Original Article

Explaining the Unexplained: Examining the Predictive Value of Semen Parameters, Sperm DNA Fragmentation and Metal Levels in Unexplained Infertility

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Abstract

Background: There is ongoing research to find an optimum modality to predict male fertility potential. **Aims:** To compare the semen parameters, sperm DNA damage and seminal metal levels of Zinc, Lead and Aluminium among the male partners of couples with unexplained infertility and men with proven fertility. **Settings and Design:** Prospective case–control study at a tertiary level teaching hospital. **Materials and Methods:** One hundred male partners of couples with unexplained subfertility and 50 men with proven fertility were included in the study. Male partners of unexplained infertility couples and fertile men were compared for their semen parameters, sperm DNA Fragmentation Index (DFI) and seminal metal levels in semen. **Statistical Analysis Used:** Chi-square test, Student's *t*-test, sensitivity and specificity analysis, binomial logistic regression analysis. **Results:** Fertile men had statistically significantly higher mean progressive sperm motility than male partners of unexplained infertility (53.12 ± 9.89% vs. 44.81 ± 19.47%, $P = 0.005$). Semen volume and sperm concentration were comparable among the cases and control population. The mean sperm DFI was significantly lower among fertile men (10.83 ± 6.28 vs. 21.38 ± 10.28, $P < 0.0001$). Plotting the receiver-operating characteristic curve the threshold for discrimination was calculated to be 18% DFI. The sensitivity specificity and overall accuracy were 43%, 84% and 56.67%, respectively when the DFI cut-off was set at 18%. Zinc concentration in the semen had a strong positive correlation (Point Biserial correlation coefficient = 0.831) with fertility, whereas lead and aluminium had a moderate negative correlation. **Conclusion:** Conventional semen analysis had limited differentiating ability for unexplained infertility. The sperm DFI may be employed for explanatory purposes among couples with unexplained subfertility. A lower discriminatory threshold of DFI (18%) has better overall accuracy as opposed to a 30% cutpoint for unexplained subfertility. Among metals, Zinc was strongly correlated with fertility status.

Keywords: *Metal concentration, semen parameters, sperm DNA fragmentation, unexplained infertility*

Introduction

Semen analysis has remained the cornerstone for evaluating male reproductive potential while being far from an ideal diagnostic modality.[1,2] Increasing

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evidence is emerging on the role of sperm in embryo gene expression, embryo development and miscarriage risk.[3] Inopportunely, standard semen examination does not provide any prognostic information on these parameters. The biological inherent variability of semen parameters and the inability to assess the post-ejaculatory changes such as capacitation, hyperactivation or acrosomal reaction are the possible factors for the poor predictive value of standard semen analysis for reproductive outcomes.[4,5]

Researchers worldwide have been exploring novel functional and molecular-level diagnostic modalities. One such test is the estimation of sperm DNA damage and the most imperative of these changes is single or double-strand breaks also known as sperm DNA fragmentation (SDF). SDF can be the result of ineffective packing of DNA during spermatogenesis, apoptosis or oxidative stress secondary to environmental or lifestyle deterrents.[6] Furthermore, many heavy metals and metalloids have been studied in relation to sperm chromatin DNA damage by producing oxidative stress.[7-9]

In the present study, the aim was to compare the semen parameters, SDF and semen metal concentrations among male partners of fertile couples and couples experiencing unexplained infertility to establish the parameters that more efficiently predict male fertility status.

Materials and Methods

Study population and participants

The prospective, comparative case–control study was conducted at a tertiary-level teaching hospital following approval from the institutional research board (Certificate number: AIIMS/RES/2018/1916) and the institutional ethical committee. The research was conducted in accordance with ethical principles charted out in Helsinki Declaration (2013). The sample size was regulated by feasibility obligations of fertile men willing to participate in the study. Hence, it was decided to identify 100 eligible subjects to enable us we will us to estimate a participation rate of 50% to within a 95% confidence interval of ± 10%.[10] Therefore, 100 male partners of the couples diagnosed with unexplained subfertility constituted the case arm of the study while 50 age-matched male partners of couples who have conceived within the last 12 months attending ante-natal outpatient department were enrolled in the control arm of the study following written informed consent. Couples were diagnosed with unexplained infertility after thorough evaluation and if they had normal ovarian function, patent tubal status, absence of ejaculatory concern or pelvic pathology and semen parameters within normal reference ranges as advocated by the WHO 2010 semen examination manual.[11] Whilst, few studies have put forth a possible association between sperm DNA damage and miscarriages, couples with conception beyond 20 weeks of gestation were approached for the control group.[12] Non-obese (body mass index < 30), non-smoker healthy men free from significant addictions and medical co-morbidities aged 20–45 years were recruited for the study. Men with a documented history of genital trauma, testicular mass, previous genital infection or varicocele were excluded from the study. Similarly, couples with female partners aged more than 37 years were not enrolled in the study to adjust for the confounding effect of female age. The seminal plasma concentrations of zinc, aluminium and lead were assessed in 78 male partners of unexplained infertility couples and 33 fertile men.

