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ASSISTED REPRODUCTION TECHNOLOGIES

# Personalized prediction model for miscarriage: in-depth sperm DNA fragmentation

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## Abstract

**Purpose** We investigated how different types of sperm DNA fragmentation (SDF) in males, in conjunction with varying levels of female reproductive potential, jointly impact ICSI outcomes.

**Methods** We retrospectively analyzed 195 couples undergoing ICSI, categorizing them by normal or poor prognosis according to POSEIDON criteria. Female factors included age, anti-Müllerian hormone (AMH), and oocyte retrieval numbers. Male factors included semen parameters, total SDF, and specific double-strand breaks (DSBs). Reproductive outcomes were followed up at different gestational stages, including clinical pregnancy, early gestation failure, live birth, and miscarriage. Risk factors were identified using univariate and multivariable logistic regression, and their predictive power was assessed via the receiver operating characteristic (ROC) curve.

**Results** In the normal group, female factors were primarily associated with reproductive failures. Non-pregnancy cases had lower AMH (4 ng/mL vs. 3.2 ng/mL), and miscarriage cases had fewer oocytes retrieved (15 vs. 10.5). However, the risk factor profile was distinct in poor prognosis. Male factors, including reduced sperm motility (68% vs. 54.5%), lower normal sperm morphology (5.5% vs. 2.5%), and elevated DSBs (7.5% vs. 18.8%) were linked to miscarriage. DSBs presented as the independent predictor of miscarriage risk (odds ratio: 1.19, 95% CI: 1.04–1.36), with a DSB cutoff of 19% providing 81% accuracy in predicting miscarriage.

**Conclusion** Paternal effect is pronounced in women with poor prognosis, where elevated DSBs are linked to an increased risk of miscarriage. We propose a refined pipeline in which DSB testing is considered as initial evaluation before assisted reproductive treatments, especially for infertile couples with poor prognosis.

**Keywords** Advanced semen analysis · Miscarriage · Prediction · Paternal effect · DNA damages

## Introduction

In standard pipeline of assisted reproductive technology (ART), clinicians refer Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number (POSEIDON) stratification to provide an optimal ovarian stimulation countering different ovarian conditions [1]. The personalized stimulation protocol is effective to increase the probability of obtaining at least one euploid embryo due to a higher oocyte retrieval count [2, 3]. However, it remains a significant challenge to enhance ART success rates in patients with poor ovarian response. To increase the likelihood of live birth, research is being directed toward uncovering the mechanisms of male infertility as a promising approach to improving outcomes in these challenging cases, since female risk factors such as advanced age cannot be modified.

Chun-I. Lee and Tse-En Wang are joint first authors by virtue of their contributions to this work.

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Male infertility is basically categorized based on sperm phenotypic abnormalities, such as oligozoospermia, asthenozoospermia, teratozoospermia, and combined factors. However, relying solely on basic semen parameters underestimates the etiology of male infertility. A significant proportion of men are affected by sperm DNA fragmentation (SDF) with approximately half of men with idiopathic infertility exhibiting a high DNA fragmentation index (DFI). Additionally, 20% of men with normal semen parameters also have elevated DFI results [4].

Sperm DNA damage could originate from abortive apoptosis initiated post-meiotically, inadequate chromatin remodeling during spermiogenesis, and oxidative stress [5, 6]. SDF profiles are typically characterized by the presence of single-strand breaks (SSBs) and double-strand breaks (DSBs). Standard methods for assessing SDF, including TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL), sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA), and comet assay, enable quantification of total SDF, encompassing both single-strand breaks (SSBs) and double-strand breaks (DSBs) [7]. Elevated levels of total SDF associated with reduced fertilization rate [8, 9], impaired blastocyst formation [10], and reduced clinical pregnancy [11, 12]. On the other hand, studies employing a modified comet assay under neutral conditions have identified significant associations between DSBs and recurrent pregnancy loss [13], delayed embryo development [14, 15], and implantation failure [15]. The neutral comet assay was performed to analyze the unique electrophoretic molecular kinetics of DSBs under neutral buffer conditions. DSBs are identified as migrated DNA fragments detached from the sperm nucleoid during electrophoresis [16]. A recently developed technique, sperm DNA fragmentation releasing assay (SDFR), applies a refined gel network rather than relying on electrophoresis, to specifically target DSBs [17]. Due to the characteristic break length of 50 kb for DSBs, the refined polyacrylamide gel confines DSBs near the nuclear core, resulting in a halo-positive presentation; in contrast, intact chromatin remains spatially restricted and does not form a halo. A method comparison table was shown in Supplementary Table 1. Using the SDFR rapid test, a strong association was demonstrated between DSBs and elevated rates of embryonic aneuploidy [18]. We believe that an in-depth analysis of sperm DNA fragmentation, distinguishing between total SDF and DSBs, holds significant potential to enhance diagnostic sensitivity for male infertility and to predict reproductive failure outcomes; however, clinical studies are lacking.

In this retrospective cohort study, we first classified ICSI couples into two groups based on the POSEIDON criteria. We then investigated the relationship between various male SDF profiles (total SDF and DSBs) and reproductive outcomes in cases of normal and poor prognosis population. By examining how SDF interacts with female conditions, we aimed to refine and optimize treatment strategies for poor ovarian responders, considering both maternal and paternal factors.

