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Original Article

Genetics Section

Sperm DNA Fragmentation Index in Infertility Patients vs Normal Individuals: A Cross-sectional Study

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ABSTRACT

Introduction: Infertility is a major problem among married couples. The male factor contributes to around 30-40% of all cases of infertility. With advancing age, reducing sperm count may magnify the problem. The awareness of qualitative change along with quantity is important for people seeking advice in infertility.

Aim: To find the prevalence of sperm Deoxyribonucleic Acid (DNA) fragmentation with Sperm Chromatin Dispersion (SCD) test.

Materials and Methods: The present study was prospective cross-sectional study which was carried out on semen sample given for routine check-up in department during the period from August 2017 to August 2018. A total of 180 couples, including 88 infertile couples and 92 couples with known fertility were enrolled in the study. Sperm Count and sperm DNA Fragmentation Index (DFI) were calculated and determined by SCD test by kit method. Comparison between groups was done according to distribution of data using Mann-Whitney rank-sum (two tailed) statistical test.

Results: Among 180 subjects, (88 cases and 92 control) no significant difference in the age was found. The sperm count was not significantly different in cases (group of men of infertile couple) and controls (group of men of proven fertility). The highest value for DFI was 27% in controls and 97% in cases. Statistically significant difference was found in DFI (p=0.008) in both groups. DFI and sperm count showed negative correlation both in cases and controls with a correlation coefficient 0.213 (p-value <0.001) and 0.754 (p-value <0.005) respectively. A significant difference was observed in the median value of sperm DFI of men of infertile couple as compared to men of fertile couple.

Conclusion: On comparing, significant difference was observed in the median value of sperm DFI in infertile couple. Sperm DFI at cut-off value 27% has higher diagnostic significance. SCD method is simple, easiest and standard tool to assess DFI.

Keywords: Deoxyribonucleic acid, Normozoospermia, Oligozoospermic, Sperm chromatin dispersion test, Sperm count

INTRODUCTION

Infertility is an important and common problem in our society. This is also quite common in affluent and learned group. This problem affects men and women equally. Out of total males, every six men in our society are infertile, which is quiet a depressing figure [1-3]. The problem becomes more, as many men may not accept infertility.

To diagnose infertility due to male factor, the sperm count is an important but not a sufficient test. Once sperm count is reported normal, a man feels happy that he has no problem. However, it's not so because the quality of sperm is also very important to give positive outcome in the form of pregnancy. To test sperm parameters, usually a doctor asks for sperm count, morphology and motility. However, to test the quality of sperm, it is also worthwhile to check for sperm DNA fragmentation (SDF). The SDF reflects the integrity of genetic material of the gamete [4]. The intact sperm DNA in turn is responsible for zygote integrity and pregnancy outcome. SDF can be a result of many factors: smoking, alcoholism, high local temperature, varicocele etc. Some of them are modifiable by adding antioxidants and astaxanthins [5]. Hence, identification of SDF, prior counseling and appropriate interventions may help in improving the fertility outcome [6].

In last three decades, many techniques have been developed to evaluate sperm chromatin quality in reproductive molecular biology [7,8]. The sperm chromatin quality is assessed in the form of sperm DFI. In a country like India, people try to avoid expensive test. DFI can be assessed by Sperm Chromatin Structure Assay (SCSA), TUNEL assay (TdT-mediated –dUTP nick end labelling), COMET assay (single gel electrophoresis) and Acridine Orange-staining Technique (AOT). The newer and economic technique is SCD test. This assay is based on the principle that sperm with fragmented DNA fail to produce characteristic halo of dispersion that is observed in sperm with non fragmented DNA following acid denaturation [9].

Many studies have concluded that sperm DNA fragmentation as one of the factor responsible of male factor infertility [10,11]. However, it is not yet incorporated or recommended as a part of routine investigation of infertility. The aim of present study was to find the prevalence of sperm DNA fragmentation in semen sample of male partner of infertile couple and its correlation with conventional semen parameters with special reference to sperm count in the central India population.