Methodology

Semen samples were collected by masturbation with an abstinence period ranging between 2 and 7 days, into sterile non-toxic wide-mouth containers for the analysis. The semen parameters were assessed and reported using methodology as prescribed by the WHO 2010 laboratory manual for the examination and processing of human semen.[13] An aliquot of well-liquefied seminal ejaculate was used to assess DNA damage through the sperm chromatin dispersion (SCD) method using the commercially available kit (Qwik Check TM DFI kit) within 2 h of ejaculation. The SCD method is based on the principle that an undamaged sperm DNA will form a halo even after treatment with denaturing and lysing agent while a fragmented DNA is susceptible to denaturation and will show no or very small halo. The air-dried stained final slide was observed under 40X and sperms with fragmented DNA were identified by the presence of small or no halo and sperms with non-fragmented DNA were identified by a large and medium-sized halo. A minimum of 200 sperms were evaluated and DNA Fragmentation Index (DFI) was calculated as the fraction of sperms with fragmented DNA out of the total sperms evaluated and was expressed as a percentage.

For metal level quantification, an aliquot of semen was digested with 65% nitric acid and 30% hydrogen peroxide, which was diluted to 5 ml to estimate the concentration of Pb, Al and Zn using atomic absorption spectrometry.[14]

Outcome measures and statistical analysis

The demographic, fertility, clinical data, semen parameters, DFI and seminal metal concentration were recorded for the study participants and fed into an Excel sheet and compared among the cases and controls. To ensure additional transparency of results, 95% confidence limits

were computed. As the seminal metal concentrations had a nonparametric distribution the difference amongst the two study groups was compared by the Mann–Whitney U-test. To assess the overall diagnostic performance of DFI and to evaluate the optimum cuff off of DFI differentiating between the male partners of unexplained couples and fertile population, a receiver operator characteristic curve and area under the curve (AUC) were computed. As the male fertility status in the study could assume one of the two possible outcomes (unexplained infertility or proven fertility), to establish the strength and pattern of association between fertility status and sperm DNA damage, binary logistic regression modelling was used. To determine the odds of establishing unexplained infertility based on DFI, DFI was used as a categorical covariable in the regression model with DFI <18% being the reference category.[15] Pearson correlation coefficient was computed for fertility, DFI and metal concentration in the semen to study the congruence between these parameters. Statistical software IBM SPSS Statistics for Windows (Version 21.0, IBM Corp., Armonk, NY, USA), was employed for the statistical analysis and a $P < 0.05$ was considered statistically significant.

Results

In the case arm of the study, the couples have been trying for conception for a median duration of 3 (range of 1–11 years) years. On average, the men aged 30.08 ± 3.9 years in the case arm and 30.1 ± 4.3 years in the control group which was not statistically significantly different ($P = 0.274$). Other demographic parameters observed were comparable within the two groups [Supplementary Table 1].

Semen parameters and fertility

Among the semen parameters, average semen volume and sperm concentration were not statistically significantly diverse among the unexplained infertility group and men with proven fertility. The mean sperm concentration of the two study groups was not statistically significantly different with a $P = 0.169$ [Table 1]. However, the mean active progressive motility (Grade A) was 44.81 ± 19.47% and 53.12 ± 9.89% among the cases and controls correspondingly, which was statistically significantly different ($P = 0.005$).