## Methods and materials

### Ethics statement

The Institutional Review Board of Chung Shan Medical University approved this study (CS2-20,012). All patients underwent comprehensive counseling and provided informed written consent before entering the study.

### Study population

We conducted a retrospective cohort study at the Lee Women's Hospital (Taichung, Taiwan) from May 2020 to March 2023. Patients who underwent autologous ICSI cycles without pre-implantation genetic testing for aneuploidy (PGT-A) were identified. A total of 195 ICSI cycles with confirmed clinical outcomes for pregnancy check and/or implantation assessment were included. Male aged > 60 years were excluded. Eligible cases were classified according to the POSEIDON stratification criteria, considering female age, AMH levels, and the number of retrieved oocytes [19]. Normal prognosis cases ($n = 61$) included females younger than 35 years with an antral follicle count (AFC) ≥ 5 or an Anti-Müllerian hormone (AMH) level ≥ 1.2 ng/mL, and more than nine retrieved oocytes. The remaining 134 cases were classified as poor prognosis, including POSEIDON group 1 [15/134 (11%)], group 2 [31/134 (23%)], group 3 [11/134 (8%)], and group 4 [77/134 (57%)].

### Basic semen analysis

Semen samples were collected after 2 – 5 days of sexual abstinence by masturbation and deposited into sterile cups. After liquefaction at room temperature (RT) within 30 min, an aliquot of the semen sample was loaded into a 10-μm deep chamber slide. Semen parameters, including sperm concentration (M/mL), total motility (%), and progressive motility (%), were measured using a CEROS II device (Hamilton-Thorne, Danvers, MA, USA) according to the manufacturer's instructions.

### Assessment of total SDF

Total SDF was performed following sperm chromatin dispersion (SCD) methodology. Initially developed by Fernández et al. [20], the SCD technique was later modified into the LensHooke® R10 Sperm Chromatin Dispersion Assay

(Bonraybio, Taichung, Taiwan), which offers a rapid test for total SDF. We conducted the test according to the manufacturer's instructions [21]. Briefly, aliquots of liquefied semen sample (10 M/mL) were mixed with de-solidified agarose and acidic denaturation solution. A 25-μL admixture was placed on a microscope slide and covered by a 22 × 22-mm coverslip. Then, the slide was horizontally placed at 4℃ for 5 min. After carefully removing the coverslip, the slide was incubated with lysis solution at RT for 10 min. Afterward, the slide was washed with distilled water for 5 min and dehydrated with 95% methanol for 1 min. Finally, the slide was stained with a Wright-Giemsa solution, followed by calculating the DFI under a bright-field microscope (Olympus BX53, Tokyo, Japan). Regarding the indication of non-fragmented DNA, a large halo was defined by a width similar to or greater than the diameter of the sperm core, while a medium halo had a width ranging from one third the diameter of the sperm core to the entire sperm core. Sperm with a small halo surrounding the sperm core or lacking a halo indicated fragmented DNA. The DFI was determined by counting the percentage of sperm cells with fragmented DNA. All slides were read blind by an experienced technician who produced consistent and reliable results. A minimum of 500 sperm cells per test were examined for analysis.

## Assessment of DSBs

Sperm DSBs were assessed by the LensHooke® R11 Sperm DNA Fragmentation Releasing Assay (Bonraybio) as described below [18]. Aliquots of 70 μL semen sample (10 M/mL) were mixed with 70 μL 30% (w/v) acrylamide/bis-acrylamide solution, 15 μL 1% (w/v) ammonium persulfate, and 15 μL of tetramethylethylenediamine to initiate gel polymerization in a 1.5-mL microcentrifuge. Aliquots of 15 μL mixture were dispensed onto the microscope slide and covered with a 24 × 40-mm coverslip. Then, the slide was horizontally placed at RT for 5 min. Following the careful removal of the coverslip, the slide was incubated with lysis solution at RT for 10 min. Subsequently, the slide was washed with distilled water for 5 min. Staining was performed using the Diff-Quik method, followed by de-staining with 75% ethanol. Sperm without a halo indicated non-DSBs, while sperm with a halo indicated DSBs. The DFI specific to DSB was calculated as the percentage of spermatozoa with halo per test. A minimum of 500 spermatozoa were scored per test sample.

## Ovarian stimulation

All the procedures were conducted following the previously described standard protocols [22]. Briefly, controlled ovarian stimulation was applied using either the gonadotrophin-releasing hormone (GnRH) agonist protocol or the GnRH antagonist protocol. Stimulation medications included leuprolide acetate (Lupron, Takeda Chemical Industries, Osaka, Japan) and exogenous gonadotropin (Gonal-F; Serono, Modugno, Italy or Menopur; Ferring, São Paulo, Brazil) for GnRH agonist protocol. Additional cetrorelix acetate (Cetrotide, Merck, Serono, Geneva, Switzerland) was used for the GnRH antagonist protocol. Oocyte maturation was triggered by human chorionic gonadotropin (hCG, Ovidrel, Serono), administration, and ultrasound-guide oocyte retrieval was performed approximately 36 h after hCG injection. Oocytes were allocated to either in vitro fertilization (IVF) or ICSI dish according to patients' condition.

