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Original Article

Obstetrics and Gynaecology Section

Role of Sperm DNA Fragmentation Index in Semen Analysis in Couples with Unexplained Recurrent Pregnancy Loss: A Case-control Study

RUCHI HOODA[1], G GEETA DEVI[2]

ABSTRACT

Introduction: Infertility has been on the rise, and male factor infertility has gained attention in cases of Recurrent Pregnancy Loss (RPL). Traditional Semen Analysis (SA) has been the main diagnostic tool, but with advancements in Assisted Reproductive Technology (ART) and the need for more accurate testing, there is a demand for improved diagnostic tests that correlate with reproductive outcomes. Sperm DNA Fragmentation Index (DFI) is a parameter used to assess the degree of sperm DNA damage and is considered crucial in evaluating semen quality.

Aim: To investigate the role of Sperm DNA Fragmentation (SDF) in patients presenting with RPL compared to a control group.

Materials and Methods: The study was conducted over a two-year period from January 2021 to December 2022 at the Centre for Infertility Management and Assisted Reproduction (CIMAR), Edappal Hospital, Edappal, Kerala, India. The control group (Group A; n=31) comprised males aged between 21-45 years whose partners had no history of recurrent abortions. The case group (Group B; n=31) included males with two or more pregnancy losses. Sperm DFI analysis and routine SA were performed in both groups to assess semen parameters such as volume, concentration, progressive and non progessive motility, morphology, and DFI. Statistical analysis was performed using the Independent student t-test.

Results: In the present study the mean age of males in Group A was 37.03±5.416 years and in Group B was 35.44±4.552 years. There were no significant differences observed between the case and control groups in terms of sperm volume (p=0.301), concentration (p=0.155), progressive motility (p=0.207), non progessive motility (p=0.178), and morphology (p=0.362). However, a statistically significant difference was found between the RPL and control groups for DFI (p<0.001), with a mean value of ±8.15 in the control group and ±19.35 in the case group.

Conclusion: The present study demonstrates that SDF is an important factor in RPL, with couples experiencing RPL showing a higher incidence of SDF. Therefore, incorporating SDF analysis alongside routine SA should be considered, particularly in patients with a history of RPL.

Keywords: Assisted reproductive treatment, Deoxyribonuleic acid, Sperm chromatin dispersion

INTRODUCTION

The definition of RPL is not uniform. The American Society of Reproductive Medicine defines RPL as two or more pregnancy failures [1]. The Royal College of Obstetricians and Gynaecologists defines RPL as three or more consecutive losses with the same sexual partner, occurring before 24 weeks of pregnancy [2]. The European Society of Human Reproduction and Embryology (ESHRE) guideline group defines RPL as the occurrence of three or more consecutive pregnancy losses before 20 weeks of gestation [3]. The aetiology of RPL is unknown in about 50% of cases [4]. The causes of RPL are heterogeneous and include endocrine dysfunction, autoimmune disorders, maternal and paternal age, genetic abnormalities, infectious diseases, congenital or structural uterine anomalies, and exposure to environmental toxins [4].

Infertility is on the rise, with an incidence of 10-15% in couples of reproductive age [5]. Among these cases, 50% involve male factors, and 20% exclusively have "male factor" infertility [6]. Recently, the evaluation of male factors has also gained importance in cases of RPL. The diagnosis of male factor infertility is heavily reliant on traditional SA. However, traditional SA is found to be normal in only about 15% of male patients experiencing infertility [7]. This highlights the limitation of SA in making a diagnosis of male fertility, as it merely serves as a surrogate marker and provides limited information on sperm concentration, motility, and morphology, without revealing sperm functional competence and reproductive potential.

With advancements in ART and the increasing needs of at-risk couples, traditional SA alone has become insufficient. There is a demand for more advanced diagnostic tests that can correlate with reproductive outcomes. Sperm DFI is used to assess DNA damage, which is considered an important parameter in evaluating semen quality. It has been observed that sperm DNA fragmentation can affect fertilisation, embryonic development, and the transmission of paternal genetic information during both spontaneous and ART pregnancies [1,8].

