Unveiling the Novel Correlation between Sperm DNA Fragmentation Index and Other Sperm Parameters in Males with COVID-19: A Prospective Longitudinal Cohort Study

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KEYWORDS
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ABSTRACT
Background:SARS-CoV-2 (COVID-19) negatively affects sperm parameters and DNA integrity. Routine semen analysis offers limited insights into male reproductive potential. Oxidative stress during COVID-19 may cause DNA damage in sperm. The sperm DNA fragmentation index (DFI) is an essential parameter for detecting oxidative stress and sperm DNA damage. The effects of COVID-19 on the Sperm parameters and sperm DFI remain poorly understood. **Aims:** To study (i) the extent to which COVID-19 affects semen quality and sperm DFI and (ii) the association between semen quality and sperm DFI. **Methods**:This prospective, longitudinal cohort study was conducted at the National Health Institute for Tertiary Care. Thirty COVID-19 males aged 19-45 were involved in the study between October 2020 and April 2021. Detailed semen analysis, including the sperm DNA Fragmentation Index, was performed based on the WHO laboratory manual for the examination and processing of human semen (5th edition). All the aforementioned tests were conducted again on the same participants after 74 days. **Results**:During COVID-19, all semen parameters, including DFI, deviated from their optimal levels. After 74 days, except for DFI, improvements in sperm parameters were observed; however, these were not optimal for pregnancy planning. DFI values remained higher than those of other sperm parameters. All outcomes were statistically significant ($P < 0.05$) for all sperm parameters. Thus, COVID-19 negatively affected sperm parameters, including the sperm DNA fragmentation index. The sperm DFI was not correlated with other sperm parameters. This indicated that the DFI values remained affected for a prolonged period of time. **Conclusion**:COVID-19 had a negative influence on all sperm parameters. Although routine sperm parameters improved after COVID-19, sperm DFI values remained elevated for an extended period, indicating no significant correlation between sperm parameters and sperm DFI. Therefore, post-COVID-19, performing a sperm DNA fragmentation assay is crucial as an additional reliable test to investigate male infertility. Monitoring DFI values can guide ART Clinicians in making treatment decisions for infertile couples.

INTRODUCTION

Millions of couples are affected by infertility worldwide. Male factor infertility (MFI) refers to the inability of a sexually mature man to impregnate a fertile woman within 12 months of unprotected intercourse. According to Sharlip et al., among infertile couples, 50% of infertile couples are due solely to a female factor, while the pure male factor accounts for 20-30%, and the rest of 20-30% are attributable to both male and female factors.[1]At the beginning of the COVID-19 pandemic, a few studies have proposed that SARS-CoV-2 may be shed in the seminal fluid due to the high-level expression of angiotensin-converting enzyme-2

(ACE-2) receptors on the germ cells, Leydig cells, Sertoli cells, and epididymis of the testis.[2]Recent studies confirmed by reverse transcription-polymerase chain reaction (RT-PCR) tests showed that SARS-CoV-2 does not shed into the semen of males suffering from COVID-19; however, semen quality decreased to subfertile levels.[3]

Therefore, we investigated the impact of COVID-19 on semen quality and sperm DFI (< 30%) during COVID-19 and after 74 days (one sperm cycle) of recovery from COVID-19. In cases of infertility, routine semen analysis, including sperm concentration (≥ 15 million/ml), total sperm count (≥ 39 million), vitality (≥ 58%), progressive motility (≥ 32%), total motility (≥ 40%), and normal morphology (≥ 4%), was performed as a standard diagnostic tool to assess semen quality.[4] However, it does not provide information on the DNA integrity of sperm or unexplained male infertility. Despite this, fertilization can occur even with damaged DNA, but the chances of miscarriage are high. The relationship between sperm parameters and the degree of sperm DNA damage during COVID-19 is not clearly understood. Therefore, semen analysis alone in infertile males does not explain the cause of infertility.[5] A study conducted by Kim et al. observed a high sperm DFI in men with routine semen analysis. Sperm DFI provides more information about abnormal genetic material within sperm than does routine WHO-based semen analysis.[6]

Sperm with increased DNA damage leads to nuclear instability in the embryo, resulting in developmental arrest, implantation failure, higher miscarriage rate, genetic mutations causing abnormalities in the offspring, and increased susceptibility to childhood cancers. European Society for Human Reproduction and Embryology (ESHRE 2017) guidelines recommend sperm DFI testing for men whose partners experience recurrent miscarriages.[7]Studies conducted during COVID-19 have shown that semen quality decreases in males suffering from COVID-19; however, studies regarding the effect of COVID-19 on DFI are not available.[8] Our study aimed to investigate semen quality and sperm DFI in males suffering from COVID-19 and to study the correlation between sperm parameter values and DFIs.

