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ORIGINAL ARTICLE

Male Infertility

Sperm DNA fragmentation in Chinese couples with unexplained recurrent pregnancy loss

Xiao-Bin Zhu*, Qian Chen*, Wei-Min Fan, Zhi-Hong Niu, Bu-Fang Xu, Ai-Jun Zhang

We aimed to study the association between sperm DNA fragmentation and recurrent pregnancy loss (RPL) in the Chinese population via a retrospective observational study of Chinese couples who had experienced RPL between May 2013 and August 2018. The study population included 461 men from couples with RPL and 411 men from a control group (couples with clinical pregnancy via *in vitro* fertilization owing to female causes). Routine semen analysis, sperm chromatin analysis, and microscopic (high-power) morphological analysis were performed using semen samples. Semen samples were assessed for volume, sperm count, and motility. The sperm DNA fragmentation index (DFI) was calculated, and the median DFI was obtained. Men were categorized as having normal (37.8%; DFI ≤15.0%), moderate (33.6%; 15.0% < DFI < 30.0%), or severe (28.6%; DFI ≥30.0%) DNA fragmentation levels. The percentage of men with severe DNA fragmentation was significantly higher in the RPL (42.3%) group than that in the control group (13.1%), whereas the percentage of men with normal levels of DNA fragmentation was significantly lower in the RPL group (22.8%) than that in the control group (54.7%). Subsequent analysis also demonstrated that the sperm DNA fragmentation rate had a moderate reverse correlation with the sperm progressive motility rate ($r = -0.47$, $P < 0.001$) and the total motile sperm count ($r = -0.31$, $P < 0.001$). We found a positive correlation between RPL and sperm DNA fragmentation. The results suggest that increased sperm DNA damage is associated with RPL.

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Keywords: DNA fragmentation index; recurrent pregnancy loss; sperm chromatin structure assay

INTRODUCTION

Recurrent pregnancy loss (RPL) is defined as two or more consecutive miscarriages according to the American Society for Reproductive Medicine.[1] The known etiological causes of RPL include genetic factors, uterine anatomy, hormonal factors, immune system dysfunction, and thrombosis. However, the etiology remains unidentified in about 50% of the RPL couples.[2] In the past, among couples with RPL, it was always the women who primarily underwent numerous medical examinations, with the male contribution being commonly associated with karyotype abnormalities alone. However, evidence suggests that patients with karyotype 46,XY may present with a high percentage of spermatozoa with aneuploidies.[3] Male gametes provide 50% of the genetic material, and there is a chance that these sperm with genetic abnormalities or epigenetic changes may fertilize the oocyte. However, this would lead to chromosomal damage, thereby seriously affecting early embryonic development.[4]

Following extensive research, it has been confirmed that sperm DNA damage is associated with infertility, lower rate of pregnancy following artificial insemination, and reduced rates of high quality embryos and blastocysts after both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection.[5–10] The testing of sperm DNA fragments has opened a new era in the comprehensive evaluation of infertility. It may also potentially contribute to the increasing success of assisted reproductive technology (ART) in the future.[11] There have also been sporadic reports on the correlation between sperm DNA damage and RPL.[12–16] These studies present conflicting results, which may be related to the detection methods employed or the sample size assessed. However, there are no large sample data to support this correlation.

There is increasing evidence that sperm DNA fragmentation (SDF) abnormalities not only severely affect fertility, but also have a close relationship with RPL. Therefore, the aim of this study was to examine a possible relationship between SDF and RPL due to no specific reason in Chinese women and as well as to evaluate the routine sperm parameters and SDF in their husbands' semen using the sperm chromatin structure assay (SCSA) method.