The aetiology of sperm DNA damage can include abnormal chromatin packing, apoptosis, and elevated levels of Reactive Oxygen Species (ROS) [9]. Several tests are available to assess sperm DNA integrity by evaluating strand breaks In-situ [10]. Methods such as the single-cell gel electrophoresis assay (comet assay), Terminal Deoxynucleotidyl Transferase d-UTP Nick End Labelling (TUNEL) assay, and Sperm Chromatin Structure Assay (SCSA) are commonly utilised [11]. Although, sperm DFI is not a part of the basic semen analysis, it is useful in cases of unexplained infertility with normal semen parameters. The evaluation of the male factor has been given less importance in couples with RPL, and a study was conducted to assess the role of the male factor and the role of DFI in semen analysis in association with RPL. The study reinforces that sperm DNA fragmentation testing could be used to provide explanations in couples with unexplained RPL [12]. There is evidence supporting an independent association between RPL and sperm DNA fragmentation, regardless of female factors, according to the ESHRE guidelines. However, the guidelines

highlight the need for further investigation to understand the impact of sperm DNA fragmentation on RPL [13].

Therefore, studies are needed to examine other aspects such as sperm DNA maturity and condition, as well as focusing on molecular factors implicated in male fertility, such as oxidative stress molecules, sperm DNA fragmentation, and Sperm Chromatin Density (SCD) [14]. The aim of the present study was to investigate the role of sperm DNA fragmentation in patients presenting with RPL compared to a control group.

MATERIALS AND METHODS

The present case-control study was conducted over a period of two years, from January 2021 to December 2022, at CIMAR, Edappal Hospital, Edappal, Kerala, India. The study was approved by the Research Ethics Board of Edappal Hospital Pvt., Ltd., Edappal, Kerala, India (IEC-13/19). The control group (Group A; n=31) consisted of males aged between 21-45 years whose partners had no history of recurrent abortions. The case group (Group B; n=31) included males who had two or more pregnancy losses. Sperm DFI analysis and routine SA were performed in both groups to assess semen parameters such as volume, concentration, progressive and non progessive motility, morphology, and DFI of the sperm.

Sample size calculation: Sample size was calculated based on a prevalence (P) of unexplained RPL of 1%, a significance level of 5%, a power (1-β) of 90%, and an effect size of 0.5, with a 10% allowance for loss to follow-up, using G power 3.1 software.

Inclusion criteria: males aged between 21-45 years whose partners had two or more pregnancy losses and in the control group, couples with unexplained infertility were included.

Exclusion criteria: Male partners with female partners having uterine anomalies, known antiphospholipid syndrome, inherited thrombophilias, endocrine causes, or balanced translocations were excluded from the cases. Patients with any known cause for male and female infertility leading to recurrent losses were excluded from the study.

Study Procedure

Basal and demographic data of the recruited patients were collected, including the age and BMI of the partners. Male partners underwent SA and DFI testing after an abstinence period of 2-7 days. Morphological abnormalities were assessed using Papanicolaou stain and categorised based on the location of the defect involving the head, neck, or tail. The Sperm Chromatin Dispersion (SCD) test was used to assess the DFI. After assessing the concentration, the semen sample was diluted with a culture medium to a concentration of 5-10 million/mL. Agarose was melted at 90° Celsius by placing it in the sperm chroma warmer 1 for five minutes. The melted agarose was then transferred to the sperm chroma warmer 2, which was maintained at 37° Celsius, and allowed to equilibrate for five minutes. Approximately 25 microliters of the diluted semen sample was added to the agarose and mixed thoroughly. The sperm cell suspension was immediately placed onto pretreated slides, and a cover slip was carefully placed, taking precautions to avoid the formation of air bubbles. The slide was then kept at 4° Celsius for five minutes, and the cover slip was gently removed by sliding it off. Throughout the procedure, the slide was maintained in a horizontal position.

The Sperm Chroma Kit from Cryolab International, which included four solutions (A, B, C, and D), was used following the protocol provided by the company. The slides were incubated horizontally in solution A for seven minutes, followed by lysis solution for 25 minutes. Afterward, the slides were immersed in distilled water for five minutes, 70% ethanol for two minutes, 90% ethanol for two minutes, and finally, 100% ethanol for two minutes. The slides were allowed to dry at room temperature. A mixture of solutions C and D in a 1:1 ratio was prepared and a layer of the stain was deposited horizontally on the slides, which were then left for 15-20 minutes. The stain was decanted, and the slides were gently washed with distilled water and dried at room temperature. The slides were visualised under a bright field microscope using a 20X or 40X objective. At least 300-500 sperm were counted to calculate the DFI. Sperm without DNA fragmentation exhibited a large 2 halo or medium halo, while sperm with DNA fragmentation appeared small, without a halo, or degraded.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 20.0. Continuous variables were presented as mean value±Standard Deviation (SD), and comparisons between groups were determined using Independent t-tests. The Shapiro-Wilk test or Kolmogorov-Smirnov test was used to assess normality. All tests were two-sided, and a p-value <0.05 was considered statistically significant. Kurtosis was used to describe the degree to which scores cluster in the tails or the peak of a frequency distribution.