## Embryo culture and embryo quality evaluation

ICSI were performed according to the previously described standardized protocol [23]. Briefly, ICSI were conducted in a fertilization medium (SAGE Biopharma, USA) supplemented with a 15% serum protein substitute (SPS, SAGE Biopharma, USA). At 12 h after inseminations, the oocytes were examined for the presence of pronuclei. The fertilization rate was determined as fertilized oocyte with two pronuclei (2PN) per total number of inseminated oocytes. At 70–72 h after inseminations, embryos were moved to a dish equilibrated with blastocyst medium (SAGE) containing 15% SPS. Embryos with > 7 cells on day 3 after oocyte retrieval were defined as good cleaving embryos, and the number of good cleaving embryos over the total number of inseminated oocytes was referred to as a good embryo rate.

## Pregnancy test and miscarriage

Clinical pregnancy was defined as the presence of a gestational sac with accompanying fetal heartbeat, which was verified by transvaginal ultrasound scans 5 weeks after embryo transfer. Miscarriage was defined as a spontaneous expulsion of the gestational sac up to 20 weeks of gestation, which was verified by transvaginal ultrasound.

## Statistical analysis

The dataset with normal distribution verified by the Shapiro–Wilk test was analyzed using one-way ANOVA and presented as means ± standard deviations. The results with skewed distribution were analyzed by the Kruskal–Wallis test and presented as a median (interquartile range). Summary statistics were computed based on the Chi-square. Spearman's correlation analysis was used to assess the relationships between factors. A top-down approach was applied to select independent variables for multivariable logistic regression analysis. Factors with Spearman's correlation coefficients between −0.5 and 0.5 were considered independent, qualifying for inclusion in the multivariable

analysis model. The ability to predict miscarriage using semen parameters was assessed using the area under the curve (AUC) of the receiver operating characteristic (ROC) and Youden statistics. Statistical analysis was performed by Prism software, version 6.01 (GraphPad Software, Inc).

## Results

As shown in Fig. 1, out of 195 enrolled ICSI cases, 61 cases (31.3%) were identified as normal prognosis, while 134 cases (68.7%) were characterized as poor prognosis. As expected, the comparison between normal and poor prognosis groups showed significant differences in female age: 35 (33–37) vs. 38 (36–43), retrieval oocyte count: 12 (7–17) vs. 4 (2–6), AMH: 3.6 (2.5–4.9) vs. 0.8 (0.4–1.5), and embryo transfer count: 2 (1–3) vs. 2 (2–4) (all $p < 0.05$). Comparing ART outcomes, there was no significant difference in fertilization rate (81% vs. 83%). Nevertheless, the poor prognosis group demonstrated a lower clinical pregnancy rate: 66.7% vs. 44%, and a higher miscarriage rate: 14.6% vs. 31.0% compared to the normal group (Supplementary Table 2).

### Comparison of parental factors across different clinical outcomes in group of normal prognosis

Reproductive outcomes were recorded at different gestational stages, including clinical pregnancy, early gestation failure, live birth, and miscarriage. At early gestation stage, a reduced AMH in non-pregnancy was indicated as compared to that in clinical pregnancy: 4 (2.6–5) vs. 3.2 (1.9–3.8) ($p < 0.05$). At late gestation stage, a lower retrieval oocyte count in miscarriage was observed as compared to that in live birth: 15 (9.5–20) vs. 10.5 (6–11.5) ($p < 0.05$). Semen parameters such as normal morphology rate and progressive motility achieved statistical significance in the comparison according to the Mann–Whitney U test. However, unexpected correlations were observed: the non-pregnancy group showed a higher normal morphology rate: 2 (1–4.8) vs. 5 (1.5–7) and higher progressive motility: 16 (11–27) vs. 24 (10–35) (both $p < 0.05$) compared to the clinical pregnancy group. We suggested that maternal factors, including low AMH and advanced female age, primarily influence clinical outcomes in normal population, present in Table 1.

### Comparison of parental factors across different clinical outcomes in group of poor prognosis

At early gestation, an advanced female age in non-pregnancy was found as compared to clinical pregnancy: 37 (35–41) vs. 41 (36–44) ($p < 0.05$). At late gestation, a reduced retrieval oocyte count: 5 (3.3–7) vs. 3.5 (2–5.3) and AMH levels: 1.1 (0.7–1.5) vs. 0.4 (0.1–0.8) (both $p < 0.05$) in miscarriage were found as compared to those in live birth. Additionally, the impaired total motility: 68 (62.2–84.5) vs. 54.5 (45.1–66.2), lower normal morphology rate: 5.5 (3–7.8 vs. 2.5 (1–5.5), and higher DSBs: 7.5 (6–13.5) vs. 18.8 (6.6–30.4) (all $p < 0.05$) were found in miscarriage, shown in Table 2. To sum up, besides female effects, male factors such as poor total motility, reduced normal morphology, and elevated DSBs were associated with miscarriage in group of poor prognosis.