Comparison of DNA Fragmentation Index among the study groups

The presence of DFI was significantly lower among fertile men than in male partners of the unexplained group (21.38 ± 10.28% vs. 10.83 ± 6.28%, $P < 0.0001$). The construction of receiver receiver-operating characteristic curve enabled the assessment of the diagnostic performance of DFI over varied cutpoints and optimum sensitivity and specificity of 75% and 71% correspondingly were observed at a cutpoint of 18% DFI [Figure 1]. The computed AUC was 0.83 denoting a good overall diagnostic accuracy of DFI. This in effect, suggests an 83% chance of correctly distinguishing fertile men from male partners of unexplained infertility couples based on DFI. In the unexplained infertility group, 41% of the males (41 out of 100) had a DFI >18% and approximately 20% (19 out of 100) had a DFI of more than 30. The corresponding figures were 16% and 0% among the fertile males. On further analysis, the DFI cutpoint of 18% had maximum test accuracy as compared to a cutoff of 30%. The sensitivity, specificity and overall accuracy were 43%, 84% and 56.67%, respectively when the DFI cut-off was set at 18% [Table 2]. When applying the binomial logistic regression model, DFI added significantly to the prediction model of unexplained infertility (Wald test = 10.99, $P \leq 0.001$). Wald test also known as Wald Chi-squared test is a statistical measure of significance of contribution of each independent variable to the overall model. The value of Wald test signifies the deviance from the null hypothesis. The higher the deviance or in other word the higher the value of Wald test, the better is the predicative value of the variables. The odds of male fertility decreased by 17% with each percentage increase in DFI. When using DFI as a categorical variable, men with DFI > 18% had 4.7 times higher odds of being classified in the unexplained group as opposed to men with DFI < 18% (odds ratio = 4.74, 95% confidence interval = 2.09–10.78) [Table 3].

Seminal metal concentration among the study groups

The mean seminal concentration of zinc was statistically significantly lower among the male partners of unexplained couples (57.95 ± 23.75) as compared to men with proven fertility (148.51 ± 35.22). On the

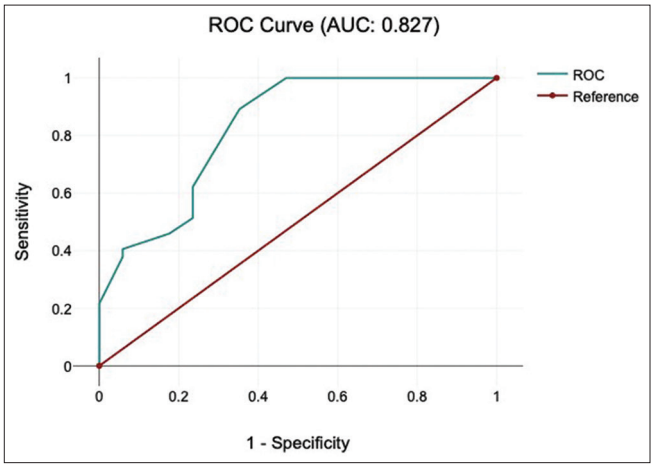

Figure 1: Receiver operator characteristic curve for sperm DNA fragmentation

Table 1: Comparison of clinical and pertinent demographic data among the male partners of couples with unexplained infertility couples and fertile controls

Outcome parameters	Cases (unexplained infertility)	Controls (fertile men)	*P*
Age	30.08±3.9	30.1±4.3	0.274[a]
BMI	24.29±3.68	23.47±3.469	0.173[a]
Mean semen volume (mL)	2.01±0.82 (1.85–2.17)	1.95±0.76 (1.74–216)	0.665[a]
Mean semen concentration (million/mL)	54.31±25.95 (49.22–59.40)	62.12±20.62 (56.41–67.84)	0.169[a]
Mean active progressive motility (Grade A) (%)	44.81±19.47 (40.99–48.63)	53.12±9.89 (50.38–55.86)	0.005[a]
Mean DFI (%)	21.38±10.28 (19.36–23.39)	10.83±6.28 (9.09–12.57)	<0.0001[a]
Semen zinc (mg/L)	57.95±23.75 (52.68–63.22)	148.51±35.22 (136.49–160.53)	<0.0001[b]
Semen aluminium (µg/L)	510.58±206.39 (464.78–556.38)	296.54±84.93 (267.56–325.52)	<0.0001[b]
Serum lead (µg/L)	7.98±5.12 (6.84–9.12)	3.85±1.25 (3.42–4.28)	<0.0001[b]

[a]Student's *t*-test, [b]Mann–Whitney *U*-test. BMI=Body mass index, DFI=DNA fragmentation index

contrary, the levels of lead and aluminium in the semen of the unexplained infertility group were considerably higher than in fertile men [Table 3]. A logistic regression model performed to ascertain the effect of DFI and seminal metal concentrations was statistically significant. Increasing zinc level was associated with an increased likelihood of male fertility, whereas rising lead and aluminium concentrations were associated with a reduced likelihood of fertility [Table 2]. One possible hypothesis could be that these metal concentrations in the semen are influencing fertility status by impacting DFI. To put this hypothesis to a test we computed point biserial 2-tailed Pearson correlation (fertility status being a dichotomous outcome) of these parameters with fertility status. Zn levels explained 69% (R^2 = 0.69) variation in the male fertility status while DFI accounted for 30% (R^2 = 0.30) variation only [Table 4]. The Pearson correlation coefficient illustrated a moderate negative association of DFI with Zn levels, but the correlation with Pb and Al concentration was weak.