PATIENTS AND METHODS

This retrospective observational study analyzed the data of couples with RPL who visited the Center of Reproductive Medicine of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine in Shanghai, China, as outpatients between May 2013 and August 2018. Among the RPL cases, 339 couples had miscarried twice, 102 couples had miscarried 3 times, and 20 couples had miscarried 4 or more times. The average number of miscarriages was 2.32. We identified 461 couples who had normal karyotypes and tested negative for the presence of endocrine disorders, antiphospholipid and lupus antibodies, and coagulation defects. Women with uterine structural abnormalities or age above 40 years were excluded. Likewise, the men presenting for

Reproductive Medical Center of Ruijin Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, China.
*These authors contributed equally to this work.
Correspondence: Dr. AJ Zhang (zhaj1268@163.com)
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fertility evaluation were reviewed, and those included in this study had no history of orchitis, toxic exposure, chronic illness, or radiation exposure in the last 3 months. The control group comprised 411 IVF patients who visited our center between May 2013 and August 2018, and their data were retrospectively analyzed. Only women with tubal factors for infertility were recruited and the exclusion criteria were the same as those for the women in the RPL group. Furthermore, the analysis was restricted to women who had given birth to live babies or carried a pregnancy for more than 3 months following IVF treatments. To better compare the differences in SDF and high DNA stainability (HDS) between the two groups, as described in several other studies conducted in recent years,[11,17] we divided our study population into three groups based on the SDF levels. DNA fragmentation equal to or lower than 15% was regarded as normal, DNA fragmentation more than 15% and lower than 30% was considered as moderate, and DNA fragmentation equal to or more than 30% was considered as severe. Furthermore, we also divided the study population into two groups based on sperm HDS levels, with the normal cutoff value being <15.0%.[18] Informed consent was obtained from all participants. This study has been approved by the Research and Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Semen test

Semen samples were collected by masturbation after 3–7 days of abstinence. After liquefaction, the semen volume was measured, and sperm concentration, total motility, and progressive motility were analyzed using a Computer-assisted Sperm Analysis (CASA) system (Sperm Class Analyzer; Microptic, Barcelona, Spain), and the total motile sperm count (TMSC)[19] (semen volume × sperm concentration × progressive motility) was calculated. Sperm morphology was determined according to the 5th World Health Organization guidelines.[20] Morphological assessment was based on high-power microscopic evaluation of the sperm for intactness of membranes of the acrosome, head, neck, midpiece, and tail. The semen smears were fixed on slides, stained using Diff-Quik (Biomart, Shenzhen, China), and then observed through oil immersion light microscopy (BX41, Olympus, Center Valley, PA, USA) with a magnification of ×100.

SCSA test

Samples were run using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), recording 5000 events. The SCSA protocol has been described elsewhere. Briefly, thawed samples (100 μl) were combined with 200 μl of acid detergent (pH 1.2) for 30 s. The sample was then stained with 600 μl of acridine orange (AO) staining solution (CellPro Biotech Co., Ltd., Ningbo, China) (600 μl AO 1.0 mg ml^{-1} to 100 ml staining buffer pH 6.0) and allowed to rest for a total of 3 min. Under AO stain, double-stranded DNA fluoresces green and single-stranded DNA fluoresces red. The extent of DNA damage was expressed as the DNA fragmentation index (DFI), which was calculated by assessing the ratio of red to total fluorescent cells using the flow cytometer software (DFIView 2010 Alpha11.15, CellPro Biotech Co., Ltd., Ningbo, China). HDS represents immature spermatozoa with incomplete chromatin condensation stained with the most intense green color.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software, version 15 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean value ± standard deviation (s.d.), and comparisons between the RPL and control groups were determined using the Student's *t*-test; categorical variables were presented as count (percentage), and comparisons between the RPL and control groups were determined using the chi-squared test or Mann–Whitney U test. The Spearman's correlation test was used to determine correlations between the DFI and the semen parameters. Receiver operating characteristic (ROC) curve analysis was applied to obtain the cutoff value of DFI to differentiate patients from controls, and the sensitivity and specificity for the best cutoff point were then assessed. Moreover, multivariate logistic regression analysis was used to determine the factors predicting RPL. All tests were two-sided. $P < 0.05$ was considered statistically significant.

RESULTS

Sperm DFI analysis and routine semen analysis for semen parameters such as volume and pH of the sample as well as progressive motility, morphology, and HDS of the sperm were performed in 872 men. The relationship between the two groups was analyzed as shown in **Table 1**. Regarding semen parameters, there was a statistically significant difference between the RPL and control groups only for DFI ($P < 0.001$) and sperm progressive motility ($P = 0.047$). Those enrolled in the study included 461 women in the age range of 21–39 (mean: 30.88 ± 3.62) years who had experienced RPL and 411 women in the age range of 22–39 (mean: 30.40 ± 3.58) years as controls. There were no statistically significant differences between the ages of the women ($P = 0.056$).