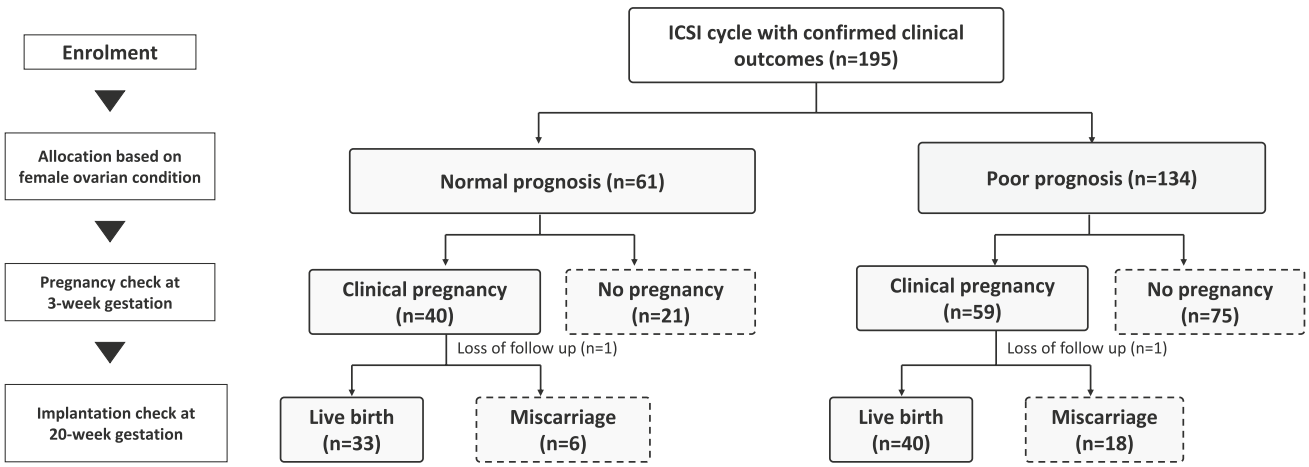


**Fig. 1** Retrospective cohort study flow diagram. Patients ($n$=195) who underwent autologous ICSI cycles without pre-implantation genetic testing for aneuploidy (PGT-A) were recruited. Enrolled cases were classified into normal prognosis and poor prognosis according to POSEIDON criteria. Clinical outcomes were followed up at 3-week and 20-week gestation, respectively

**Table 1** Comparison of parental factors across different clinical outcomes in group of normal prognosis

| *n* | Early Gestation | | | Late Gestation | | |
|---|---|---|---|---|---|---|
| | Clinical pregnancy | Non-pregnancy | P value | Live birth | Miscarriage | *P* value |
| | 40 | 21 | | 33 | 6 | |
| Female age (years old) | 35 (32.3–37) | 35 (33–37) | 0.819 | 35 (32–36.5) | 38 (33.8–41.3) | 0.082 |
| Retrieval oocyte (n) | 13.5 (9.3–18) | 10 (4–16.5) | 0.055 | 15 (9.5–20) | 10.5 (6–11.5) | 0.039 |
| AMH (ng/mL) | 4 (2.6–5) | 3.2 (1.9–3.8) | 0.019 | 4 (2.6–5) | 4.2 (2–5.7) | 0.392 |
| Male age (years old) | 38 (34–42) | 38.1 (33.9–40.1) | 0.742 | 37.5 (33.9–41.5) | 42.5 (32.2–45.8) | 0.36 |
| Sperm concentration (M/mL) | 33.7 (14.4–64.1) | 39.9 (19.2–52.7) | 0.796 | 33.3 (13.7–62.6) | 41.1 (22.8–111.6) | 0.392 |
| Total motility (%) | 45.6 (32.5–64.1) | 53.6 (40–73) | 0.182 | 44 (32–63.4) | 52.5 (46.9–81.7) | 0.139 |
| Progressive motility (%) | 10 (4.3–24.8) | 12 (8–24.5) | 0.362 | 10 (4.5–27.5) | 10.5 (7.3–22.3) | 0.907 |
| Normal morphology (%) | 2 (1–4.8) | 5 (1.5–7) | 0.047 | 2 (1–4.5) | 3 (1.8–5.8) | 0.322 |
| Total SDF (%) | 19 (11–39.5) | 13 (10.5–19.6) | 0.125 | 21 (12.2–41.4) | 13.5 (8–29) | 0.243 |
| DSBs (%) | 15 (7.9–31) | 9.3 (5.5–13) | 0.052 | 15.5 (7.9–33.5) | 8.8 (3.8–22.6) | 0.133 |

*total SDF, total sperm DNA fragmentation; *DSBs*, sperm double-stranded breaks

**Table 2** Comparison of parental factors across different clinical outcomes in group of poor prognosis

| *n* | Early Gestation | | | Late Gestation | | |
|---|---|---|---|---|---|---|
| | Clinical pregnancy | Non-pregnancy | *P* value | Live birth | Miscarriage | *P* value |
| | 59 | 75 | | 40 | 18 | |
| Female age (years old) | 37 (35–41) | 41 (36–44) | 0.002 | 36 (34–39) | 38.5 (36–42.5) | 0.05 |
| Retrieval oocyte (n) | 5 (3–6) | 4 (2–6) | 0.602 | 5 (3.3–7) | 3.5 (2–5.3) | 0.04 |
| AMH (ng/mL) | 1 (0.4–1.5) | 0.8 (0.4–1.3) | 0.476 | 1.1 (0.7–1.5) | 0.4 (0.1–0.8) | 0.001 |
| Male age (years old) | 39 (36–45) | 41 (37.9–45) | 0.1 | 39 (34.9–45) | 38.7 (36.9–49) | 0.278 |
| Sperm concentration (M/mL) | 47.8 (23.2–77.1) | 48.5 (27.3–86.8) | 0.509 | 53.8 (26.1–81.9) | 36.6 (14.2–68.7) | 0.207 |
| Total motility (%) | 66 (54.8–84.4) | 72.2 (57.3–83.5) | 0.355 | 68 (62.2–84.5) | 54.5 (45.1–66.2) | 0.01 |
| Progressive motility (%) | 16 (11–27) | 24 (10–35) | 0.017 | 16 (11–28) | 15.5 (10.3–24) | 0.567 |
| Normal morphology (%) | 5 (2–7) | 5 (2–9) | 0.477 | 5.5 (3–7.8) | 2.5 (1–5.5) | 0.025 |
| Total SDF (%) | 19.6 (11.6–28) | 18.4 (13–33.8) | 0.457 | 21 (11.5–27.2) | 18.8 (12.8–40.4) | 0.668 |
| DSBs (%) | 8.2 (6–18) | 9.5 (4.5–16) | 0.435 | 7.5 (6–13.5) | 18.8 (6.6–30.4) | 0.013 |