Table 2: Diagnostic evaluation analysis of DNA fragmentation index at various cut-offs

	DFI cut-offs (%)		
	18%	25%	30%
Sensitivity	43	37	19
Specificity	84	88	100
Positive likelihood ratio	2.69	3.08	
Negative likelihood ratio	0.68	0.72	0.81
Positive predictive value	84.31	86.05	100.00
Negative predictive value	42.42	41.12	38.17
Accuracy	56.67	54.00	46.00

DFI=DNA fragmentation index

Discussion

The present study evaluated the effect of semen parameters, DFI and metal levels on unexplained male infertility. In our study, we report significantly higher mean progressive sperm motility in fertile men as opposed to the male partners of unexplained couples. Other semen parameters, including ejaculate volume and sperm concentration, were not significantly different between the two groups.

There has been ongoing research to develop an optimum diagnostic and predictive modality for unexplained male infertility.[12,16,17] Towards this pursuit, researchers have studied the association of sperm DFI with fertilisation rate, implantation rate and reproductive outcomes with contentious results.[13,17,18] Our study findings are in agreement with the study conducted by Zandieh *et al*.[19] who found significantly lower sperm motility among the male partners of unexplained infertility couples than fertile men. Other semen parameters were found comparable among the groups. On the other hand, Verit *et al*.[20] observed no statistically significant difference in any of the semen parameters among men with unexplained infertility and with proven fertility, although semen parameters in this study were not assessed using the WHO 2010 laboratory manual for the examination and processing of human semen.

DNA damage was found to be significantly higher in unexplained infertility couples. The odds of male fertility decreased by 17% with each percentage increase in DFI; however, the overall accuracy of DFI as a diagnostic test for unexplained infertility was average at the most. Our study findings were comparable to Feijo *et al.* found a mean DFI of 20.6% using the SCD method among 20 male partners of couples with unexplained fertility.[21] Similarly, Oleszczuk *et al.* observed a mean DFI of 16.2% among male partners of unexplained infertility couples.[22] Our study found a mean DFI of 10.83% in the fertile arm of the study which is comparable to previous published literature.[23-26] Published literature proposes a DFI cut-off of 30% for distinguishing infertile men from fertile controls.[27-30] In the present study, the best discriminatory cut-point of DFI between male partners of unexplained couples and fertile males was estimated to be 18%. Our findings are similar to Gill *et al.* who

found a very similar cut-off of DFI while comparing men with normal and abnormal semen parameters.[31] Similar discriminating threshold values have been established by many others for the infertile male population and fertile men.[22-24] Of note is the finding that various studies have employed diverse methodologies for the estimation of sperm DNA damage.

Current evidence has suggested an association between high-sperm DNA damage and poor reproductive outcomes for intrauterine insemination treatment (IUI)[29,32,33] and natural conception.[30,34,35] Nonetheless, the evidence on the predictive ability of DFI in assisted reproduction (IVF/ICSI) is weak and debatable at best.[36,37] A recent meta-analysis by Ribas-Maynou *et al*., has detected no statistically significant impact of sperm DNA damage on reproductive outcomes of patients undergoing ICSI treatment while a negative tendency on outcomes was observed with IVF treatment it was not statistically significant.[38] Correspondingly, the majority of current studies have negated any predictive value of sperm DNA damage on assisted reproduction.[39,40]

Semen Zn levels showed a strong positive correspondence with male fertility which was much superior to its correlation with sperm chromatin DNA integrity. Similarly, the negative association of Pb and Al with male fertility was stronger than that with DFI. This leads us to postulate that the seminal concentrations of these metals are independently associated with male fertility. Other authors have also shown higher lead levels among infertile men as opposed to their fertile counterparts.[41,42] However, few other researchers have refuted any association of lead levels with semen quality.[43] The present study results are consistent with Wdowiak *et al*., who have also observed higher Zn levels among fertile men.[44] Furthermore, the present study is in

Table 3: Binary logistic regression analysis between various fertility status and clinical parameters

Parameter	*B*	Wald	df	Significant	Exp (B)	95% CI for Exp (B)	
						Lower	Upper
DFI	−0.185	10.996	1	0.001	0.831	0.745	0.927
DFI (1)	1.55	13.801	1	0.000	4.74	2.086	10.778
Cut-off 18%							
Zn level	0.105	19.928	1	0.000	1.111	1.061	1.163
Al level	−0.015	13.580	1	0.000	0.986	0.978	0.993
Pb level	−0.643	10.530	1	0.001	0.525	0.356	0.775
Constant	10.842	21.323	1	0.000	51144.34		