To study the distribution of DFI in the RPL population, the RPL and control couples were subdivided into three groups on the basis of the cutoff of DFI, that is, 15.0% and 30.0%. Of the 461 RPL and 411 control samples analyzed, 105 (22.8%) and 225 (54.7%) had a sperm DFI of 0.0%–15.0%, 161 (34.9%) and 132 (32.1%) had a sperm DFI of 15.0%–30.0%, and 195 (42.3%) and 54 (13.1%) had a sperm DFI of 30.0%–100.0%, respectively. The RPL and control couples were also subdivided into two groups on the basis of the cutoff HDS of 15.0%. Of the 461 RPL samples analyzed, 424 (92.0%) had a sperm HDS of 0.0%–15.0%, and 37 (8.0%) had a sperm HDS of more than 15.0%. In the control group, 386 (93.9%) samples had sperm HDS of 0.0%–15.0% and 25 (6.1%) samples had a sperm HDS of more than 15.0%. The different DFI and HDS ranges in the two groups were compared, as shown in **Table 2**. There was a statistically significant difference in the percentage of DFI between the normal subgroup and the severe subgroup. With an increase in the DFI, the percentage of RPL also increased (**Figure 1a**). There was no significant difference observed on comparison of the HDS ranges of the male partners in the RPL and control groups (**Figure 1b**).

Table 1: Characteristics of recurrent pregnancy loss and control groups

Characteristics	*RPL group (n=461)*	*Control group (n=411)*	*P*
Age of male (year)	32.41±4.56	32.67±4.52	0.391
Age of female (year)	30.88±3.62	30.40±3.58	0.056
Volume of semen (ml)	3.46±1.81	3.42±1.53	0.753
pH	7.44±0.14	7.45±0.11	0.785
Sperm concentration (10^6 ml^{-1})	85.54±57.69	86.54±58.21	0.798
Progressive motility (%)	47.54±18.96	49.99±17.19	0.047
Total motile sperm count (10^6 ml^{-1})	153.31±188.39	155.27±156.28	0.871
Normal forms (%)	7.55±4.32	7.64±3.31	0.725
DFI (%)	25.88±12.58	17.09±10.08	<0.001
HDS (%)	8.37±4.25	8.49±3.80	0.667

Data are presented as mean value±s.d.. Comparison between RPL group and control group was determined by Student's *t*-test. *P*<0.05 indicated a significant difference. RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; HDS: high DNA stainability; s.d.: standard deviation

Table 2: The comparison of different DNA fragmentation index and high DNA stainability ranges in recurrent pregnancy loss male partners and controls

Items	*DFI (%)*			*P*	*HDS (%)*		*P*
	<15	*15–30*	*>30*		*<15*	*≥15*	
RPL group (*n*=461)	105 (22.8)	161 (34.9)	195 (42.3)	<0.001	424 (92.0)	37 (8.0)	0.265
Control group (*n*=411)	225 (54.7)	132 (32.1)	54 (13.1)		386 (93.9)	25 (6.1)	

Data are presented as count (percentage). Comparison between RPL group and control group in DFI and HDS was determined by Mann–Whitney U test and Chi-squared test, respectively. *P*<0.05 indicated a significant difference. RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; HDS: high DNA stainability

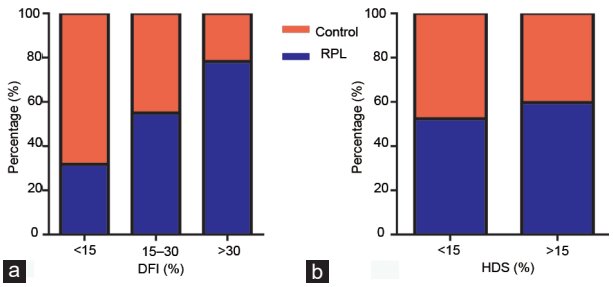

Figure 1: Tendency regarding different DFI and HDS ranges for the RPL and control groups. (**a**) Sperm of men from couples with a history of RPL tended to have a higher DFI than do sperm of men from the control group. (**b**) There was no tendency for different HDS ranges for the RPL and control couples. with a history of RPL and the controls. RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; HDS: high DNA stainability.