*total SDF, total sperm DNA fragmentation; *DSBs*, sperm double-stranded breaks

## Risk factors associated with miscarriage by univariate and multivariable logistic regression analyses

Based on Spearman's correlation coefficient of 0.5 indicating a fair correlation, the number of retrieved oocytes showed a significantly positive correlation with AMH ($r = 0.52$) among female factors (Fig. 2). Sperm concentration was correlated with total motility ($r = 0.51$). Total motility was positively correlated with both progressive motility ($r = 0.53$) and normal morphology ($r = 0.63$), and negatively correlated with DSBs ($r = -0.5$). Additionally, progressive motility was positively correlated with normal morphology ($r = 0.54$) (Fig. 3). Based on univariate regression analysis (Table 3), total motility (odds ratio [OR]: 0.96, 95% confidence interval [CI]: 0.92–0.99; $p = 0.02$) and DSBs (OR: 1.09, 95% CI: 1.03–1.17, $p = 0.01$) were significantly correlated with miscarriage in poor prognosis group. Furthermore, we considered factors as independent when the factor-to-factor Spearman's correlation coefficient was $< 0.5$ or $> -0.5$, and subsequently applied these independent factors to multivariable regression analysis. The regression model incorporated six independent variables: female age, retrieved oocyte count, male age, sperm morphology, total SDF, and DSBs. Among these, only DSBs (OR: 1.19, 95% CI: 1.04–1.36, $p = 0.01$) showed a significant correlation. For every 10% increase in DSBs, the probability of miscarriage increased by 19%. Given the potential collinearity between total SDF and DSB, two independent

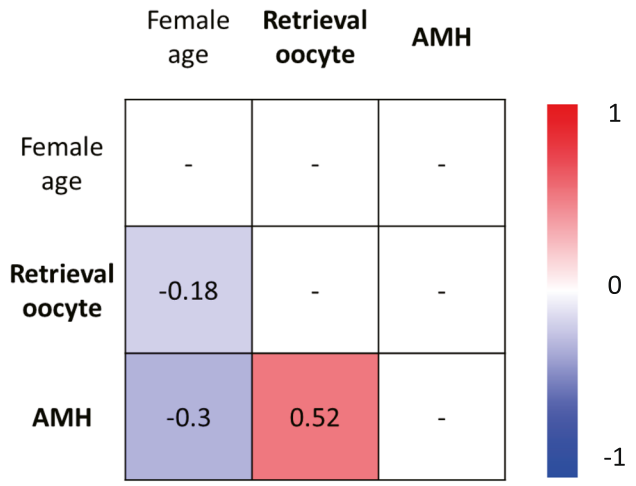


**Fig. 2** Spearman correlation of maternal variables. Heatmap of Spearman's rank correlation coefficient between female factors denotes the strength of monotonic relationship between subsequent variable pairs

multivariable logistic regression models were performed: Model 1 for total SDF and Model 2 for DSBs (Supplementary Table 3). The findings from both models align with those presented in Table 3, demonstrating that DSBs are an independent factor associated with an increased probability of miscarriage.

## Evaluation of DSB values in predicting miscarriage

Figure 4 illustrates a notable predictive value of DSBs according to the ROC analysis. An DSBs cutoff of > 19% showed strong predictive performance for miscarriage (AUC: 0.701, $p = 0.0205$). In contrast, the total SDF indication did not show significance in predicting miscarriage (AUC: 0.533, $p = 0.7073$) with cutoff of > 30% By applying cutoff values for miscarriage prediction, the present study suggests 19% for DSBs, while 30% for total SDF [24]. Compared to total SDF, DSBs demonstrated better performance across several metrics: sensitivity (50%), specificity (95%), positive predictive value (PPV) (82%), negative predictive value (NPV) (81%), and accuracy (81%) in detecting miscarriage. In contrast, the sensitivity, specificity, PPV, NPV, and accuracy of total SDF in detecting miscarriage were 33%, 85%, 50%, 74%, and 69%, respectively (Table 4).

