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HUMAN SPERM DNA FRAGMENTATION INDEX AND ITS CORRELATION WITH VARIOUS SEMEN PARAMETERS.

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Introduction

Infertility is defined as any couple, not being able to conceive after one year (or longer) of unprotected sexual intercourse. Approximately 40–50% of all infertility cases are related to "male factor" infertility, with as many as 2% of all males having inadequate sperm parameters. Low sperm concentration, poor sperm motility, or aberrant morphology are all possibilities. In less developed countries, rates of infertility are significantly higher, and infectious illnesses account for a bigger proportion of infertility (Kumar and Singh, 2015) (Elbardisi et al., 2020).

Male fertility begins to decline with age, when sperm quality deteriorates. Children with aged fathers are also more likely to suffer mental health issues (although this is still rare). Children whose fathers are 40 or older are five times more likely than children whose fathers are under 40 to acquire autism spectrum disorder (McGrath et al., 2014) Although there is less evidence of a link between paternal age and the incidence of miscarriage, it is scientifically feasible that an increasing number of genetic and epigenetic sperm abnormalities in older males lead to miscarriage (du Fossé et al., 2020).The classical semen analysis (sperm concentration, motility, morphology and vitality) has typically been used as the first step in the diagnosis of male infertility. However, its capacity to uncover the underlying pathways that produce the condition has major limits. Sperm DNA fragmentation has been identified as a probable cause of reproductive failure and its assessment has been proposed as a valuable complement to the laboratory approach of male infertility evaluation, particularly prior to the introduction of assisted reproduction technologies (ART) (Esteves et.al,2016). During spermatogenesis, human sperm chromatin appears to be highly susceptible to structural changes, which also occur as a result of DNA filament cuts, as well as sensitive to environmental factors such as temperature changes, oxidative stress, and environmental pollution, all of which are involved in chromatin structure alterations. The ability to repair physiological defects in sperm chromatin is particularly efficient during the early stages of spermatogenesis, but it is already relatively limited in mature spermatids due to chromatin compaction and loss of cytoplasmic components that impede repair. Several studies have revealed that chromatin integrity is a crucial need for a spermatozoon to effectively fertilize an egg and convey paternal genetic information (Ferrigno et al., 2021). Few epidemiologic studies have recently indicated that arsenic exposure promotes infertility, low sperm quality, and erectile dysfunction in men. Several experimental studies have shown that arsenic accumulates significantly in the testes and accessory sex organs such as the epididymis, seminal vesicle, and prostate gland (Kim and Kim, 2015).

DNA sperm fragmentation and its implications on fertility has been the subject of a lot of recent research. To date, evidence suggests that as people get older, their fragmentation rate increases (Gao et al., 2021). This is thought to be the result of increasing oxidative stress throughout time, and also due to the epididymis antioxidant capacity which decreases with growing .Fragmentation of sperm DNA is seen in males of all ages (Harris et.al, 2011). In order to transmit genetic material to the following generation correctly, sperm DNA must be intact. Reduced fertilization rates, early embryo development, embryo quality, pregnancy rates, and increased rates of spontaneous miscarriage have all been linked to high sperm DNA fragmentation index (Sergerie et.al,2005).

So, many reproductive clinics have begun to include fragmentation testing as part of their examination for unexplained infertility or male infertility.

In order to transmit genetic material to the following generation correctly, sperm DNA must be intact. Reduced fertilization rates, early embryo development, embryo quality, pregnancy rates, and increased rates of spontaneous miscarriage have all been linked to high sperm DNA fragmentation index (DFI) percentages. Among the various tests that could improve the prediction of natural conception through routine sperm analysis, sperm DNA fragmentation (sDF) analysis looks to be promising. (Muratori et al., 2015)

Material and Methodology

A cross-sectional study was conducted on 33 men from infertile couples of Bhagalpur, Bihar with unexplained infertility and some with altered semen parameters where included. All of the patients voluntarily volunteered to participate in the study. The factors addressed in this study were age and the following sperm parameters: volume (ml); sperm concentration (millions/ml); total sperm count (millions); sperm progressive motility A+B (percent); sperm morphology (percent normality); and sperm DNA fragmentation (percent). The WHO guidelines were used to define normal values (World Health Organization, 2010).

