Impact of SARS-CoV-2 on the Male Reproductive Tract: Insights from Semen Analysis and Cryopreservation

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Abstract

SARS-CoV-2, the virus behind the COVID-19 pandemic, affects multiple organs, including the male reproductive system. The male reproductive tract expresses ACE-2, the primary viral entry receptor, in spermatogonia, Leydig, and Sertoli cells. While viral infections can harm male fertility through cytokine storms, the effects of SARS-CoV-2 on fertility are still unclear. Thus, this study aimed to examine the impact of COVID-19 on the male reproductive tract. Semen samples from 20 patients, collected 3 months post-SARS-CoV-2 infection, revealed the presence of ACE2 and TMPRSS2 receptors in both plasmatic and spermatozoa fractions. Five patients showed viral RNA-dependent RNA polymerase (RdRp), indicating potential viral persistence. Elevated levels of inflammatory cytokines and chemokines were observed in the plasmatic fraction, suggesting a persistent inflammatory condition affecting sperm vitality. Immune cell populations associated with viral clearance were identified in the semen fraction, correlating with receptor expression and inflammatory cytokines. To address SARS-CoV-2 uncertainties in reproductive medicine, we assessed the virus's presence in seminal fluid before and after cryopreservation using slow freezing and vitrification. Our study confirms the safety of both techniques in preserving male fertility for assisted reproductive technology programs. In conclusion, our study highlights the impact of SARS-CoV-2 on male reproductive health, emphasizing the persistence of viral entry receptors, potential viral RNA presence, the inflammatory environment, and the involvement of immune populations in the male reproductive tract post-infection. Importantly, we confirm the safety of conventional freezing and vitrification techniques for preserving male fertility in assisted reproductive technology programs amidst the COVID-19 pandemic.

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Running Head: "SARS-CoV-2 and Male Reproductive Health"

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Abstract

SARS-CoV-2, the virus behind the COVID-19 pandemic, affects multiple organs, including the male reproductive system. The male reproductive tract expresses ACE-2, the primary viral entry receptor, in spermatogonia, Leydig, and Sertoli cells. While viral infections can harm male fertility through cytokine storms, the effects of SARS-CoV-2 on fertility are still unclear. Thus, this study aimed to examine the impact of COVID-19 on the male reproductive tract.

Semen samples from 20 patients, collected 3 months post-SARS-CoV-2 infection, revealed the presence of ACE2 and TMPRSS2 receptors in both plasmatic and spermatozoa fractions. Five patients showed viral RNA-dependent RNA polymerase (RdRp), indicating potential viral persistence. Elevated levels of inflammatory cytokines and chemokines were observed in the plasmatic fraction, suggesting a persistent inflammatory condition affecting sperm vitality. Immune cell populations associated with viral clearance were identified in the semen fraction, correlating with receptor expression and inflammatory cytokines.

To address SARS-CoV-2 uncertainties in reproductive medicine, we assessed the virus's presence in seminal fluid before and after cryopreservation using slow freezing and vitrification. Our study confirms the safety of both techniques in preserving male fertility for assisted reproductive technology programs.

In conclusion, our study highlights the impact of SARS-CoV-2 on male reproductive health, emphasizing the persistence of viral entry receptors, potential viral RNA presence, the inflammatory environment, and the involvement of immune populations in the male reproductive tract post-infection. Importantly, we confirm the safety of conventional freezing and vitrification techniques for preserving male fertility in assisted reproductive technology programs amidst the COVID-19 pandemic.

Keywords

SARS-CoV-2, male reproductive sytem, inflammation, viral clearance, cryopreservation

Introduction

In late 2019, an outbreak of pneumonia caused by a newly discovered coronavirus called Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in Wuhan, China . This virus spread quickly worldwide and let to a severe acute respiratory syndrome known as "COVID-19" as designated by the World Health Organization (WHO). The rapid transmission of this novel virus resulted in the WHO declaring it a pandemic, causing significant changes in social behavior. COVID-19 has had a profound impact on the health of affected individuals and has also greatly affected healthcare delivery for all patients, not just those with COVID-19.

