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Biological Science

TO STUDY THE IMPACT OF SMOKING ON MALE FERTILITY OF BHAGALPUR DISTRICT OF BIHAR.

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ABSTRACT The purpose of this study was to determine the impact of tobacco use on male fertility in the Bhagalpur district of Bihar. A total of 20 men from infertile couples were enrolled in this cross-sectional study, and their sperm samples were collected along with their general information. General characteristics such as sperm concentration, count, motility, and morphology were observed. The sperm chromatin dispersion (SCD) assay was used to calculate the sperm DNA fragmentation index (DFI). A DFI 30% threshold was used to classify groups as normal (DFI< 30%) or abnormal (DFI > 30%). The smoking habit was found to be significantly related to sperm motility, morphology, and DFI. However, there was no correlation with sperm count. In this study, 5 out of 20 sperm samples had abnormal motility (< 32% progressive motility) and 9 out of 20 had abnormal sperm morphology (Teratozoospermia). Our findings revealed no link between DFI and motility or morphology. The sperm DNA fragmentation index did not have a strong correlation with other sperm parameters. As a result, as an additional step in determining sperm fertility, a sperm DNA fragmentation assay should be performed.

KEYWORDS : Sperm, DNA fragmentation, smoking, motility, morphology, DFI, SCD, Male fertility and semen parameters.

Introduction-

"The inability to conceive after 12 months of unprotected intercourse" is defined as infertility. This suggests that after a year of trying, a couple will be unable to conceive. Infertility is commonly defined as the inability to conceive after 6 months for women 35 and older. Ten percent to fifteen percent of couples struggle with infertility. This makes it one of the most prevalent diseases among adults aged 20 to 45. Furthermore, the longer a woman attempts to conceive without success, the lesser her odds of becoming pregnant without medical intervention. Most couples with normal fertility (85 percent) will conceive within a year of trying. If a couple fails to conceive during the first year, their chances of conceiving decrease with each passing month. As a woman gets older, this happens more quickly. Ten percent to fifteen percent of couples struggle with infertility. This makes it one of the most prevalent diseases among adults aged 20 to 45. Furthermore, the longer a woman attempts to conceive without success, the lesser her odds of succeeding. Infertility is a psychological, economic, and physiological disease that causes pain and stress, especially in a society like ours that places a heavy priority on child-bearing. Primary and secondary infertility are two types of infertility. When a guy has never been able to have a child, he is said to have primary infertility. Secondary infertility occurs when a guy has previously impregnated a woman, even if she is not the current couple's partner. Subfertility refers to male infertility that is either total or partial. It could be caused by a decrease in the number of spermatozoa (oligozoospermia), a decrease in sperm motility (asthenozoospermia), a decrease in sperm vitality (necrozoospermia), aberrant sperm morphology (teratozoospermia), or a combination of these factors *(Sharma A, 2017).*

The most essential aspect in standard sperm analysis

Males with sperm parameters below the WHO normal value were declared infertile. Low sperm concentration (oligospermia), poor sperm motility (asthenospermia), and aberrant sperm morphology are the most common (teratospermia). Other seminal markers are epididymal, prostatic, and seminal vesicle functions, as well as semen volume, are less well related with infertility *(Zegers-Hochschild F,et. al. 2009)*. Less sperm concentration is the most common cause of infertility; 90 percent of male infertility issues are connected to count, and there is a link between aberrant semen characteristics and sperm count. The problem with sperm count, motility, and morphology is caused by a malfunction in the control mechanism, which includes pre-testicular, testicular, and post-testicular components *(Nand K, et. al. 2015)*.

As a result, with a sensitivity of 89.6%, semen analysis remains the single most useful and fundamental investigation, detecting 9 out of 10 men with a serious problem of male infertility. This assay provides important information for the initial examination of infertile men, but it is not a fertility test. It doesn't reveal anything about the spermatozoon's functional capacity to go through the maturation stages that lead to fertilization. It's a straightforward test that determines sperm production and maturation, as well as how sperm interact in the seminal fluid. It also reveals sperm quality (motility, morphology) in addition to sperm production (count) *(R H Mehta, et. al. 2006).*

The lower reference limits for semen analyses have been changed by the WHO. The acceptable 5th percentile is represented by the parameters listed below *(TG Cooper, et. al. 2010)*:

- Volume: 1.5 millilitres (95 percent CI: 1.4-1.7)
- Sperm concentration: 15 million spermatozoa per millilitre (95 percent confidence interval: 12-16).
- Total number of spermatozoa per ejaculate: 39 million (95 percent CI: 33-46)
- Morphology: 4% normal forms (95 percent confidence interval: 3-4), adopting the "strict" Tygerberg technique.
- Vitality: 58 percent of the population is alive (95 percent CI: 55-63)
- Progression of motility: 32% (95 percent CI: 31-34)
- Total motility (progressive + non-progressive): 40% (95 percent CI: 38-42)

Tobacco smoke has been shown to be harmful to reproduction. In cigarette smoke, benzo[a]pyrene (B[a]P) is a powerful carcinogen. Its reactive metabolite forms complexes with DNA, causing mutations *(M. T Zenzes. et. al. 1999)*. Infertility can occur in both men and women as a result of this. Smoking has been linked to lower sperm counts, worse sperm motility, more defective sperm, and lower testosterone levels in men, which could lead to congenital defects and asthma in their children. Heavy father smoking has been linked to an increased risk of childhood cancer in offspring. Inhaling cigarette smoke causes nicotine, carbon monoxide, and heavy metals to be absorbed throughout the body, where they can end up in smokers' seminal plasma via multiple routes of diffusion and active transport *(BN Ames. et. al, 1994)*. According to reports, the sperm cell differentiation and maturation process involves a continuous and significant number of cell divisions. Simultaneously, cigarette smoking has an effect on sperm quality, especially in heavy smokers or those who have been smoking for a long time.

According to *Yang H. et.al 2019,* Sperm DFI is negatively correlated with sperm density, viability and normal sperm morphology, though it is positively correlated with age, abstinence time and unhealthy lifestyle habits (smoking and alcohol drinking). Another research suggested that as compared to the non-smoking group, the smoking group had relatively decreased sperm viability (*Wang Z. et.al, 2016)*. Smoking also disrupts reproductive hormones, impairs spermatogenesis and maturation, and impairs spermatozoa function *(Saleh et al., 2002).* Tobacco smoke contains a number of harmful and mutagenic chemicals, including nicotine, which is a psychoactive

drug. Nicotine and its metabolite, cotinine, can pass the blood-testis barrier, causing injury to germ cells in varying degrees. Tobacco smoke has been linked to a decrease in sperm motility (though this is debatable), sperm concentration, and defective sperm morphology, as well as abnormal protein expression and genetic and epigenetic abnormalities in spermatozoa *(Pereira et al. 2014)*.

Bhagalpur is a city in the Indian state of Bihar, located on the Ganges' southern bank. It is the state's third-largest city and the administrative center of the Bhagalpur district and division. It's known as Silk City, and it's a significant educational, commercial, and political hub that's been identified for development under the Smart City program, government-industry collaboration. Rice, wheat, maize, barley, and oilseeds are the principal crops grown on the Gangetic plains that surround the city. It is situated at a height of 141 feet above sea level on the plains of the Ganga basin. It has a total size of 2569.50 square kilometres. It is located between 25o 07' and 25o 30' north latitude and 86o 37' and 87o 30' east longitude.

Methodology-

Data collection- A cross-sectional study was conducted on 20 women from an infertile marriage whose husbands smoked regularly. Records were checked for studies that were eligible, and data was retrieved from the patients through personal interviews and counseling. Men from infertile couples diagnosed with infertility according to WHO guidelines, with semen analysis and halosperm test findings, were included in the study. All of the patients voluntarily volunteered to participate in the study.

Clinical approaches- Age, occupation, geography, duration of infertility, history of any internal ailments, food habits, lifestyle, smoking or non-smoking, address, and contact number were all recorded as general information.

Laboratory procedure-

Semen analysis- The concerned and counselled patients' sperm samples were obtained and examined. Sperm motility, vitality, sperm concentration, sperm count, sperm morphology, head defect, midpiece defect, tail defect, pus cell, and agglutination were all evaluated under the microscope.

To test sperm concentration and motility, a Makler chamber was employed. The volume enclosed between the two layers when the cover slip is placed on the quartz pins in the Makler chamber is exactly 1 million part of ml *(W Maya-Cardona et. al, 2008)*.