Sample collection

All the patients where counselled for DFI test along with routine semen analysis. The semen samples were collected by masturbation into a sterile container after a sexual abstinence period of 3– 7 days.

Laboratory procedure

Semen Analysis

Semen analysis is done in two parameters i.e. macroscopic (volume, color, pH, viscosity and liquefaction time) and microscopic (count, motility, viability and morphology). The sperm parameters were assessed in accordance with WHO recommendations (WHO, 2010).

A Makler chamber a device introduced by Dr.Amnon Makler in 1978 is used to measure sperm concentration and motility. When the cover slip is placed onto the quartz pins in makler chamber, the volume enclosed between the two layers is exactly 1 million part of mL.

Count - The number of spermatozoa counted in any strip of 10 squares of the grid indicates their concentration in millions/mL.Spermatozoa in any three alternate column is counted and the average value is taken as sperm count.

Motility- Progressive (PR), Non Progressive (NPR) and Non Motile is counted separately and percentage is calculated by the formula 100* (PR + NPR) /Total Count.

Morphology is calculated by smear made by Eosin- nigrosin staining (Agarwal, Gupta and Sharma, 2016) (Bjorndahl, 2003), and 200-300 sperm is counted in different focus on 100X.

The following World Health Organization reference values are compared to the results of the semen analysis performed as part of an initial assessment.

- volume: 1.5 ml or more
- semen pH:≥ 7.2
- sperm concentration:≥ 15 million spermatozoa per ml
- total sperm number: ≥39 million spermatozoa per ejaculate
- total motility (percentage of progressive motility and non-progressive motility): 40% or more motile or 32% or more with progressive motility
- vitality: ≥58%
- Sperm morphology (percentage of normal forms): ≥4%.

The quantitative examination of normal and aberrant sperm morphological forms in an ejaculate is possible using the staining of a seminal smear and observing under magnification of 100x.

A nigrosin-eosin stain is often employed because it is effective, simple, and, in addition to allowing for easy visualization of sperm, it is a "live-dead" stain, allowing for the assessment of membrane integrity as well as morphology. The nigrosin-stain creates a dark background against which the sperm appear as light coloured objects. Normal live sperm do not take up the eosin stain, due to intact plasma membrane and appears white, whereas dead sperm appears pinkish because of lost in membrane integrity (Bjorndahl, 2003).

The impression of the sample is normozoospermia when all of the parameters are within the normal range; oligozoospermia when the count is less than the reference value; asthenozoospermia when the motility is less; tetratozoopermia when the morphology is less than the normal range; azoospermia when there are no sperm; and Oligoasthenoteratozoopermia when all the parameters are below the normal range (OATS).

SCD Assay

The Sperm Chromatin Dispersion (SCD) test is based on the principle that sperm with fragmented DNA cannot form a typical halo of dispersed DNA loops as seen in sperm with non-fragmented DNA after acid denaturation and removal of nuclear proteins. So, normal spermatozoa create halos formed by DNA loops at the head of sperm, which are absent in spermatozoa with damaged DNA, these dispersed DNA loops are stained ,which can be measured using conventional bright-field microscopy.

Procedure

After assessment of semen sample, there concentration is adjusted to 5-10 *10^6 sperm/mL by diluting the sample in culture medium.

Agarose is melted at 90˚C in DNA warmer for 5 minutes and kept at 37˚C.After then 25 micro liters of semen sample is added and mixed well to the melted agarose. Then prepared sperm cell suspension is placed on pre coated agarose slide and carefully placing the coverslip avoiding formation of air bubble, the slide is then kept at 4˚C for 5 minutes. After 5 minutes slide is removed from refrigerator and coverslip is removed gently by sliding it off. During all these procedure the slide is maintained at horizontal position. Slide is incubated horizontally in solution “A” for 7 minutes. Again slide is incubated in lysis solution for 25 minutes. Next that slide is kept in distilled water for 5 minutes. Now slide is placed in 70% ethanol for 2 minutes, followed by 90% ethanol for 2 minutes and finally placed in 100% ethanol for 2 minutes and then slide is dried at room temperature. Solution C and D is mixed in the ratio 1:1, then a layer of the mixed solution is deposited on the slide in horizontal position for 20 minutes. After then the stain is drawn off and the slide is washed with distilled water and is air dried at room temperature. Now the slide is ready to visualize under Bright field microscopy under 40X.And 200-300 spermatozoa are graded and counted for DFI calculation.