The relationship between semen parameters and DNA fragmentation in 461 RPL couples is shown **Figure 2**. Using Spearman's rank correlation coefficient, a moderate inverse relationship was seen between sperm progressive motility and DNA fragmentation ($r = -0.47$, $P < 0.001$). SDF also showed a moderate inverse correlation with TMSC ($r = -0.31$, $P < 0.01$). A mild inverse correlation was seen between sperm concentration and DNA fragmentation ($r = -0.16$, $P < 0.01$), whereas a mild correlation was observed between sperm DNA fragmentation and male age ($r = 0.15$, $P < 0.01$). SDF showed no correlation with semen volume, sperm morphology, HDS, and number of RPL.

The area under the curve was 0.713 ($P < 0.001$; 95% CI: 0.679–0.747), with 81.8% sensitivity and 54.2% specificity (**Figure 3**). According to the ROC curve analysis, a sperm DFI of approximately 24.6% was used as the threshold to distinguish between the RPL group and the control group. **Table 3** shows the results of regression models of the association between variables predicting RPL. A higher risk of RPL was observed with increased SDF (OR = 1.096, 95% CI: 1.078–1.115, $P < 0.001$), older age of the female partner (OR = 1.120, 95% CI: 1.062–1.182, $P < 0.001$), and lower progressive motility (OR = 0.905, 95% CI: 0.866–0.945, $P = 0.003$).

DISCUSSION

In this study, we investigated the relationship between the rate of RPL in women and the sperm DFI in their respective partners in a relatively large sample in China. The main finding from this study was that increased SDF may be a risk factor in couples experiencing RPL. Two different statistical analyses showed that sperm from men in the RPL group have a higher percentage of DNA damage than do sperm from men in the control group, as shown by two different statistical analyses ($P < 0.001$). These data were in accordance with the results of other studies[3,12,14–16,21] and suggest an association between increased SDF and a history of RPL. As for the other semen parameters, there was no significant difference between the two groups except for sperm progressive motility, with lower progressive motility being associated with an increased risk of RPL; older age of the female partner was also found to be associated with increased RPL. An inverse relationship was found between sperm progressive motility and DNA fragmentation, which may explain the reason for the a statistically significant difference between the RPL group and the controls in sperm progressive motility.

Although it is well known that sperm DNA integrity plays a vital role in the development of the embryo and in fetal wellbeing, and while there has also been growing interest in the use of DFI as a marker of evaluating RPL, the correlation between DFI and RPL has remained highly controversial. This diversity in opinion may be due to the limited caseload as well as the different methods used in the evaluation having different sensitivities and specificities. The most commonly used methods are SCSA, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and sperm chromatin dispersion (SCD).[17,22–24] The TUNEL assay detects both single- and double-stranded DNA breaks by labeling the free 3'-OH terminus with the large terminal deoxynucleotidyl transferase (TdTA) enzyme.[25] The SCSA test determines the percentage of sperm stained with AO in a semen sample that fluoresces red (broken DNA) or green (intact DNA) following an acid denaturation step.[17] The SCD test is based on the principle that DNA fragments of sperm cannot produce a "halo" of dispersed DNA rings after acidic denaturation and nucleoprotein removal.[26] The number of sperm without DNA fragments was assessed by microscopy.

A meta-analysis of seven eligible papers on the research carried out by Zini *et al*.[27] concluded that spontaneous pregnancy loss is associated with sperm DFI. The results were based on SCSA and TUNEL array, and the sample size ranged from 50 to 388. Leach *et al*.[16] indicated that 108 couples with a history of RPL showed sperm with high levels of DNA fragmentation on evaluation using SCSA. In addition, Kumar *et al*.[15] and Kamkar *et al*.,[28] using the same method, observed that the DFI was higher in the RPL group of 42 patients compared to that in a control group of 45 patients. Carlini *et al*.[12] investigated 112 men from RPL couples, 114 infertile men with 1 or more impaired semen parameters, and 114 fertile men with high-quality semen by analyzing the SDF using TUNEL, and they found that the DFI was higher in the RPL group than that in the fertile controls (18.8% ± 7.0% *vs* 12.8% ± 5.3%, $P < 0.001$), and similar to that in infertile patients. Most of these studies used either the SCSA or the TUNEL method, and the study population was relatively large. Our results are in accordance with the above-mentioned research. However, there are some studies that have observed that the DFI was higher in the RPL group of patients than that in the control group using the SCD method.[14,29]