METHODS

Study setting:This was a prospective, longitudinal cohort study.

Study sample size:As this pilot study was conducted during the COVID-19 lockdown, the minimum sample size was 30.

Study participants:COVID-19-positive males, diagnosed by RT-PCR at the AllIndia Institute of Medical Sciences (AIIMS) Patna, a dedicated COVID-19 hospital, were included in the study. After obtaining IRB approval (Institutional Ethics Committee, AIIMS Patna, RC/AIIMS/Pat/2020/53), participants were isolated from October 2020 to May 2021. Their ages ranged from 19 to 45 years. We contacted the participants telephonically to obtain their informed verbal consent. After they tested negative for COVID-19, written informed consent was obtained.

Inclusion criteria: Mild COVID-19 men withoutrespiratory distress, sexually active young men having at least one child, and those who had a sexual abstinence period of two to seven days.

Exclusion criteria:Patients with a history of infertility, COVID-19 with respiratory distress, negative RT-PCR test results for SARS-CoV-2, sexually transmitted diseases based on history and earlier investigations, history of immunosuppression, history of smoking, alcohol intake, diabetes, and varicocele were excluded.

STUDY TOOLS

Safety measures:Semen collection and analysis were performed following all recommended precautions and biosafety level-2 practices.

History of participants: We collected the history of COVID-19 progress and identified sexually active males willing to participate in the study. We collected other clinical histories and information regarding the investigations, such as complete blood counts, blood biomarkers, and chest X-ray findings.

Basic semen analysis:Semen samples were collected in sterile containers by masturbation. After the semen had liquefied at room temperature, it was analyzed in the andrology laboratory following the guidelines outlined in the 5th Edition of the WHO Laboratory Manual for Examination of Human Semen, published in 2010.[9]

Test for leukocytospermia:The study utilized a pre-prepared glass slide coated with a dye mixture comprised of methylene blue-N and cresyl violet acetate. A single drop of semen specimen was placed in the centre of the coated microscope slide. A transparent cover glass was then placed on top of the slide, and the sample was observed under a microscope. After 15 minutes, the slide was gently stirred to prevent the

accumulation of cells that could affect the microscopic examination. Leukocytospermia was considered to be present if the concentration of white blood cells in the sample exceeded one million per milliliter.[10]

Sperm vitality test:A small drop of semen was placed on a clean glass slide. Add two drops of 1 % aqueous Eosin Y-solution, mix well, and wait for 15 s. Two drops of a 10% aqueous nigrosin solution were added to the mixture. Again, mix well and take a ten-microliter drop of the mixture on a new glass slide. Make a thin, uniform smear and allow it to air-dry. It was observed under an oil immersion objective lens, and white- and pink-stained sperms were counted. Live sperms remain unstained and look white, while dead sperm were stained pink due to the loss of cell membrane integrity. We counted 200 sperm and estimated the percentage of white-stained sperm. According to the WHO manual for semen analysis, normal sperm vitality is more than 58%. [11,12]

Sperm DFI test:Sperm DFI was assessed using a kit-based sperm chromatin dispersion test using Qwik Check DFI kits manufactured by Medical Electronic Systems India Private Limited (Chennai, Tamil Nadu State, India). In normal sperm, halos formed by the loop strands of DNA in the head are visible; however, in fragmented sperm, halos do not form in the loop strands of damaged DNA. The ratio of fragmented sperm to the total number of sperm was analyzed and expressed as the DFI ratio using a selection of 300 sperms. Elevated (15-29.9%) and severely elevated (≥30%) levels of DNA fragmentation in sperm indicate a potential impact on fertility.[13]

STATISTICAL ANALYSIS

All statistical tests were performed using SPSS software (version 21.0; IBM SPSS Chicago, USA). All numerical data were interpreted as mean values ± standard deviation (SD). A comparison of the medians for any significant differences between group variables was performed. Wilcoxon rank-sum test was used to compare median values. Differences between semen values were considered statistically significant at $P<$ 0.05.