Coronaviruses have a spherical shape awith spike proteins on their envelope, giving them a club-like appearance. The virus contains a single-stranded RNA genome with a positive sense, which is enclosed within a helical nucleocapsid. The entry of SARS-CoV-2 into cells is mediated by the viral glycoprotein Spike binding to the enzymatic domain of angiotensin-converting enzyme 2 (ACE-2) on the cell surface . The virus is then internalized through endocytosis by another membrane bound protease called transmembrane serine protease 2 (TMPRSS2) . Several testicular cells, including Sertoli, Leydig cells and spermatogonia cells, express the ACE-2 receptor on their surface along with proteins belonging to the angiotensin pathway (ATR1, ATR2, ACE1, and TMPRSS2) . There receptors and proteins are crucial for SARS-CoV-2 infection. Given that other RNA viruses such as Ebola, Marburg, and Zika, have been found to breach the blood-testis barrier and cause inflammation , it was hypothesized that the male reproductive system could also be a target for SARS-CoV-2. Additionally, numerous studies have reported that viral infections significantly contribute to long-term male infertility. Viruses like HPV (papillomavirus), HSV (herpes simplex virus), and HIV (immunodeficiency virus) can infect the male reproductive system, leading to severe consequences for reproductive health , possibly due to triggering an inflammatory response that result in the release of inflammatory cytokines and subsequent oxidative stress.

In light of these considerations, we conducted an investigation into the inflammatory status of the testis by evaluating the presence of inflammatory cytokines and immune cell populations in seminal fluid after viral clearance.

To date, there has been limited research on the mechanisms responsible for the observed effects of COVID-19 infection on semen quality. However, a recent study has provided valuable insights, confirming that COVID-19 infection can reduce semen volume, progressive motility, morphology, sperm count, and DNA integrity .

Preserving male reproductive cells, including semen, epididymal, and testicular spermatozoa, through cryopreservation, is a crucial approach for managing permanent or temporary infertility in young men and children. Cryopreservation technology, in particular, plays an important role in preserving fertility for these patients . It remains uncertain whether viral particles persist after cryopreservation, and this question is especially relevant for cryopreserved samples processed during the COVID outbreak. Therefore, we investigated the presence of SARS-CoV-2 genome in seminal fluid before and after cryopreservation using two methods: conventional slow freezing and vitrification. While slow freezing is a commonly employed technique in assisted reproductive technology, seminal fluid vitrification may offer a more efficient and faster approach for sperm vitrification. However, due to the risk of contamination from direct contact with liquid nitrogen, sperm vitrification is considered a hazardous procedure .[13,271113–19]

Our aim was to investigate the effect of COVID-19 on the male reproductive tract. We collected semen samples from 20 patients, with a median interval of 3 months from SARS-CoV-2 infection. Our findings demonstrated that both ACE2 and TMPRSS2 were detectable in both the plasma and sperm fractions even months after the viral infection. Additionally, we found viral RNA-dependent RNA polymerase (RdRp) in 5 out of 20 patients, indicating the potentional presence of the virus in the body three months after the initial infection. The study also revealed high levels of inflammatory cytokines and chemokines in the plasma seminal fraction of individuals exposed to SARS-CoV-2-, suggesting the persistence of an inflammatory condition that could impact spermatozoa vitality. We also identified immune populations in the semen fraction that are known to play a role in clearing viral infection, and these populations positively correlate with the expression of of SARS-CoV-2 receptors and inflammatory cytokines.

Furthermore, we investigated the potential implicaitons of SARS-CoV-2 in reproductive medicine, particularly in medically assisted reproduction and gamete cryopreservation. We examined the presence of SARS-CoV-2 genome in seminal fluid before and after cryopreservation using two methods: conventional slow freezing and vitrification. Our findings demonstrate that both conventional freezing and vitrification are safe procedures for preserving male fertility in assisted reproductive technology programs.

Methods

Semen Sample Collection

Semen sample were collected by masturbation with an ejaculatory abstinence of 3 to 5 days for all patients. Semen analysis was performed according to the WHO 2010 guidelines, evaluating sperm volume, concentration, motility, viability, morphology. Susequently, semen samples were treated using conventional freezing and vitrification methods. For each patient, a semen sample was used to perform both conventional freezing and vitrification. Additionally, a partion of the sample was treated to separate seminal plasma from pellet for the molecular and cellular analysys. In more detail, we recruited 20 patients from January to April 2021. The average age of 20 patients was 39 years (**Table 1**) and none of the patients were hospitalized due to the SARS-CoV-2 infection. The median interval from viral infection to provide semen samples was 3 months.

Semen Preparation

The semen sample was divided, with 0,5 mL being used for cryopreservation. Another 0.5 mL was centrifuged at 2000 rpm for 10 minutes, to separate seminal plasma and the pellet. Furthermore, 1 mL of the sample was stratified using a 80% / 40% of a solution of Pure sperm 100 Nidacon in Sperm Washing Medium (Irvine Scientific, Santa Ana, CA) to separate round cells from the remaining semen. The resulting fraction was then centrifuged at 1600 rpm for 15 minutes, and the pellet containing spermatozoa was resuspended in TRIZOL (Thermo Scientific, Waltham, MA) and stored at -80C. The layer between the gradients, which contained round cells, was recoverd, centrifuged at 1800 rpm for 10 minutes, and resuspended in 500 mL of FBS10% DMSO.