## Discussion

In this retrospective study, for the first time, we explored and discussed different types of DNA fragmentation upon varied female age and ovarian reserve, concerning the complexity of SDF effects. Analyses contingent on parental factors demonstrated that sperm DSBs are uniquely linked to miscarriage for female partners with poor prognosis. Specifically, the likelihood of miscarriage significantly rises in the

**Fig. 3** Spearman correlation of paternal variables. Heatmap of Spearman's rank correlation coefficient between male factors denotes the strength of monotonic relationship between subsequent variable pairs

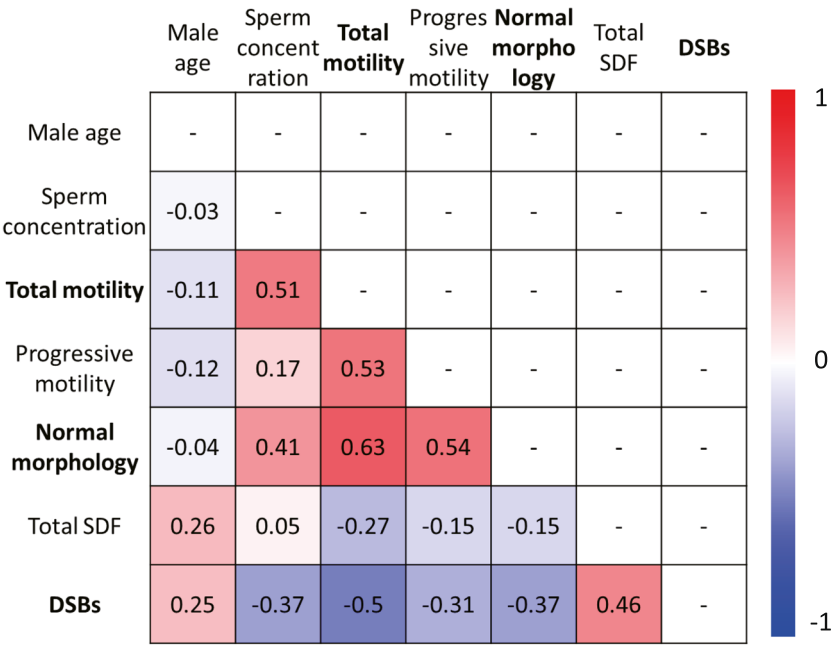

**Table 3** Risk factors associated with miscarriage by univariate and multivariable logistic regression analyses

| Variables | Univariate | | Multivariable | |
|---|---|---|---|---|
| | Odd ratio (95% CI) | *P* value | Adjusted Odd ratio (95% CI) | *P* value |
| Female age (years) | 1.15 (1–1.32) | 0.06 | 1.21 (0.96–1.54) | 0.11 |
| Retrieval oocyte (n) | 0.79 (0.62–1.02) | 0.07 | 0.84 (0.64–1.11) | 0.22 |
| AMH (ng/mL) | 0.48 (0.22–1.09) | 0.08 | — | — |
| Male age (years) | 1.06 (0.97–1.16) | 0.17 | 0.91 (0.75–1.1) | 0.33 |
| Sperm concentration (M/mL) | 0.99 (0.98–1.01) | 0.39 | — | — |
| Total motility (%) | 0.96 (0.92–0.99) | 0.02 | — | — |
| Progressive motility (%) | 0.98 (0.93–1.04) | 0.5 | — | — |
| Normal morphology (%) | 0.85 (0.72v1.01) | 0.07 | 0.97 (0.77–1.21) | 0.76 |
| Total SDF (%) | 1.02 (0.99–1.06) | 0.24 | 0.93 (0.84–1.02) | 0.11 |
| DSBs (%) | 1.09 (1.03–1.17) | 0.01 | 1.19 (1.04–1.36) | 0.01 |

*Total SDF, total sperm DNA fragmentation; *DSBs*, sperm double-stranded breaks

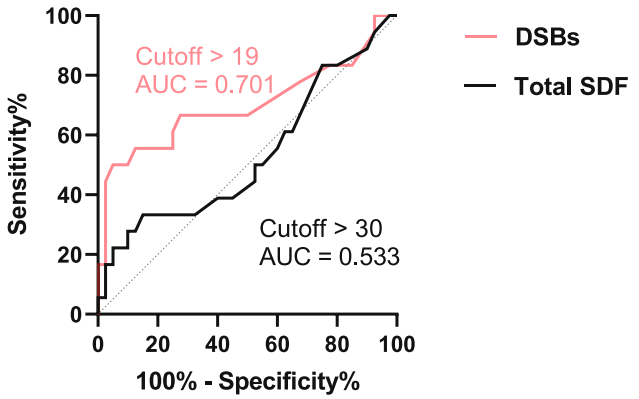


**Fig. 4** Evaluation of double-stranded break (DSB) values in predicting miscarriage. Receiver operating characteristic (*ROC*) analysis was used to evaluate total sperm DNA fragmentation (*SDF*) and double-stranded break (*DSB*) in predicting incidence of miscarriage. **AUC*: area under curve

**Table 4** Accuracy metrics for diagnosing miscarriage

| | Total SDF > 30% | DSBs > 19% |
|---|---|---|
| Sensitivity | 33% | 50% |
| Specificity | 85% | 95% |
| Positive Predictive Value | 50% | 82% |
| Negative Predictive Value | 74% | 81% |
| Accuracy | 69% | 81% |

*Total SDF, total sperm DNA fragmentation; *DSBs*, sperm double-stranded breaks

poor prognosis group when DSBs exceed 19%, whereas this association is not observed in the normal prognosis group.