RESULTS

Our comprehensive review of recently published studies revealed a unique finding regarding SARS-CoV-2 shedding in the semen. Surprisingly, only one study has reported its presence, confirming that it is neither secreted into semen nor sexually transmitted.

Table 1. shows that the mean sperm DFI during COVID-19 was 74.25±8.93 and 66.93±11.97 during post-COVID-19, respectively. The difference in mean DFI was highly significant (p≤ 0.000). For each semen parameter tested, there was a statistically significant difference (P ≤ 0.005) between the obtained values in the Covid-19 and post-Covid-19 periods regarding Volume, LT, Viscosity, SC, Total SC, %Vitality, WBC, Agglutination, %progressive motility, %total Motility, %Morphology-N, %Head-D, %Cytoplasmic D. However, the pH, %Neck-D, and %Tail-D showed no significant difference between COVID-19 and post-COVID-19 semen samples. (Table 1, Fig. 1, and Fig. 2).

The dependence of sperm parameter variables during COVID-19 on DFI is shown in Table 2. There was no statistically significant correlation between DFI and sperm parameters, except for cytoplasmic-D, which exhibited a statistically significant negative correlation with DFI (r = -0.358, $P \leq 0.05$). There were positive correlations between DFI and LT, SC, %vitality, WBC, Agglutination, %Morphology-N, and %Tail-D and negative correlations between DFI and volume, pH, viscosity, total SC, %progressive motility, % total motility, % head-D, and %Neck-D.

Table 1:Comparison and correlation analysis of sperm parameters and DFI between COVID-19 and post-COVID-19

SemenParameters (Unit)	**Mean±SD** (min–max)	**Median(25–75)** (min–max)	**Z-value**[a]	**Significance *P*-Value**
Volume_CoV (≥1.5 ml)	2.24±0.79	2.5(1.5-3)	-3.008[b]	0.003**
Volume_post-Cov	2.91±0.67	2.95(2.5-3.5)		
pH_CoV (7.2-8)	8.18±0.35	8(8-8.5)	-0.815[c]	0.415
pH_ post-Cov	8.1±0.44	8(8-8.5)		
LT_CoV (≤30 min)	54.8±37.98	40(33.75-60)	-2.545[c]	0.011*
LT_ post-Cov	35.23±19.38	30(24.5-40)		

Viscosity_CoV (≤2 cm)	9.3±7.52	4.5(3-20)	-2.925[c]	0.003**
Viscosity_ post-Cov	4.2±2.68	3(2-5.25)		
SC_CoV (≥15 X 10^6/ml)	28.65±18.46	26.85(13.05-39.7)	-4.206[b]	0.000***
SC_ post-Cov	57.88±25.35	58.75(41-74.075)		
Total_SC_CoV (≥39 X 10^6)	64.19±52.83	46.45(29.72-82.27)	-4.186[b]	0.000***
Total_SC_ post-CoV	168.59±88.57	161.6(100.62-205.57)		
Vitality_CoV (≥58 %)	33.86±14.81	35.5(28-44)	-2.312[b]	0.021*
Vitality_ post-Cov	43.93±14.1	47.5(38-55.25)		
WBC_CoV (≤1X10^6/ml)	3.9±8.05	2(6-13.5)	-3.038[c]	0.002**
WBC_ post-Cov	2.1±3.36	6.5(4.75-9)		
Agglutn_CoV 9 (≤1grade)	3.4±0.62	3(3-4)	-2.827[c]	0.005**
Agglutn_ post-Cov	2.83±0.87	3(2-3)		
Prog. Motility Cov (≥32 %)	20.2±9.05	20(5.4-8.2)		
Prog. Motility_post-Cov	34.4±2.36	34(2.3-3.6)	-1.963[b]	0.003*
Total Motility_CoV (≥40 %)	26.4±11.07	27(19.5-34.5)	-2.931[b]	0.003**
Total Motility_ post-Cov	38.43±13.48	39(30-46.25)		
Morpho_N_CoV (≥4 %)	8.43±8.51	5(3-10)	-2.120[b]	0.034*
Morpho_N_ post-Cov	11.93±6.14	10(7.75-15.25)		
Head_D_CoV (%)	43.6±8.01	44.5(39.5-49)	-2.727[c]	0.006**
Head_D_ post-Cov	38.23±6.9	38(32-45)		
Neck_D_CoV (%)	15±5.45	13.5(10-20)	-1.268[b]	0.205
Neck_D_ post-Cov	16.93±6.74	16.5(11.5-23)		
Tail_D_CoV (%)	26.16±5.98	27.5(22-29)	-1.094[c]	0.274
Tail_D_ post-Cov	23.93±8.03	21(18.75-27.25)		
Cytopl_D_CoV (%)	6.56±2.2	6(5-8.25)	-2.262[b]	0.024*
Cytopl_D_ post-Cov	9.26±5.54	9(5.75-12)		
DFI_CoV (≤30%)	74.25±8.93	72.6(66.82-77.65)	-4.782[c]	0.000***
DFI_ post-Cov (%)	66.93±11.97	68.5(60.1-73)		