Others, in contrast, reported that there was no significant correlation between DNA fragmentation and RPL and concluded that DFI was not an important cause and predictive factor for RPL. Gil-Villa *et al*.[3] evaluated the DFI in a control group (18.5% ± 4.2%) and RPL group (16.3% ± 4.0%) using the SCSA test and found no significant difference between 23 couples with history of RPL and 11 men with

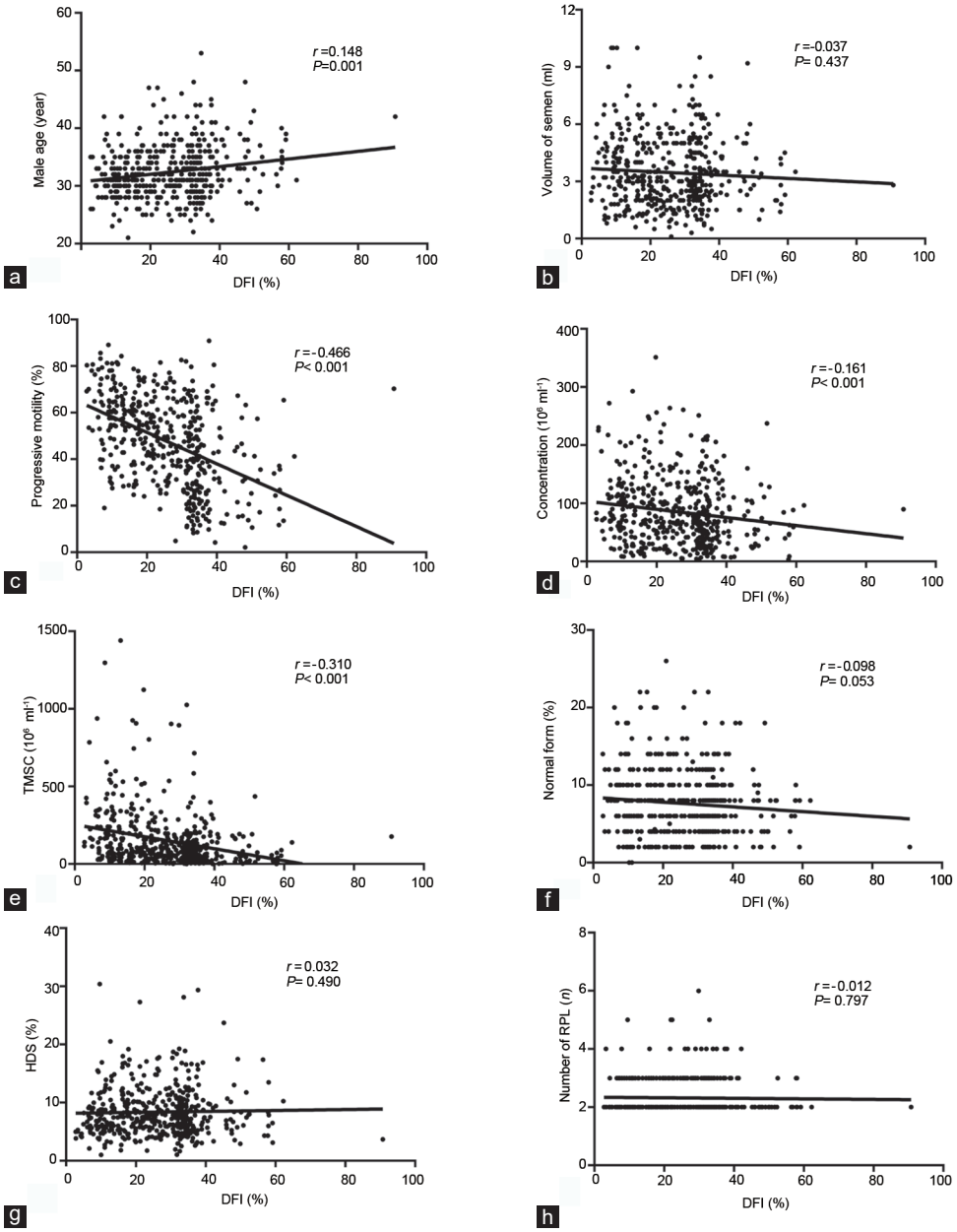

Figure 2: Correlation between sperm DFI and semen parameters, age, and the number of RPL in recurrent pregnancy loss patients. The relationship between male age, semen variables, and sperm DFI was analyzed by Spearman's correlation test. (**a**) A mild correlation was seen in male age, (**b**) no correlation in semen volume, (**c**) a moderate inverse relationship in sperm progressive motility, (**d**) a mild inverse relationship in mean sperm concentration, (**e**) a moderate inverse relationship in TMSC, (**f**) no correlation in mean sperm normal forms, (**g**) sperm HDS, and (**h**) number of RPL with sperm DFI. RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; HDS: high DNA stainability; TMSC: total motile sperm count.

recent fertility. Bellver *et al.*[30] found that there was no statistically significant difference in the DFI (using the SCD test method) between a group of 30 patients with RPL and the 30 controls, and Coughlan *et al.*[21] came to the same conclusion using the SCD test in 16 RPL patients.

To our knowledge, the present study is the first investigation of the male factor in RPL following natural conception in a large cohort of Chinese patients. We also analyzed the correlation between semen parameters and DFI. Progressive motility of sperm, TMSC, sperm concentration, and male age showed correlation to the DFI, with no significant differences for other semen parameters. Previous studies conducted to demonstrate the correlation between SDF and semen parameters showed mixed results in RPL patients. Zhang *et al.*[31] showed that there were no significant differences in ejaculate volume, sperm concentration, or percentage of abnormal forms between 111 RPL men and 30 healthy fertile controls. Brahem *et al.