While exploring the impact of SDF on reproductive outcomes, two potential confounding factors have been discussed. One of the dominant factors is oocyte repair capability, which masks the effects of paternal DNA abnormalities. The repair mechanisms depend on the quality of the oocyte's cytoplasm and genome; hence, sperm DNA damage can be appropriately repaired in high-quality oocytes [25, 26]. In a study comparing clinical outcomes between oocytes from young female patients and women of advanced age exposed to comparable levels of SDF, significant reductions in embryo development rate, implantation rate, and pregnancy rate were observed in ICSI cycles from women with advanced maternal age, but not in the younger controls [25]. Similarly, Meseguer et al. reported that increased SDF significantly affected the likelihood of pregnancy in autologous cycles, but not in egg-donation cycles [27]. The other confounding factor in interpreting the effects of SDF is the complexity of different types of DNA damage (SSBs vs. DSBs). Considering the two aforementioned variables, our findings align with the nature of oocyte DNA repair capability: SDF effects are primarily observed in the poor prognosis cohort but not in the normal prognosis group. Furthermore, we demonstrated a unique association of total SDF and DSBs with clinical outcomes. DSBs are linked to an increased likelihood of miscarriage. Although there was no correlation between total SDF and clinical outcomes following embryo transfer, the significantly higher total SDF levels were found in the group with low embryo development rates on day 3 compared to those with normal development. This effect of total SDF was observed only in the poor prognosis cohort as well (Supplementary Table 4).

In 2023, Sakkas et al. pointed out the interplay between SDF and oocyte repair capability and proposed a hypothetic model. It implicated that the progression of embryos through various developmental milestones depends on remaining DNA damage [28]. Embryos with extensive DNA damage may encounter reproductive failure at early stages, such as during cell division and blastocyst formation, whereas lower residual DNA damage may manifest later in development. This corresponds with our discovery that high SDF stress (total SDF) correlates with preimplantation embryo

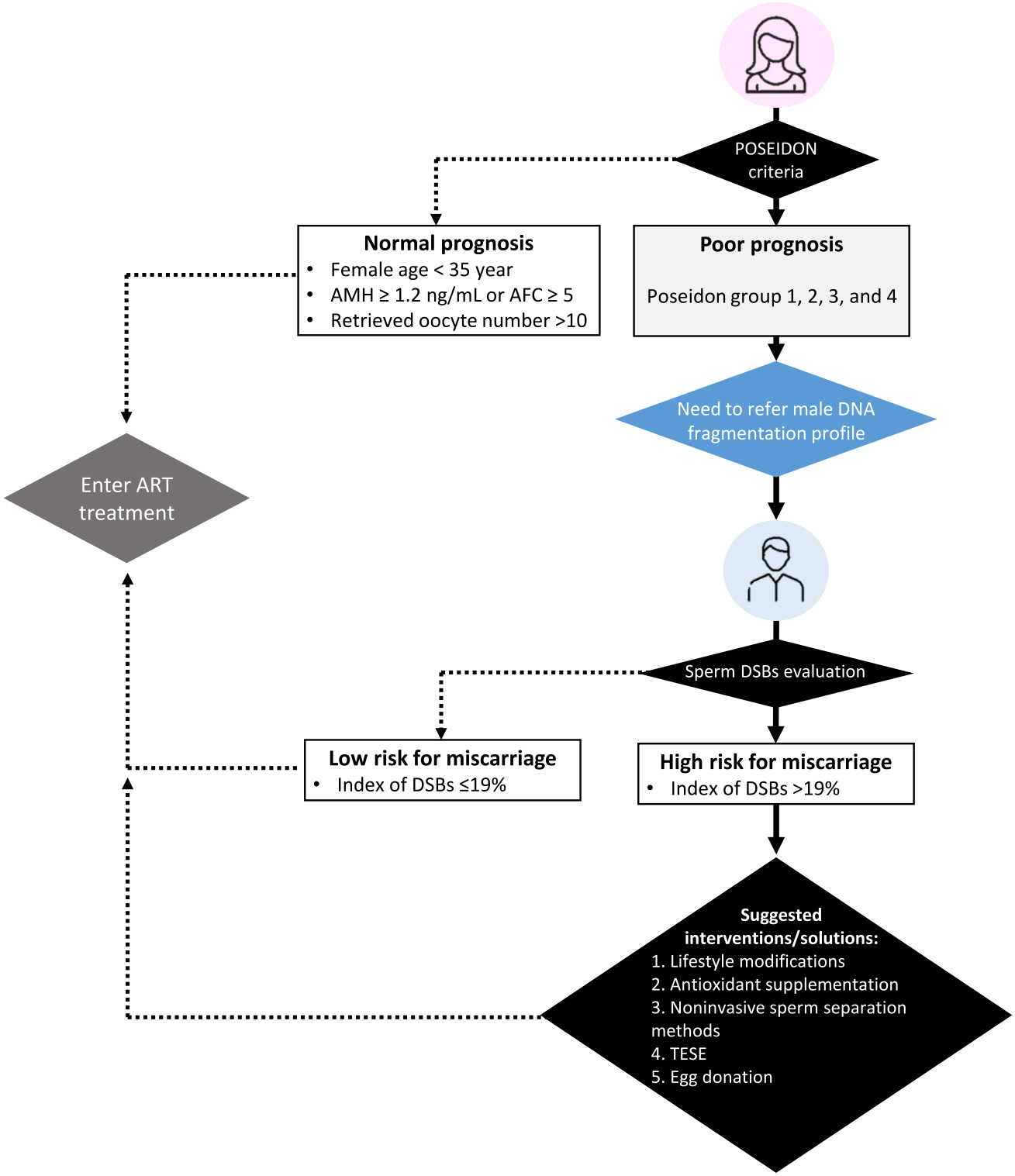


**Fig. 5** Optimized pre-ART guidelines incorporating parental factors. Clinicians use ovarian reserve markers, including antral follicle count (*AFC*), Anti-Müllerian hormone (*AMH*), and age, to estimate the minimum number of oocytes. In cases of high risk for poor prognosis where the probability of live birth is low due to limited ovarian reserve, advanced assessment of sperm double-stranded breaks (*DSBs*) in the male partner is highly recommended. A high risk of miscarriage is estimated if DSB levels exceed 19%. In such cases, treatments to reduce DSBs—such as lifestyle modifications and antioxidant supplementation—are advised. Alternatively, interventions like non-invasive sperm preparation techniques, surgical sperm extraction, and/or egg donation may be considered. **ART*: assisted reproductive technologies; *TESE*: testicular sperm extraction