Values are expressed as ± SD, **P*<0.05 is considered statistically significant and high; ** *P*<0.01 is considered statistically significant and higher; *** *P*<0.001 is considered statistically significant and highest.

a. Wilcoxon rank sum test to compare the median values; b. Based on negative ranks; c. Based on positive ranks.

Table2:Correlation analysis between DFI and semen parameters during COVID-19

SpermParameters (COVID-19)	**Mean±SD** (min–max)	**Median(25–75)** (min–max)	**Correlationwith DFI - Pearson's(*r*)**	**Significance(*P* - Value)**
Volume (≥1.5 ml)	2.24±0.79	2.5(1.5-3)	-0.293	0.115
pH (7.2-8)	8.18±0.35	8(8-8.5)	-0.156	0.411
LT (≤30 min)	54.8±37.98	40(33.75-60)	0.213	0.258
Viscosity (≤2 cm)	9.3±7.52	4.5(3-20)	-0.038	0.841
SC (≥15 X 10^6/ml)	28.65±18.46	26.85(13.05-39.7)	0.14	0.459
Total SC (≥39X10^6)	64.19±52.83	46.45(29.72-82.27)	-0.09	0.637
Vitality (≥58 %)	33.86±14.81	35.5(28-44)	0.112	0.557

WBC (≤1X10^6/ml)	11.9±8.05	9(6-13.5)	0.059	0.757
Agglutin (≤1grade)	3.4±0.62	3(3-4)	0.035	0.856
Prog. Motility (≥32 %)	20.2±9.05	20.1(5.4-8.2)	-0.142	0.263
Total Motility (≥40 %)	26.4±11.07	27(19.5-34.5)	-0.163	0.39
Morpho_N (%)	8.43±8.51	5(3-10)	0.078	0.682
Head_D (%)	43.6±8.01	44.5(39.5-49)	-0.182	0.337
Neck_D (%)	15±5.45	13.5(10-20)	-0.058	0.76
Tail_D (%)	26.16±5.98	27.5(22-29)	0.291	0.118
Cytopl_D (%)	6.56±2.2	6(5-8.25)	-0.358	0.052

*Correlationissignificantatthe$P \leq 0.05$level

Table3:Correlation analysis between DFI and sperm parameters during post-COVID-19