*[32] found that sperm motility was higher in the control group ($P < 0.001$), and they did not find any statistically significant difference in other semen parameters between the RPL and control groups. Bhattacharya *et al.*[33] studied 74 RPL men and 65 fertile men and found no significant difference in age and semen parameters. Further, Khadem *et al.*[14] also did not find any statistically significant difference in semen parameters except for a negative correlation between SDF and progressive motility ($r = -0.613$; $P < 0.001$) and percentage of abnormal forms ($r = -0.764$; $P < 0.001$). Carlini *et al.*[12] found that SDF had a positive correlation with sperm

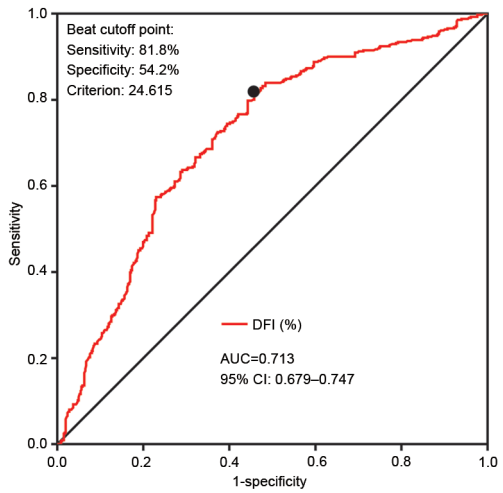

Figure 3: Receiver operating curve analysis of DFI in PRL and control group. Using receiver operating characteristic curve analysis, a threshold value of 24.6% was obtained to discriminate from the control group. The area under the curve was 0.713 ($P < 0.001$; 95% CI: 0.679–0.747), with 81.8 % sensitivity and 54.2 % specificity. AUC: area under the curve; RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; CI: confidence interval.

Table 3: Multivariate logistic regression analyses of factors affecting recurrent pregnancy loss

Items	*Multivariate logistic regression*			
	P	*OR*	*95% CI of OR (lower)*	*95% CI of OR (higher)*
DFI	<0.001	1.096	1.078	1.115
Age of male	0.095	0.891	0.847	0.934
Age of female	<0.001	1.120	1.062	1.182
Volume of semen	0.400	0.947	0.835	1.075
pH	0.544	0.691	0.209	2.281
Sperm concentration	0.450	0.998	0.994	1.003
Progressive motility	0.003	0.905	0.866	0.945
Total motile sperm count	0.292	1.001	0.999	1.003
Normal forms	0.444	1.015	0.977	1.054
HDS	0.126	0.971	0.936	1.008

Factors predicting RPL were determined by multivariate logistic regression analyses. $P<0.05$ was considered statistically significant. RPL: recurrent pregnancy loss; DFI: DNA fragmentation index; HDS: high DNA stainability; CI: confidence interval; OR: odds ratio

morphology, although this was not statistically significant. However, there was a moderate inverse correlation between SDF and progressive motility ($r = -0.41$, $P < 0.001$). Besides, they found no correlation between SDF and the total sperm count. The inconsistency in the results of different studies is due to the differences in the techniques used, sample size, and cutoff values of DFI.