Calculation of DNA Fragmentation Index

DNA fragmentation index of spermatozoa was determined by Sperm Chromatin Dispersion Assay (SCD Assay) (Fernandez et al., 2003; 2005) with Sperm Chroma Kit (CryoLab International, SAR Healthline). The test was carried out strictly according to the kit's standard protocol.

SDFI (%) = 100 × No. of spermatozoa with fragmented DNA/ No. of spermatozoa counted

Study Area

Bhagalpur is a city on the southern banks of the river Ganges in the Indian state of Bihar. It is the 3rd largest city of Bihar. Bhagalpur is also known as silk city. It is a major educational, commercial, and political center, and listed for development under the Smart City program, a joint venture between Government and industry. The Gangetic plains surrounding the city are very fertile and the main crops include rice, wheat, maize, barley, and oilseeds. According to the 2011 India census, the Bhagalpur Urban Agglomeration has a population of 410,210 people, with men numbering 218,284 and females numbering 191,926. In terms of urban population, it is Bihar's third largest city. There are 55,898 people in the age bracket of 0 to 6 years. There are 286,125 literates in total, with 160,720 men and 125,405 females. The effective literacy rate of the population aged 7 and up is 80.76 percent, with male literacy at 84.95 percent and female literacy at 75.95 percent. The sample for this study was collected with various clinics of Bhagalpur.

Result

Statistical analysis was performed using MedCalc Software Ltd. A *P* value less than 0.05 was considered statistically significant.

Table 1. DFI value of patients with Normal and Abnormal semen parameters.

Sample Group	DFI (%)
Normal Semen parameter	
Sample 1	33.50%
Sample 2	30%
Sample 3	19%
Sample 4	15%
Sample 5	7%
Sample 6	26%
Sample 7	8%
Sample 8	25.50%
Sample 9	18%
Sample 10	11%
Sample 11	15%
Sample 12	21.50%
Sample 13	13
Sample 14	24%
Sample 15	12.50%
Abnormal Semen Parameter	
Sample 16	11%
Sample 17	39.50%
Sample 18	45.50%
Sample 19	28%
Sample 20	17.50%
Sample 21	64%
Sample 22	49.50%
Sample 23	33%
Sample 24	38.50%
Sample 25	33%
Sample 26	17%
Sample 27	21%
Sample 28	41%
Sample 29	32%
Sample 30	14%
Sample 31	43%
Sample 32	37.50%
Sample 33	56.28 %

In order to determine DNA fragmentation levels in semen samples obtained from both groups by SCD assay, semen samples were evaluated against their normal semen parameter and DFI.As shown in Table 1, the DFI value of semen samples ranged from 7% to 56.28%.In

addition, 45.5% of the sample from patient with normal semen parameter of which only 6.7% of them had high DNA fragmentation and 54.5% of the sample from patient with abnormal semen parameter of which 61% patients were teratozoospermia with 63.6% of high DFI and 38% patient were asthenoteratozoospermia with 71.42% of high DFI value. Overall 66.6 % of high DFI was found in semen with altered semen parameter. And combinedly only 42.42 percent of semen samples showed normal DFI values (less than 30 percent) and 57.57 percent of samples had abnormal DFI values (greater than 30 percent).The age of patients ranged from 29 to 52 years (mean±SD) 38.9±5.9.

Table 2.Descriptive statistics and comparison between Normal and Altered semen parameter patients.