MATERIALS AND METHODS

The present study was a prospective cross-sectional study in which 88 infertile couples visiting infertility clinic in Department of Obstetrics and Gynaecology, AIIMS, Raipur, Chattishgarh, India, and 92 couples with known fertility were included. Study was conducted from August 2017 to October 2018. Institutional Ethical Clearance (IEC) (AIIMSRPR/IEC/2016/046) was obtained.

Inclusion criteria: Normozoospermic infertile couple, Oligozoospermic infertile couple in age group 22-45 years were included as a case. For the comparable control, age was same and either normozoospermia or oligozoospermic having child.

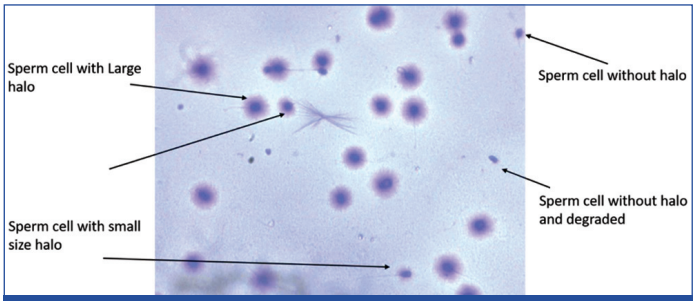
Exclusion criteria: Men with history of testicular maldescent, testicular malignancy or testicular injury including torsion were excluded.

Study Procedure

Eighty-eight men from infertile couples and 92 men from couples with known fertility were enrolled in the study. Men from infertile couples were taken as cases and men with known fertility were taken as control group. Patient information sheets were given to all and explained about the outcome. After obtaining written consent

for participation, patients were asked to give semen sample usually after three days of abstinence. Each sample was allowed to liquefy for 20-30 minutes at 37°C. After evaluating volume, samples were aliquoted in two parts. One part of the sample was used to evaluate basic sperm parameters including sperm count as per to World Health Organisation (WHO) guideline [12] in Department of Pathology and Laboratory Medicine. Based on the total sperm count, the cases were divided in two subgroups; normozoospermic (>15 million/mL) and oligozoopermic group (<15 million/mL).

The second part was used to assess sperm DNA fragmentation in Department of Anatomy. The sperm DNA fragmentation was carried out by SCD test: Sperm DNA fragmentation test was done by kit sperm 360 DNA fragmentation (Sperm Processor Pvt. Ltd., Aurangabad, India). This kit is based on protocol illustrated by Fernandez JL et al., and further modified by Chauhan KR et al., using 0.4 M Dithiothreitol (DTT) [9,13]. An aliquot of semen sample is mixed with 1% low melting agarose at 37°C. Aliquots of 60 µL of the mixture were pipetted and spread on slide and covered with coverslip. Slides were left to solidify at 4°C. After that the slide was treated with reagent I (lysis solution) for seven minutes. Then the slide was treated with reagent II (neutralising and lysis solution 2) then washed with distilled water. The slide was then dehydrated sequentially in 70%, 90% and 100% ethanol, keeping for two minutes in each. Slide was stained with stain provided in kit for microscopy. Thereafter, the slide was examined under bright field microscope. On each slide, 500 sperms were evaluated for halo size and dispersion pattern as described by Fernandez JL et al., [9] for: 1) nuclei with large DNA dispersion halos; 2) nuclei with medium sized halos; 3) nuclei with small sized halos; and 4) nuclei with no halo. The nuclei with large to medium size halo were considered as non fragmented DNA (dispersed nuclei). The nuclei with small size halo and no halo at all were considered as fragmented DNA (non dispersed nuclei) [Table/Fig-1]. DFI were calculated as percentage of number of spermatozoa with fragmented DNA out of number of spermatozoa counted. The subjects were further divided according to DFI with cut-off values 25%, 15%-25% and <15% [14].



[Table/Fig-1]: Sperm Chromatin Dispersion (SCD) test: sperm with different size of halo using Wright stain and magnification (40x).