In our findings, SDF showed moderate inverse correlations with progressive motility ($r = -0.47$, $P < 0.001$) and TMSC ($r = -0.31$, $P < 0.001$) and mild inverse correlations with sperm concentration ($r = -0.16$, $P < 0.001$), indicating that sperm DNA damage might be a key factor leading to the decrease in semen quality. It has been described by some authors that men with a history of RPL had a higher incidence of sperm with poor motility than that of men from a control group.[32,34] Furthermore, Xue *et al*.[35] found that sperm DFI, as an independent factor, could predict male fertility even better than routine semen parameters.

Some studies have shown that the sperm DFI tends to increase with increasing paternal age.[36–38] Cohen-Bacrie *et al*.[37] reported a significant correlation between SDF and paternal age in a prospective study of 1633 patients using TUNEL. Our data also suggest that increasing male age is correlated with decreasing sperm DNA integrity ($r = 0.15$, $P < 0.01$), which is consistent with the findings of these studies.

Despite a number of studies showing that RPL and sperm DFI have a strong correlation, the potential mechanism of the effects of high sperm fragmentation on RPL remains unclear. Sperm DNA integrity is an important requisite for the correct transmission of genetic material to the offspring, and its impairment increases the risk of abortion. The production of DNA fragments in sperm is usually caused by external factors, such as reactive oxygen species (ROS), rather than programmed cell death.[39,40] This is supported by the fact that the use of antioxidant therapy in men reduced oxidative DNA damage. In their study, Menezo *et al*.[41] reported that the use of oral antioxidant therapy could reduce the sperm DFI, especially in the setting of oxidative DNA damage, and significantly improve sperm DNA quality. DNA strand breaks usually occur during meiosis, and oxidative stress induces DNA degeneration, which results in single- and double-stranded breaks. The spermatozoa carrying damaged DNA can fertilize and bind to oocytes, but with the paternal genome activated, it may interfere with the development of the embryo, leading to regulation failure of paternal genes in early embryos.[42–46] Another factor that has a possible role in RPL with high sperm fragmentation is the repair mechanism of the oocyte on sperm DNA damage. Hamatani *et al*.[47] reported that sperm DNA may be repaired by oocytes up to a threshold of female age ≤35 years. Our data also suggest that female age could be a risk factor for RPL (OR = 1.120, 95% CI = 1.062–1.182, $P < 0.001$).

ROC curves and the area under the ROC curve (AUC) were used to assess the feasibility of DFI in distinguishing RPL and control cases. The value (25%) of DFI from RPL cases by using ROC curve analysis was lower than the 30% value previously reported threshold for male infertility.[48] A minor limitation of this study is that the two groups of women underwent different treatments, with the women from the control group having received ovarian stimulation. However, the control group completed the IVF cycle treatment within 1 month after the DFI test, so the quality of sperm was less likely to be affected by environmental and lifestyle factors.

On the basis of the results above, our study demonstrated that SDF is an important cause of RPL, and couples with a history of RPL showed a higher incidence of SDF and poor progressive motility of the sperm. These findings indicate that testing for DNA fragmentation has a certain predictive value in the assessment of the prospective risk of RPL; moreover, the higher the level of sperm DFI, the higher the risk of RPL. It is necessary to perform an SDF test in couples experiencing RPL.

AUTHOR CONTRIBUTIONS

XBZ and QC designed the project, recruited the patients, reviewed and analyzed the data, and wrote the paper. WMF conducted semen tests and SCSA tests, and ZHN conducted the statistical analysis. BFX also recruited the patients and analyzed the data. AJZ conceived this study, performed data analysis, and prepared the manuscript. All authors have read and approved the final manuscript.

COMPETING INTERESTS

The authors declared no competing interests.

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