RESULTS

The study included 31 women in the age range of 21-45 years (mean: 32.1±5.51 years) who had experienced RPL, and 31 women (mean: 29.74±4.69 years) as controls. There were no statistically significant differences in the ages of the women (p=0.078) [Table/Fig-1].

A statistically significant difference was observed between the RPL group and the control group only for the DFI [Table/Fig-2].

Parameters	Mean	Median	SD	Kurtosis	Test of normality	p-value
Female age (years)						
Group A	32.1	31.5	±5.51	-0.1	0.348	0.078
Group B	29.74	30	±4.69	-0.32	0.165	
Female BMI (kg/m^2)						
Group A	21.94	222.2	±2.57	-1.151	0.188	0.402
Group B	22.58	22.3	±3.13	-0.187	0.199	
Male age (years)						
Group A	37.03	37.10	±5.416	-1.152	0.054	0.211
Group B	35.44	35.48	±4.552	-0.881	0.156	
Male BMI (kg/m^2)						
Group A	22.54	23.05	±4.38	1.52	0.001	0.061
Group B	24.68	24	±3.29	0.395	0.012	

[Table/Fig-1]: Characteristics of male and female factors in Recurrent Pregnancy Loss (RPL) and control group.
Independent student t-test; BMI: Body mass index; SD: Standard deviation

Semen parameters	Mean	Median	SD	Kurtosis	Test of normality	p-value*
Volume (mL)						
Group A	1.65	1.4	±0.85	10.077	0.01	0.301
Group B	1.87	1.8	±0.91	3.27	0.03	
Concentration (million)						
Group A	43.18	39.5	±24.22	-0.233	0.044	0.155
Group B	39.58	26	±34.27	4.6	0.001	
Count (million sperm/mL)						
Group A	63.633	46.5	±41.82	4.39	<0.001	0.559
Group B	72.509	48	±79.62	6.406	<0.001	
Progressive motility (A)						
Group A	7.16	6	±4.11	1.998	<0.001	0.207
Group B	7.90	4	±8.34	3.546	<0.001	
Slow progressive motility (B)						
Group A	32.53	32	±9.78	1.135	0.305	0.178
Group B	35.90	35	±9.54	1.037	0.051	

Non motility						
Group A	60.3	60	±11.13	-0.106	0.736	0.174
Group B	35.81	20	±45.88	9.154	0.088	
Total motile						
Group A	39.7	40	±11.12	-0.106	0.736	0.174
Group B	43.8	40	±12.14	-0.842	0.088	
Total Motile Sperm Concentration (TMSC) (ejaculate volume (mL)× concentration×% motility of A and B/100)						
Group A	30.07	20.5	±28.05	5.313	<0.001	0.862
Group B	56.19	60	±12.14	-0.842	<0.001	
Morphology						
Group A	9.4	9.5	±2.094	2.48	0.18	0.362
Group B	9.8	10	±1.910	0.191	0.148	
Head defects						
Group A	80.86	80	±7.8	0.345	0.51	0.64
Group B	79.96	81	±7.1	0.614	0.055	
Neck defects						
Group A	76.67	77	±7.2	0.442	0.65	0.76
Group B	74.87	79	±7.6	0.654	0.023	
Tail defects						
Group A	86.32	82	±8.4	0.67	0.54	0.59
Group B	81.23	81	±7.4	0.49	0.076	
DNA Fragmentation Index (DFI)						
Group A	18.55	16.5	±8.15	0.373	0.009	0.002
Group B	31.22	27	±19.35	2.552	<0.001	

[Table/Fig-2]: Parameters of Semen Analysis (SA) and DNA fragmentation in recurrent pregnancy group (Group B) and control group (Group A).
p-value *determined using Independent student t-test; SD: Standard deviation