STATISTICAL ANALYSIS

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) (version 21.0) software. Comparison between groups was done for values of Body Mass Index (BMI), sperm count, DFI by using Mann-Whitney rank-sum (two tailed) statistical test. Results were expressed in mean±Standard Deviation (SD). Percentage of non fragmented DNA/dispersed nuclei, fragmented DNA/non dispersed nuclei and dead nuclei were calculated in cases and control. Pearson's correlation test was used to correlate various variables in the study. The p-value of <0.05 was considered as statistically significant.

RESULTS

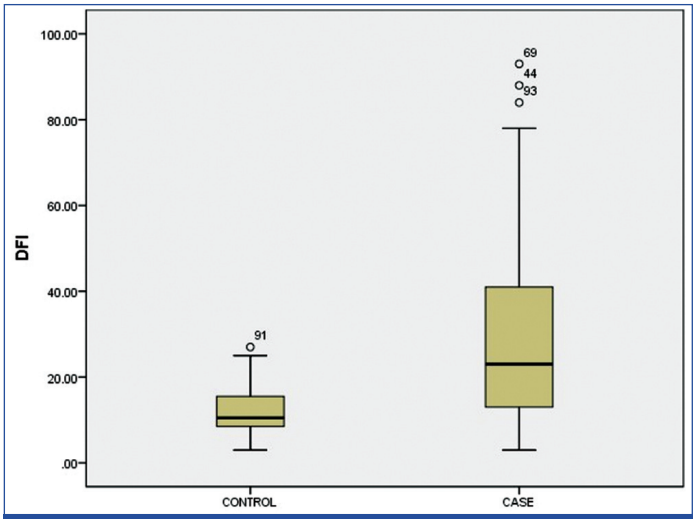
All variables tested by Mann-Whitney rank-sum test were found to be variably distributed. Results of parameters measured in the study are shown in [Table/Fig-2]. Demographic profiles were not significantly different. The cases showed decreased sperm count as compared to controls. However, this was not statistically significant. Percentage of non fragmented sperm count were significantly lower in cases than in control (p= 0.011). Percentage or fragmented sperm and dead sperm were significantly higher than control (p=0.026, p=0.05, respectively).

Parameters	Men of infertile couple (N=88)	Men of proven fertility (N=92)	p-value
Age (years)	30.32±4.56	31.33±4.92	0.481
BMI (Kg/m^2)	23.15±4.44 (13-32)	23.54±4.33 (15.56-28.91)	0.773
Sperm count million/mL	64.55±54.83 (1-210)	80.83±36.04 (30-150 million/mL)	0.321
Non fragmented DNA (Dispersed nuclei) %	70.23±22.17 (7-97%)	87.08±7.09 (73-97%)	0.011*
Fragmented sperm (Non dispersed nuclei) %	19.52±17.16 (3-89%)	8.25±2.98 (3-13%)	0.026*
Degraded sperm %	9.63±8.45 (0-54%)	4.66±4.77 (0-15%)	0.050*
DNA fragmentation index %	29.45±21.16 (3-93%)	12.83±7.06 (3-27%)	0.008*

[Table/Fig-2]: Comparison of various parameters between two groups: case and control.
To obtain p-value, unpaired t-test was used. *p-value <0.05 was significant

The highest value of DFI in cases was 93% as compared to 27% in controls. This was statistically significant (p=0.008). On comparing DFI value was significantly high in men from control group as compared to men from infertile couple.

DFI and sperm count showed negative correlation both in cases and control with a correlation coefficient being 0.213 (p-value <0.01) and 0.754 (p-value <0.005), respectively. Median value of sperm DFI in cases was significantly higher, 23% (3-93%), compared to 10.5% (3-27%) {[Table/Fig-3] box plot}.