Parameters	Normal (n=15) Mean±SD	Altered (n=18) Mean±SD	P value
Age	35.6±3.6	41.6±6.1	.0021
Volume(mL)	2.1±1.03	2.5±0.9	.2428
Count(million/mL)	70.6±20.42	46.9±21.56	.0030
Morphology(%)	4.4±0.6	2.0±0.7	<.0001
Motility(%)	64±9.8	40.8±16.6	<.0001
DFI(%)	18.6±7.9	34.5±14.6	.0007

Table 2, shows the comparison between different parameters of semen analysis between normal and abnormal semen parameter patients and the P value. The study showed that altered patient ages where significantly higher when compared to the normal patients, normal morphology, motility and count was also lower in altered patients. DFI value of normal and abnormal semen parameter patients group respectively, 18.6±7.9 vs 34.5±14.6, p=0.0007.DFI was also higher in altered semen parameter patients.

Thus it can be concluded that altered semen parameter can reflect the DNA integrity of sperm.

Discussion

Male infertility is caused by sperm DNA damage, which has a negative impact on reproductive outcomes. Recent clinical practice suggests that sperm DNA fragmentation testing may be useful in some treatment conditions. This would improve the sperm DNA fragmentation assay in global potential as a predictive and diagnostic tool in a majority of male infertility cases and treatment management. According to WHO guidelines, sperm analysis using standard criteria is the primary approach for measuring male infertility. Routine semen analysis can provide a limited prediction of male reproductive potential and cannot always explain the cause of male infertility. Many occurrences of male infertility are caused by spermatogenesis abnormalities that are undetected by standard sperm quality tests. Our study shows that To some extent, the altered semen parameter can indicate DNA integrity. The link between sperm DNA fragmentation index (DFI) and sperm parameters is unclear and need more research. While some research identified a strong association between DFI and human sperm parameters, according to (Rafighdoost et.al,2013) morphology and motility characteristics were found to be predictive of DFI in a logistic regression model. While (Fernández et.al,2003) stated that samples from infertile patients with normal or abnormal semen parameters showed no statistically significant differences. (Le et al., 2019) also showed no strong correlatiob between DFI and semen parameter.As a result, the sperm DNA fragmentation assay can be the predictive value of the chromatin damage. should be conducted as a follow-up to the male fertility study. In advanced age patients and males with risk factors such as, certain lifestyle habits (tobacco consumption,smoking,alcohol) and occupational hazard(exposure to chemical or environmental toxicants),varicocele,infection or sperm with a high prevalence of aberrant semen parameters fragmentation DNA testing is extremely important.(Sivanarayana et. al,2014) reported a significant difference in semen parameters observed between the DNA fragmentation normal and abnormal groups [count, motility and morphology ($p < 0.05$)].Muriel et.al,2006 indicated a negative correlation between cells with degraded chromatin and sperm morphology ($r = -.29$, $P = .04$).24 Furthermore, the percentage of sperm with progressive motility in semen was negatively correlated with the percentage of cells with a small halo ($r = -.22$, $P = .04$) and positively correlated with the percentage of sperm cells with a large halo ($r = .30$, $P < .01$), indicating a link between progressive motility and intact DNA. Overall, sperm DNA damage was negatively correlated with sperm motility. The findings of (Campos et al., 2021) imply that an aberrant spermogram reflects not only altered spermatogenesis but also a detrimental impact on sperm DNA, and that a high DFI is associated with considerable impairment in all seminal parameters. Sperm characteristics have low but significant associations with sperm DNA/chromatin integrity, and age has a detrimental effect on sperm quantity and quality (Gao et al., 2021).

Lastly, our findings also revealed that the sperm DNA fragmentation index was associated to traditional sperm characteristics(count,motility,morphology and age)while no significant relation was observed between volume of semen and DFI(p=.24).Although there is need of many more research on the current issue with larger sample size.

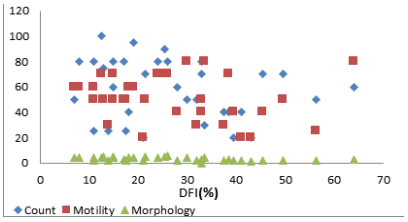

Fig 1: Correlation between semen parameters and DFI

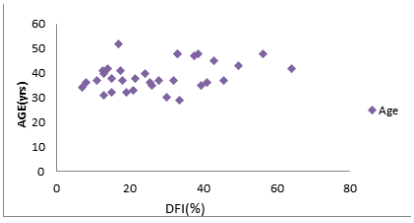

Fig 2:A graphical representaion of DFI and Age.

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