DISCUSSION

Sperm DNA integrity plays a crucial role in determining male reproductive potential and outcomes. The inclusion of sperm DNA Fragmentation (SDF) testing can be used for predictive purposes and to plan individualised treatment strategies. Unfortunately, many ART centres still overlook the evaluation of male partners facing fertility issues with normal SA or who have spermatozoa available for intracytoplasmic sperm injection without considering the potential impact of DNA fragmentation on RPL. This oversight can lead to multiple failed ART cycles, adding to the emotional distress experienced by the couple and the challenges faced by the treating physician. SDF testing in men with Oligoasthenoteratozoospermia (OAT) on routine SA could be considered, as recommended by the European Academy of Andrology (EAA) [15]. The European Association of Urology (EAU) supports SDF testing in couples with unexplained infertility or after RPL [16]. The European Society of Human Reproduction and Embryology (ESHRE) recognises the potential role of SDF testing in explaining the association with RPL [17]. Recently, the American Urological Association (AUA) and American Society for Reproductive Medicine (ASRM) published guidelines on male infertility, recommending against SDF testing in the initial evaluation of fertility but acknowledging its importance in couples experiencing RPL [18].

In the present study, sperm from men in the RPL group exhibited a higher percentage of DNA fragmentation (31.22%) compared to sperm from men in the control group (18.5%) p<0.001. These findings are consistent with other studies that have reported significantly higher levels of abnormal DNA fragmentation in the recurrent spontaneous abortion group compared to the control group [19-21]. This further supports the association between increased DNA fragmentation and RPL (p<0.001). Leach et al., conducted a study involving 108 couples with a history of RPL and found significantly higher levels of SDF using the Sperm Chromatin Structure Assay (SCSA) [22]. Similarly, Kamkar N et al., used a similar method to assess DNA fragmentation and found higher DFI in the RPL group compared to the control group [23]. Regarding other semen parameters, the present study did not find any significant differences between the two groups in relation to an increased risk of RPL.

A meta-analysis conducted by Yifu P et al., included seven studies using the SCSA, nine studies using the SCD test, and eight studies using TUNEL assay. The results showed significant differences supporting an association between sperm DFI and RPL [24]. Another meta-analysis that assessed the relationship among traditional semen parameters, SDF, and unexplained recurrent miscarriage included a total of 1182 couples with unexplained recurrent miscarriage and 1231 couples without recurrent miscarriage. The results showed significantly increased levels of SDF in unexplained recurrent miscarriage and significantly decreased levels of total motility and progressive motility compared to couples without recurrent miscarriage [25].

In contrast, De Geyter et al., suggested that sperm selection techniques used in ART to decrease SDF rates do not necessarily lead to improvements in pregnancy rates [26]. Moreover, van den Berg et al., confirmed that even a few double-strand DNA breaks are sufficient to delay cell cycle progression [27]. Another study by Gil-Villa AM et al., reported no significant correlation between DNA fragmentation and RPL using the SCSA and concluded that DFI was not an important cause or predictive factor for RPL [28]. This may be due to the fact that oxidant agents have an impact on sperm morphology, motility, and concentration, but not on the sperm nucleus, which provides genetic material to the future embryo. Under these conditions, sperm can still fertilise the oocyte.

The correlation between DFI and RPL remains highly controversial. These differences in opinion among studies may be due to the limited number of cases with RPL and the use of different evaluation methods with varying sensitivities and specificities. The most commonly used methods are SCSA, TUNEL, and SCD [29]. Although authors concluded, that routine semen analysis cannot solely be relied upon to assess the role of the male factor in the incidence of idiopathic recurrent early pregnancy loss, further additional sperm tests, such as SDF testing, are needed to search for a better diagnostic tool.

Limitation(s)

The small sample size was a limitation of present study. Additionally, the study was conducted in a single centre, so the findings cannot be generalised.

CONCLUSION(S)

Sperm DFI is recognised as an important cause of RPL, and couples with a history of RPL have been shown to have a higher incidence of SDF. It has been observed that higher levels of sperm DFI are associated with a higher risk of RPL. Therefore, SDF testing has potential predictive value in diagnosing DNA fragmentation and assessing couples with RPL. It is necessary to perform an SDF test in couples facing RPL.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Reproductive Medicine, CIMAR, Edappal Hospital, Edappal, Kerala, India.
2. Senior Resident, Department of Foetal Medicine, CIMAR, Edappal Hospital, Edappal, Kerala, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Ruchi Hooda,
Senior Resident, Department of Reproductive Medicine, CIMAR, Edappal Hospital, Edappal-679576, Kerala, India.
E-mail: hoodaruchi@gmail.com

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