Sperm count- The count of spermatozoa counted in each strip of 10 squares of the grid corresponds to their concentration in millions per millilitre. The sperm in the three alternate columns were counted, and the average value was calculated *(E Zuvela et. al, 2020)*.

Motility- Manual counting under a compound microscope at 100x total magnification was used to determine the sperm motility parameter. The percentages of progressive (PR), non-progressive (NPR), and non-motile were computed using the formula *(Nguyen, T et. al. 2019)*-

$$\frac{PR + NPR}{\text{Total count}} * 100$$

Morphology- Smears in Eosin-nigrosin stain were used to calculate morphology, and 200-300 sperm were counted in different focus at 100X. The quantitative examination of normal and aberrant sperm morphological forms in an ejaculate can be done by staining a seminal smear and examining it under a 100x magnification. A nigrosin-eosin stain is widely used because it is effective, simple, and a "live-dead" stain, allowing for the assessment of membrane integrity as well as morphology. The nigrosin-stain produces a black background on which the sperm appear as light-colored objects. Due to the intact plasma membrane, normal live sperm do not take up the eosin stain and look white, but dead sperm appear pinkish due to the lack of membrane integrity *(Gacem S. et. al. 2021)*.

DNA fragmentation test- All sperm were tested for fragmented DNA using a cryolabs sperm chroma kit (SAR healthline). The sperm chromatin dispersion (SCD) *(Fernández et. al. 2005)* approach, which comprises a controlled DNA denaturation process to facilitate the subsequent removal of the protein contained in each spermatozoan, is used to create this halosperm test. In this way, normal spermatozoa produce halos, which are produced by DNA loops at the sperm's head and are absent in those with damaged DNA. The sperm was diluted in culture medium until it reached a maximum concentration of 20 million spermatozoa per militre. To obtain sperm concentrations of 5-10 million per militre, aliquots of 0.2ml of fresh sample semen were diluted in medium. The agarose gel was melted in the sperm chroma warmer 1 for 5 minutes at 90 degrees. The agarose was transferred to sperm chroma warmer 2 and kept warm for 5 minutes at 37 degrees. A 25 microlitre quantity of sperm was added to agarose and thoroughly mixed. Avoiding the formation of air bubble, the sperm cell suspension was immediately deposited onto the pre-heated slides and covered with a cover slip. For 5 minutes, the slides were kept at 4 degrees. Following that, the slides were gently removed. After that, the slides were incubated horizontally for 7 minutes in solution A (denaturation solution). After that, the slides were horizontally incubated in lysis solution for 25 minutes. For 5 minutes, the slides were immersed in distilled water. After that, the slides were submerged in 70% ethanol for 2 minutes, followed by 90% ethanol for 2 minutes, and then 100% ethanol for another 2 minutes. After that, a layer of stain was placed horizontally and left for another 15-20 minutes after mixing solution C and solution D (1:1). The stain was decanted and gently rinsed with distilled water before being allowed to dry at room temperature. Under a bright field microscope with a 20X or 40X objective, the slides were examined. The resulting images of halos were highly contrasted and can be carefully analyzed using standard methods. As seen below, there are five SCD patterns that can be used-

- The enormous haloed sperm cell.
- The medium-haloed sperm cell.
- The small halo encircled the sperm cell.
- The degraded sperm cell.

According to the manufacturer's recommendations, 300-500 spermatozoa were counted and those with DNA fragmentation were detected by-

$$\text{DFI (\%)} = 100 \times \frac{\text{No. of spermatozoa with fragmented DNA}}{\text{No. of spermatozoa counted}}$$

RESULT:

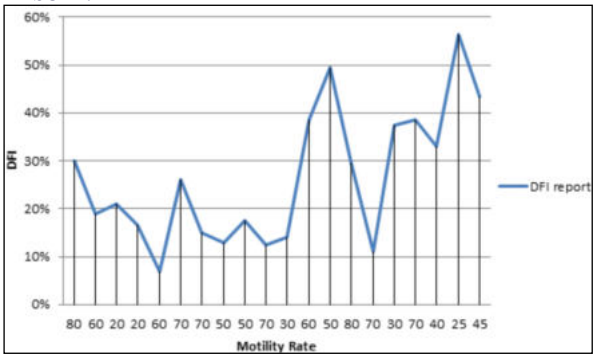

Fig 1: Line Graph Between Motility Rate And DFI

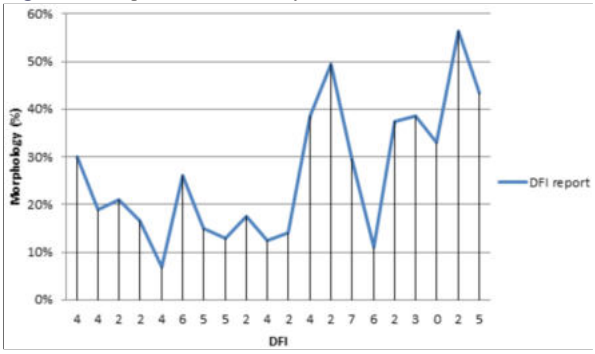

Fig 2: Line Graph Between Morphology And DFI

Patients	Age	Semen profile			
		Sperm count (million/ml)	Motility rate (%)	Morphology (%)	DFI report
1	30 Years	50	80	4	30%
2	32 Years	95	60	4	19%
3	33 Years	20	20	2	21%

4	34 Years	10	20	2	16.50%
5	34 Years	50	60	4	7%
6	35 Years	80	70	6	26%
7	38 Years	80	70	5	15%
8	40 Years	75	50	5	13%
9	41 Years	25	50	2	17.50%
10	41 Years	100	70	4	12.50%
11	42 Years	25	30	2	14%
12	42 Years	80	60	4	38.50%
13	43 Years	70	50	2	49.50%
14	43 Years	30	80	7	29.50%
15	46 Years	60	70	6	11%
16	47 Years	40	30	2	37.50%
17	48 Years	40	70	3	38.50%
18	48 Years	70	40	nil	33%
19	48 Years	50	25	2	56.28%
20	50 Years	110	45	5	43.33%

DISCUSSION-

The total of 20 infertile couples was recruited for the study group. Table 1 shows the general characteristics and DFI result. There were 7 couples with DFI > 30% and 13 couples with DFI< 30% with maximum DFI is 7% and minimum DFI is 56.28%. The sperm motility, morphology and DFI are significantly related to smoking habit. However, it did not showed any correlation with sperm count. In the year 2016, Sharma et al reported significantly negative effect on sperm count (Sharma. R. et al, 2016). In this study, 5 out of 20 semen samples showed abnormal motility (< 32% progressive motility) rate (Asthenozoospermia) whereas 9 out of 20 showed abnormal sperm morphology (Teratozoospermia) as per lower reference limit for semen analyses provided by WHO, 2010. In their study, Temidayo S Omolaoye et al, in the year 2021 showed no such effect of smoking on sperm motility and increase in abnormal sperm morphology (Omolaoye T.S. et al, 2021). Our results showed no correlation between DFI and motility or morphology. However, Le Tam Minh et al in the year 2019, reported negative correlation between DFI and progressive motility and sperm morphology (L.T. Minh. et al, 2019).

Male infertility is caused by sperm DNA damage, which has a negative impact on reproductive outcomes in couples. Recent clinical practice recommendations suggest that sperm DNA fragmentation assays may have a role in some therapeutic settings. This would increase the global potential of the sperm DNA fragmentation assay as a prognostic and diagnostic tool in a variety of male infertility scenarios and treatment management.

According to WHO guidelines, the primary method for assessing men fertility is sperm analysis using conventional parameters. It is obvious that routine sperm analysis can only provide a limited prediction of male fertility potential and cannot always explain the cause of male infertility. Indeed, many cases of male infertility are caused by sperm DNA defects that routine sperm quality analyses skip. The relationship between sperm DNA fragmentation index (DFI) and sperm parameters is still unknown and debated. While some studies found a strong correlation, others found no link between DFI and human sperm parameters.

Finally, our findings revealed that the sperm DNA fragmentation index was not strongly correlated with conventional sperm parameters. As a result, the sperm DNA fragmentation assay should be used as an additional step in the study of male fertility. Assessment of sperm fragmentation DNA is especially important for elderly patients and men who have risk factors such as smoking, alcohol consumption, or sperm with a high rate of abnormal head.

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