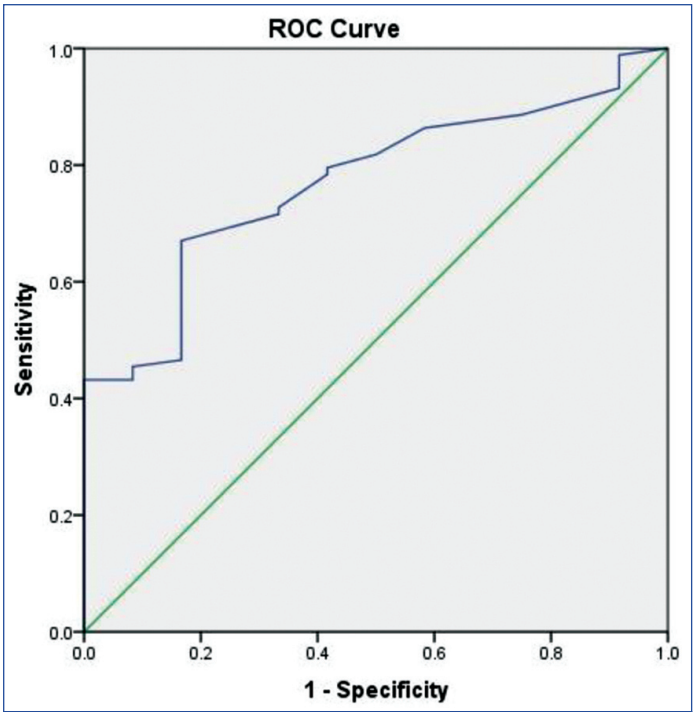


[Table/Fig-3]: Box Plot for control and case of DNA fragmentation index (DFI).

The discriminating power of DFI measured by SCD test, to identify threshold value between cases and control, was calculated by ROC curve analyses. During ROC analyses [Table/Fig-4], varying percentage of DFI values was used to calculate optimum sensitivity and specificity value for SCD test. The best area under cover ROC curve was 0.770 for 27% of DFI [Table/Fig-4]. With the cut-off point 27%, sperm DFI was able to distinguish cases and control. With this threshold had 98.91% (94.01.52% to 99.97%) specificity and sensitivity of 43.18% (95% CI 32.66 to 54.18). The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the 27% sperm DFI were 97.44% (95% CI 84.21 to 99.63) and 64.54 (95% CI 60.24 to 68.62%), respectively. The sperm DFI value >27% was found in 31% (20/65) of normozoospermic cases and 78% (18/23) of oligozoospermic cases [Table/Fig-5].

DISCUSSION

In spite of the periodic refinements in semen analysis techniques and cut-off values in the form of WHO guidelines for semen assessment, upto 30% of men with normal semen parameters remain infertile



Area under the curve

Test result variable(s): DFI

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence interval	
			Lower bound	Upper bound
0.770	0.058	0.002	0.656	0.885

[Table/Fig-4]: Receiver operating characteristics at 27% sperm DNA fragmentation showing specificity and sensitivity in infertility group (n=88).
The test result variable(s); DFI has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
a) Under the nonparametric assumption
b) Null hypothesis: true area=0.5

DNA fragmentation index	Men of proven fertility (Control)	Men of infertile couple (Case)	
	Normozoopermia (>15 million/mL) N= 92, n (%)	Normozoopermia (>15 million/mL) N=65, n (%)	Oligozoospermia (<15 million/mL) N=23, n (%)
<15%	69 (75)	24 (36.9)	3 (13.1)
15-25%	15 (16.3)	20 (30.8)	1 (4.3)
>25%	8 (8.7)	21 (32.3)	19 (82.6)

[Table/Fig-5]: Frequency of occurrence of DNA fragmentation in both case and control.
Normozoopermic Controls verses Normozoopermic cases; χ^2 value=17.67, p-value=0.001
Normozoopermic Cases verses Oligozoopermic cases; χ^2 value=1.67, p-value=0.046 N=frequency

[15]. The studies advocate testing the sperm DNA fragmentation test before going for Assisted Reproductive Technology (ART). In order to avoid an increase in budget, whom to recommend should be assessed. Studies suggest that sperm DNA fragmentation test appears to have strong correlation with, In Vitro Fertilisation (IVF) and Intracytoplasmic Sperm Injection (ICSI) outcome [16,17].

In present study, there was no significant difference in the age and BMI of two groups [Table/Fig-1]. Studies have reported that ageing decreases the function of organs hence the sperm count. With increasing infertility problem, one should go for DNA fragmentation test without any delay. Frattareli JL et al., found age related decrease in ability of spermatozoa to fertilise ova [18]. Other studies reported that age has no effect on fertilisation rate [19] and still others have shown negative correlation with paternal age for ICSI [20].