development. In contrast, DSBs exert their mild, but nonetheless significant, adverse effects during post-implantation events, primarily causing pregnancy loss.

The current clinical diagnostic methods are still limited in clarifying the etiology of different types of DNA breaks, and precision medicine is not yet available. However, we aimed to reshape the approach to enable early medical intervention. To prevent reproductive failures associated with high SDF, integrating SDF evaluation into the initial assessment before ART treatment can be valuable, particularly when the female partner has a poor prognosis. Based on SDF reports, clinicians can recommend early interventions to mitigate the effects of SDF, such as lifestyle modifications, dietary adjustments, and antioxidant supplementation [28, 29]. Sequentially, advanced sperm selection techniques, including ICSI with hyaluronan microdot selection [30], magnetic sorting [31], and MSOME [32], microfluidic selection system [33], and live sperm-sorting device [34] can be utilized to select sperm with minimal DNA damage before ICSI treatment. According to Aitken's two-step sperm chromatin damage hypothesis, oxidative stress is a major contributor to SDF [6]. Consequently, the use of testicular sperm for ICSI may serve as a treatment option for high SDF, as testicular sperm have not been challenged or exposed to the elevated oxidative stress upon epididymal transition. Previous studies showed a significant lower DFI in testicular sperm than ejaculated sperm as well [35, 36]. Additionally, since healthy and mature oocytes possess DNA repair mechanisms that act as a biological defense against paternal DNA damage, oocyte donation presents an ultimate viable alternative for infertile couples with high SDF levels, advanced maternal age, or poor oocyte quality.

The cutoff of DSBs for miscarriage prediction was determined at 19%. As shown in Supplementary Table 5, which presents the frequency of high DSBs in the study, 33% of male partners in the normal prognosis group had R11 > 19%, compared to 18% in the poor prognosis group. This pattern in poor prognosis cohort closely correlated with miscarriage prevalence. In contrast, the frequency of high total SDF (> 30%) was similar between the normal and poor prognosis groups, at 26% and 25%, respectively.

Three major limitations in the present study need to be noted. First, image-based diagnostic test is commonly challenged by low reproducibility due to manual subjectivity. Therefore, incorporating statistical measures of intra/inter-observer reliability to future validation could solidify the consistency of the findings. Second, we acknowledge that this pilot study is limited by an insufficient sample size (40 live birth cases and 18 miscarriage cases), which indicated a statistical power of only 0.54 as determined by post hoc power analysis. To ensure adequate power for detecting meaningful differences, a larger study is required. Based on an effect size of 0.5 and sufficient statistical power, the minimum sample sizes for the control (live birth) and disease (miscarriage) groups were estimated at 334 and 50 cases, respectively. These calculations were derived using the reported prevalence of live birth (81.8%) and miscarriage (12.6%), as described by Chen et al. [37]. The post hoc and priori power analyses were indicated in Supplementary Figs. 1a and 2b. Third, we acknowledge that we did not examine the SDF effects within each Poseidon category. The group of poor prognosis was generalized by combining participants from Poseidon group 1 (15/134, 11%), group 2 (31/134, 23%), group 3 (11/134, 8%), and group 4 (77/134, 57%), which may have underestimated the distinct characteristics of each group. Additionally, the unexpected correlation between normal morphology/progressive motility and miscarriage (Table 2), along with the moderate predictive power derived from the ROC analysis (Fig. 4), may be attributed to the limited sample size. Therefore, further studies with an enlarged sample size and a multi-center design are needed.

We demonstrated, for the first time, a guideline, which accounts for the female reproductive potentials based on POSEIDON criteria and advancements in SDF evaluation, offers a comprehensive management strategy (Fig. 5). The proposed pre-treatment screening workflow can help clinicians determine the optimal timing for ART treatment. Implementing this approach could potentially reduce the number of ART attempts, thereby minimizing emotional and financial burdens, and ultimately increase the success rate of ART with fewer trials.

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**Author contribution** C.I. Lee, C.C. Huang, T. H. Lee, C.T. Hsu, M.S. Lee, conceived, designed, and conducted all the experiments; H.M, Tsao, C.H. Chen, T.E. Wang performed sample collections and conducted all the experiments; T. H. Lee, T.A. Chang, and T.E. Wang constructed and wrote the manuscript.

**Data availability** The data underlying this article will be shared on reasonable request made to the corresponding author.

## Declarations

**Compliance with ethical standards** This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the local Ethics Committee of Chung Shan Medical University (No. CS2-20012).

**Consent to participate** Informed consent was obtained from all individual participants included in the study.

**Competing interests** The authors declare no competing interests.

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