SemenParameters(Post-COVID-19)	**Mean±SD**	**Median(25–75)**	**Correlationwith DFI -Pearson's (*r*)**	**Significance(*P*-Value)**
Volume (≥1.5 ml)	2.91±0.67	2.95(2.5-3.5)	0.229	0.224
pH (7.2-8)	8.1±0.44	8(8-8.5)	0.137	0.471
LT (≤30 min)	35.23±19.38	30(24.5-40)	-0.018	0.924
Viscosity (≤2 cm)	4.2±2.68	3(2-5.25)	0.286	0.125
SC (≥39X10^6)	57.88±25.35	58.75(41-74.07)	0.056	0.768
Total_SC (≥39X10^6)	168.59±88.57	161.6(100.62-205.55)	0.143	0.452
Vitality (≥58%)	43.93±14.1	47.5(38-55.25)	-0.134	0.480
WBC (≤1X10^6/ml)	7.1±3.36	6.5(4.75-9)	0.316	0.089
Agglutin (≤1grade)	2.83±0.87	3(2-3)	0.198	0.295
Prog. Motility (≥32 %)	34.48±2.36	34(2.3-3.6)	-0.101	0.421
Total Motility (≥40 %)	38.43±13.48	39(30-46.25)	-0.106	0.576
Morphology_N (≥4 %)	11.93±6.14	10(7.75-15.25)	0.195	0.302
Head_D (%)	38.23±6.9	38(32-45)	0.023	0.904
Neck_D (%)	16.93±6.74	16.5(11.5-23)	-0.018	0.926
Tail_D (%)	23.93±8.03	21(18.75-27.25)	-0.193	0.307
Cytopl_D (%)	9.26±5.54	9(5.75-12)	0.016	0.935

***Correlationissignificantatthe $P \leq 0.05$level**

Fig. 1: Comparison of sperm parameters between COVID-19 and post-COVID-19

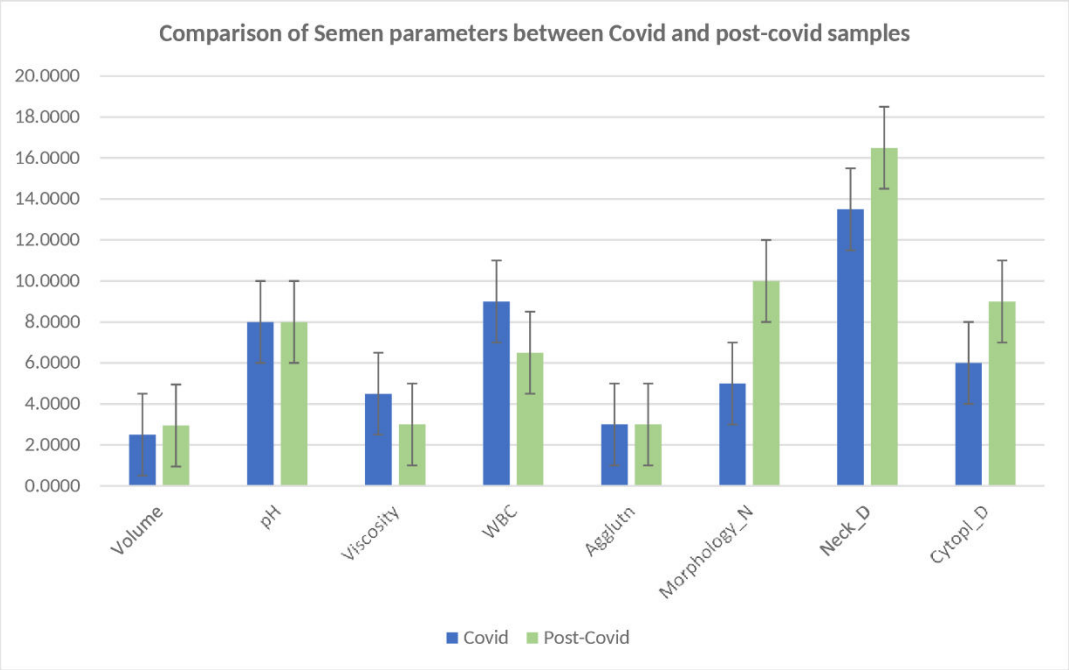
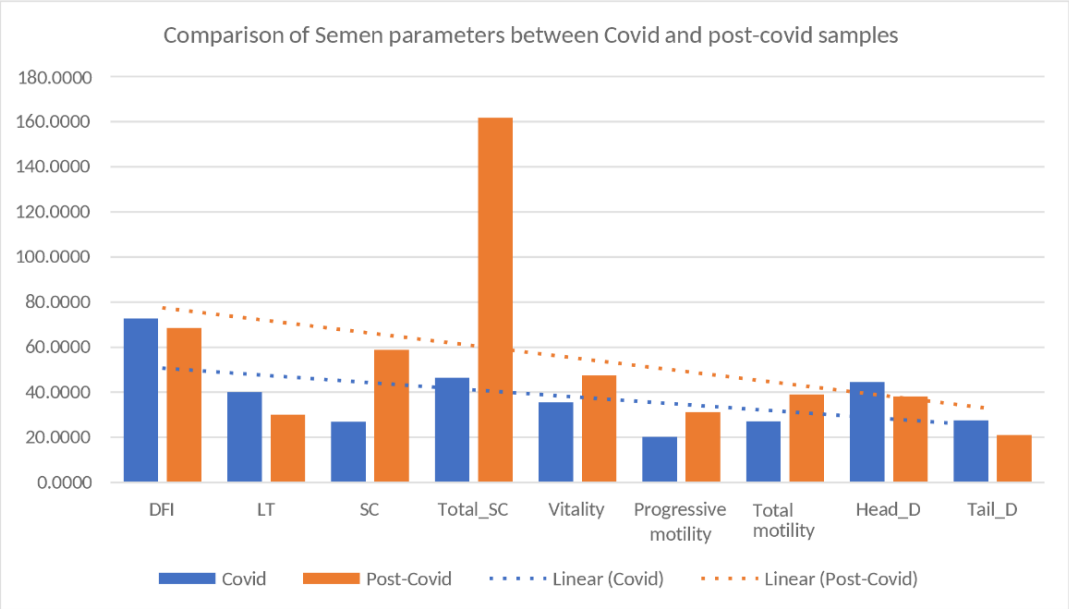