In current study, DFI value was significantly higher in men from infertile couple as compared to men from control group which suggests high DNA fragmentation is associated with infertility. Threshold DFI value (27%) showing infertility in the current study was comparable to earlier study done by Larson KL et al., [21]. They had also reported absence of clinical pregnancy at or above DFI threshold value of 27%. Also, in their study using these samples in ART i.e., IVF and ICSI cycles did not give good results. Fernandez JL et al., found statistically significant difference in sperm DFI values in healthy sperm donor and infertility patients (16.7±9.9 Vs 35.4±18.3, p<0.05), tested with SCD test [9]. Wiweko B and Utami P in their study again found significant difference in DFI in healthy fertile men and infertile men (19.9% vs 29.9%, p<0.001) [22]. Sperm nuclear protein, the protamine and Transitional Protein (TP), are responsible for integrity of compacting of sperm DNA. Sperm nuclear protein plays vital role in integrity of compact sperm DNA during spermatogenesis [23-27]. Sperm DNA damage is because of protamine deficiency. In the current study, median value of DFI was significantly higher in cases as compared to control. Wiweko B and Utami P also found similar results [22].

In the current study, 32.3% of normozoospermic cases and 83% of oligozoospermic cases had sperm DFI value >25%. In the current study, 31% of normozoospermic cases and 78% of oligozoospermic cases have sperm DFI value >27%. It means the probability of oligozoospermic cases to have high sperm DFI value was significantly higher as compared to normozoospermic cases. However, Fernandez JL et al., found no statistically significant differences in sperm DFI values from infertility patients with normal or abnormal semen parameters (32.1± 20.4 vs 38.7±16.3, p>0.05) [9].

Correlation between DFI and semen parameters was found to be only weak-to moderate (r=0.213). This was statistically significant (χ^2 value=1.67, p-value=0.046). Normozoospermic cases and control also had significant difference in preponderance of DFI (χ^2 value=17.67, p-value=0.001). Other studies also showed similar finding [28-30].

In current study, the method adopted to study the sperm DNA fragmentation was SCD. In this, on agarose fixed sperm, acid denaturation solution is used to segregate single stranded DNA [31] and is responsible for suppression of production of DNA halo. Treatment with lysis solution is responsible for disintegration of single stranded DNA and non formation of DNA halo in fragmented sperm. The result of ROC curve analysis indicated that sperm DNA fragmentation, as measured by SCD test, was a good predictive parameter to distinguish between fertile and infertile population on the basis of largest AUC {0.77; [Table/Fig- 2]} (p<0.002; 95% CI 0.656-0.885). Wiweko B and Utami P also found AUC value 0.862 (p<0.001; 95% CI 0.783-0.941) [22].

Various studies have produced correlation between sperm DFI value and clinical pregnancy outcome. Meta-analysis and systemic review by Zini A, Osman A et al., Agarwal A et al., have evidenced a significant correlation between sperm DFI value and pregnancy rates with IUI and IVF {Odds ratio (OR)= 9.9, p<0.001} [32-34]. Additionally, Zini A et al., reported correlation between sperm DFI value and miscarriage rate after IVF and ICSI (combined OR=2.48, p<0.0001) [35].

Limitation(s)

Limitations of present study design are: 1) A very small sample size was enrolled in the study; 2) Participants were selected from one Institute and therefore may not be representative of all patients with sperm defragmentation in general central Indian population. Many other environmental factors may contribute to sperm DNA damage in different parts of India.

CONCLUSION(S)

On comparing, significant difference was observed in the median value of sperm DFI of men of infertile couple. The SCD test is a simpler method for the analysis of sperm DNA fragmentation in semen. This test offers simple and reliable way to screen intact sperm, sperm quality. In the management of infertility patients, the inclusion of SCD test along with the routine semen analysis may help estimate the success of ART. Thus, it is suggested that sperm DFI with a cut-off

of 27% by SCD method may be used along with semen analysis before sending an infertile couple for ART in this population.

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