular perfusion and hormonal function [35].

Despite this compelling evidence, comorbidities were not systematically assessed in our study, an acknowledged limitation. Nonetheless, the elevated SDF and apoptosis observed in our cohort suggest the possibility of undiagnosed systemic contributors. This highlights the importance of incorporating metabolic screening and cardiovascular evaluation into fertility assessments, particularly in men with idiopathic infertility [36].

Encouragingly, many systemic conditions impacting sperm quality are modifiable. Studies have shown that improved glycemic control, weight reduction, and exercise can significantly enhance sperm parameters and reduce DNA fragmentation within a few months [37, 38]. These insights emphasize that molecular sperm testing, when combined with broader male health evaluation, can help identify treatable causes of infertility and guide personalized interventions.

In conclusion, our study reinforces the diagnostic value of sperm DNA fragmentation and apoptotic analysis in uncovering hidden male factors in UI and RPL. It also highlights a pressing need to move beyond isolated semen parameters and adopt an integrated, systemic approach to male reproductive health.

Limitations: This study offers valuable insights into the molecular quality of sperm in men with UI and RPL, but a few limitations should be considered. The sample size was relatively small, which may limit how widely our findings can be applied. Larger, multi-centre studies would help confirm and expand on these results. While we carefully excluded female partners with known risk factors, there is always the possibility that subtle or undetected conditions could have influenced the outcomes. Furthermore, our analysis focused primarily on sperm DNA fragmentation and apoptosis, while additional molecular and functional parameters such as oxidative stress markers, mitochondrial function, or epigenetic alterations were not evaluated. Incorporating these complementary assessments in future work could provide a more comprehensive understanding of the mechanisms underlying idiopathic male infertility and recurrent pregnancy loss.

5. Conclusions

This study reveals that SDF and apoptosis are significant but often overlooked contributors to UI and RPL, even when conventional semen parameters appear normal. These findings underscore the limitations of routine semen analysis and highlight the value of incorporating molecular assessments into standard fertility evaluations.

The elevated DNA damage observed in UI and RPL cases points to the need for a broader, more integrative approach to male reproductive health, one that includes advanced sperm testing and screening for systemic conditions like diabetes and metabolic syndrome.

Clinical protocols should embrace SDF testing as a complementary tool, particularly in idiopathic cases. Future research should focus on standardizing assay thresholds, linking SDF levels to artificial reproductive treatment outcomes, and evaluating how targeted lifestyle or medical interventions can improve sperm DNA integrity [39, 40].

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during the current study are not publicly available due to institutional and ethical policies that safeguard patient privacy and confidentiality. Sharing the raw patient data publicly could potentially compromise participant anonymity. However, the datasets are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

AG, NK and RS—Conceptualization. SS—methodology. AG and PP—software, writing-original draft, visualization. NK and RS—validation, supervision. AG and SS—formal analysis. FR—investigation. AG and FR—resources. AG, PP, NK and RS—writing-review & editing. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval and consent to participant: The ethical clearance was obtained from the Independent Ethics Committee, New Delhi registered (Registration ECR/222/Indt/DL/2015/RR-21) with Drug Controller General of India, Directorate General of Health Services, New Delhi as per the Rule 122D of the Drugs and Cosmetics Rules 1945. Informed consent form was obtained to every participant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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