Fig. 2: Comparison of Sperm parameters between COVID-19 and post-COVID-19



Similarly, the correlation of sperm parameter variables in post-COVID-19 samples with the DFI variable is shown in Table 3. There was no statistically significant correlation between the DFI and sperm parameters. Positive correlations were observed between DFI and volume, pH, viscosity, SC, total SC, WBC count, agglutination, % morphology-N, %head-D, and %cytoplasmic-D. Negative correlations were found between DFI and LT, %vitality, % progressive motility, % total motility, %neck-D, and %tail-D.

DISCUSSION

Many researchers, such as Peckham et al., discussed that although men and women have the exact prevalence of COVID-19, overall, their health is at high risk, leading to worsened outcomes and death.[14]Male lifestyle, such as smoking addiction, alcoholismare considered a potential risk factors for

developing COVID-19. In contrast, increased estrogen levels and the presence of XX chromosomes in females provide them with more innate immunity and a protective role against the virus.[15] In males, during COVID-19, the reproductive system is also equally affected, along with the respiratory system.

In our study, during COVID-19, sperm parameters such as vitality, progressive motility, and total motility decreased below the normal limits specified in the WHO manual. During, post-COVID-19, agglutination, viscosity, and leukocyte levels remained above the normal limits. Other semen parameters, namely Volume, SC, Total SC, and %Morphology-N levels, decreased during COVID-19 compared to post-COVID-19 but were within normal limits. In contrast, semen liquefaction time increased during COVID-19 compared to post-COVID-19 but was within the standard limit. Similar findings were confirmed by the study done by Best et al., where the total SC decreased during COVID-19.[16]In our study, sperm DFI increased to abnormal levels to 74.25±8.93 COVID-19 while it remained elevated to 66.93±11.97 with slight improvement during the post-COVID-19 period. This confirmed that COVID-19 affects the male reproductive system, decreasing semen quality to the subfertile level. We further noticed that the semen quality assessed after one sperm cycle at 74 days post-COVID-19 improved, but DFI remained at a higher level, leading to miscarriage. Our findings are supported by the studies conducted by Donders et al. [3] and Vahidi et al.[17]

The supporting pathophysiology of decreased semen quality during COVID-19 is the high expression of ACE2 receptors in the testes, which supports the entry of SARS-CoV-2 in the testes, affecting spermatogenic and endocrine functions.[18] Despite this, researchers have not obtained SARS-CoV-2 from the semen of male patients with COVID-19. Therefore, sexual transmission of SARS-CoV-2 is not possible. The blood-testis barrier (BTB) may be sufficiently strong to prevent SARS-CoV-2 entry into the testes. Basal tight junctions in the BTB formed by Sertoli cells in the seminiferous tubules prevent antigens and antibodies from disrupting spermatogenesis via viral infection.[19] This is supported by studies conducted by Gilbert et al. [3] and Guo et al. [20]