DFI=DNA fragmentation index, CI=Confidence interval

Table 4: Point biserial two-tailed Pearson correlation between various fertility status and clinical parameters

	DFI	Zn	Al	Pb	Fertility
DFI					
Correlation	1	−0.444**	0.291**	0.274**	−0.505**
Significant (two-tailed)		0.000	0.002	0.004	0.000
n	150	111	111	111	150
Zn					
Correlation	−0.444**	1	−0.504**	−0.337**	0.831**
Significant (two-tailed)	0.000		0.000	0.000	0.000
n	111	111	111	111	111
Al					
Correlation	0.291**	−0.504**	1	0.154	−0.479**
Significant (two-tailed)	0.002	0.000		0.107	0.000
n	111	111	111	111	111
Pb					
Correlation	0.274**	−0.337**	0.154	1	−0.398**
Significant (two-tailed)	0.004	0.000	0.107		0.000
n	111	111	111	111	111
Fertility					
Correlation	−0.505**	0.831**	−0.479**	−0.398**	1
Significant (two-tailed)	0.000	0.000	0.000	0.000	
n	150	111	111	111	150

**Indicates significant correlation (*P* value<0.05). DFI=DNA fragmentation index

agreement with other published literature which shows a weak to little correlation between lead and SDF.[45,46]

The foremost strength of the study lies in the fact that the unexplained couples included in the study underwent a thorough screening and investigation work-up to rule out possible female factors before recruitment in the study. Moreover, limiting the recruitment to couples with male partners aged 20–45 and female partners aged under 37 years enabled us to alleviate the impact of age-related fertility decline. Additional strong suits of the study were the best possible comparison group and a reasonably good number of participants enrolled in both groups.

The study involves a point estimation of the SDF and the study was limited to accommodate the possibility of dynamic variation of sperm DNA damage over time as with the other semen parameters. As the study included medically healthy non-smoker men, it was not possible to ascertain the role of obesity, varicocele, alcohol and smoking on DFI or seminal metal concentrations. The cross-control design of the study permits it to estimate variance and association but not causation; the ascertainment of which would require a longitudinal prospective study.

Conclusions

A considerable overlapping of semen parameters was observed between the male partners of unexplained infertility couples and fertile men. Seminal zinc concentration was significantly correlated with male fertility. Male partners of unexplained infertility couples were found to have significantly higher degrees of sperm chromatin DNA damage. However, the overall accuracy of DFI as a diagnostic tool was sub-optimal. This information puts the role of DFI as explanatory, at best, in unexplained infertility. In addition, the study proposes a lower discriminatory threshold of 18% for couples with unexplained subfertility. Equally, the emerging evidence disavows any significant negative influence of sperm DNA damage on reproductive outcomes of assisted reproduction, especially ICSI treatment. It can be, possibly extrapolated from the study results and emerging evidence that sperm DFI may be offered for couples with unexplained subfertility for explanatory purposes and potential early referral for assisted reproduction. Further research can address the impact of DFI in referral algorithms for unexplained infertility.

Author's contributions

N. K. G. conceived the idea for the study and was involved in data acquisition, statistical analysis, manuscript preparation, construction of figures and tables and submission of the manuscript. K. K. S. was involved in the conception, design of the study and acquisition of data. S. N., S. R., D. C., S. K. and P. E. were involved in data acquisition. P. K. and P. S. helped with manuscript revision and critical appraisal.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

Data related to the manuscript is the property of AIIMS Jodhpur and can be shared after permission from AIIMS Jodhpur.

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Supplementary Table 1: Baseline demographical characteristics of participants in unexplained infertility group and fertile controls

Parameter	Cases (unexplained infertility) (*n*=100)	Control (couples with proven fertility) (*n*=50)	*P*
Male partner age	30.08±3.9	30.1±4.3	0.274[a]
Female partner age	27.21±3.8	26.6±3.8	0.079[a]
Male partner BMI	24.29±3.68	23.47±3.47	0.173[a]
Female partner BMI	27.20±5.72	25.62±4.49	0.090[a]
Male education status			
Illiterate	16	6	0.628[b]
Below graduation	44	21	0.862[b]
Graduation and above	40	23	0.489[b]
Wife education status			
Illiterate	27	11	0.555[b]
Below graduation	55	30	0.603[b]
Graduation and above	18	9	0.825[b]
Occupation			
Unskilled	7	2	0.718[b]
Skilled	77	36	0.549[b]
Professional	16	12	0.269[b]

[a]Student's *t*-test, [b]Chi-square test. Data presented as mean±SD or *n* (%). SD=Standard deviation, BMI=Body mass index