The probable reasons for decreased semen quality during COVID-19 infection are high-grade fever, inflammation, and oxidative stress. [21] COVID-19 infection can trigger immune responses, such as cytokine storms and autoimmunity, affecting the sperm membrane and DNA integrity.[22] Disruption of the hypothalamic-pituitary-gonadal axis (HPGA) by SARS-CoV-2 indirectly affects the production and secretion of gonadotropins (LH and FSH) and sex steroids (testosterone and estradiol), leading to decreased semen quality and DFI.[23] Leukocyte infiltration combined with inflammatory cytokines can harm Leydig cells outside the blood-testis barrier (BTB), leading to impaired testosterone production and decreased semen quality.[24] Sperms are vulnerable to oxidative stress because they have limited antioxidant defense and DNA repair mechanisms. Severe systemic oxidative stress during COVID-19 may lead to an imbalance between the synthesis and elimination of reactive oxygen species (ROS). These high-level ROS molecules during COVID-19 can damage the sperm membrane and DNA, resulting in an increased DFI.[25] This indicates that SARS-CoV-2 indirectly affects semen quality and DFI.

The effects of COVID-19 on sperm parameters appear to be temporary and reversible. Some studies have reported a gradual improvement in semen quality three months after recovery from COVID-19. [26, 27]Our research also observed partial improvement in semen quality, with the exception of sperm DNA fragmentation index (DFI), which remained abnormally high even after 74 days. Additionally, our study did not find a correlation between sperm parameters and DFI.

Our study did not find a correlation between sperm parameters and DFI. This indicates that after COVID-19, sperm DFI remained abnormally high for more than three months. In such conditions, even if conception occurs, the chances of miscarriage are high. Pregnancy loss and failure rates of artificial reproductive therapy (ART) in ART clinics are expected to be higher.[28] However, further research is needed to understand the long-term implications and underlying mechanisms of the changes in sperm DNA during other viral infections. Sperm DFI is a measure of damage to the genetic material of the sperm cells. DFI can affect male fertility and reproductive outcomes.[29] Routine semen analysis does not assess all aspects of sperm quality. The sperm DFI test may predict male fertility and reproductive outcomes better than conventional semen analysis.[30]

CONCLUSION

Most sperm parameters were negatively correlated with sperm DNA fragmentation index (DFI), but without statistical significance. In other words, sperm DFI was not strongly correlated with conventional semen parameters post-COVID-19. However, sperm DFI has proven to be a reliable test for evaluating male

fertility, providing more information about sperm health and holding greater predictive value in assisted reproductive technology (ART) clinics. Therefore, sperm DNA fragmentation assays should be performed as an additional test to investigate male fertility and assist in pregnancy planning. ART clinics should assess both sperm DFI and semen analysis for males recovering from COVID-19 or similar diseases.

ADDITIONAL INFORMATION

Limitations of the study:small sample size.

Conflict of interest:There is no conflict of interest among authors.

Ethics approval and consent to participate:This research project was approved by the Institutional Ethical Committee (vide letter no. RC/AIIMS/Pat/2020/53. Informed consent was obtained from each participant before inclusion in the study, as per the 'National Ethical Guidelines for Biomedical and Health Research Involving Human Participants 2018' of the Indian Medical Research Council.

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Availability of data and material:All data relevant to the study are included in the article or uploaded as supplementary information. All relevant data are available upon reasonable request from the corresponding author.

List of abbreviations:

Abbreviation	Definition
COVID-19	Corona Virus Disease-2019
DFI	DNA Fragmentation Index
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
MFI	Male Factor Infertility
ACE-2	Angiotensin Converting Enzyme-2
RT-PCR	Reverse transcription polymerase chain reaction
WBC	White Blood Cells
LT	Liquefaction Time
SC	Sperm Count
%Morphology-N	Percentage of Normal Morphology
%Head-D	Percentage of Head Defects
%Neck-D	Percentage of Neck Defects
%Tail-D	Percentage of Tail Defects
HPGA	Hypothalamo-Pituitary-Gonadal Axis
LH	Luteinising Hormone
FSH	Follicle Stimulating Hormone
BTB	Blood Testes Barrier
ROS	Reactive Oxygen Species
ART	Artificial Reproductive Therapy

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