Vitrification

For vitrification aliquots of 30µl of prepared sperm mixed with a sucrose solution were directly dropped into liquid nitrogen using micropipettes. A stainless strainer was present in the liquid nitrogen to capture the formed spheres. This spheres were then transferred into pre-cooled cryovials and stored in liquid nitrogen for further storage.

The vitrification protocol followed the procedure described in "A simple method of human sperm vitrification" **.** Swim up sperm (30 microliters) combined with 0.5 M sucrose solution in a 1:1 ratio were dropped into liquid nitrogen using a micropipette, causing them to harden into spheres that sank into a stainless steel tea strainer. These spheres were quickly transferred into cryovials previously cooled in liquid nitrogen for future storage.

After vitrification, prompt transfer into cryovials in liquid nitrogen was crucial to maintain their integrity. Larger volumes necessitated the transfer of more spheres, increasing the risk of spontaneous thawing during the process. This transfer procedure was vital for achieving high post-warming survival and motility.

For devitrification/warming, the spheres were removed from storage after a week and placed in 5 ml of prewarmed gamete handling fluid supplemented with human serum albumin at 42 degrees Celsius for 10 seconds.

Conventional Freezing

The conventional freezing method was performed following the guidlines of SpermFreeze Solution: Quinn's Advantage Sperm Freezing Medium (Cooper Surgical Trumbull CT). SpermFreeze solution was added to the seminal sample in cryotube vials at a 1:1 ratio. The cryovials were kept at room temperature for10 minutes, after which they were cooled in a vapor state for 20 minutes. They were placed horizontally, approximately 3 cm above the liquid nitrogen surface, reaching a temperature of approximately -70 to -90°C. Finally, the cryovials were submerged in liquid nitrogen and stored at -196 0C.

Flow-Cytometry Analysis

Multiparameter Flow Cytometry analysis was performed on semen samples from COVID-19 patients using a BD FACSymphony A5 instrument (BD Biosciences) and analyzed with FlowJo (FlowJo LLC, Ashland,

OR). Gates were done on live cells and the following primary antibodies were used for immune cell characterization: CD163 (BD Horizon, Franklin Lakes, NJ, clone MAC2-158), CD45 (Biolegend, San Diego, CA, clone HI30), CD56 (BD Biosciences, clone MY31), CD19 (Biolegend, clone HIB19), CD16 (Biolegend, clone 3G8), CD25 (BD Biosciences, clone 2A3), CD123 (BD Biosciences, clone 7G3), HLA-DR (BD Biosciences, clone G46-6), PD-1 (Biolegend clone EH12.2H7), CD8 (BD Biosciences, clone SK1), CD95 (BD Biosciences, clone DX2), CD4 (BD Biosciences, clone M-T477), CD11c (Biolegend, clone Bu15), CD127 (eBioscience, clone eBioRDR5), CD45RO(BD Biosciences, clone UCHL1), CD3 (BD Biosciences, clone UCHT1), CD14 (Biolegend, clone M5E2), CD68 (BD Biosciences, clone Y1/82A), CD27 (BD Biosciences, clone M-T271), CCR7 (Bd Biosciences, clone 3D12).

RT-qPCR Assay

Viral RNA was extracted from plasma and sperm of recovered patients using RNA with Quick-RNA MiniPrep (ZYMO RESEARCH, Irvine CA, catalog #1054) according to the manufacturer's guidelines. Genomic DNA contamination was removed with DNase-Free DNase set (ZYMO RESEARCH, catalog #E1010). The total RNA extracted was stored at -80°. Then, 500ng RNA was retro-transcribed with ImProm-II Reverse Transcriptase kit (Promega) following manufacturer's instruction and random primers (0,5 ug/ul). qPCR assay was performed with Luna Universal qPCR Master Mix (New England Biolabs) on 10 ng cDNA template/reaction using exon-spanning primers (final concentration 1 μM). ΔΔCT and 2ˆ-ΔCT methods were used to calculate the expression levels, samples were normalized to GAPDH.

Cytometric Bead Array (CBA)

Using the BD Cytometric Bead Array (CBA) Human Kit Flex Set (BD Bioscience-Pharmingen, San Diego, CA). to measure IL-2 (catalog #558270), IL-6 (catalog #558276), IFN-γ (catalog #558269), IL-10 (catalog #558274), TNF (catalog #560112), IL-1β (catalog #558279), IFN-α (catalog #560379), GM-CSF (catalog #558335) and IL-8 (catalog #558277). The kit contains three bead populations with distinct fluorescence intensities. The beads were coated with capture antibodies specific to IL-2, IL-6, IFN-γ, IL-10, TNF, IL-1β, IFN-α, GM-CSF and IL-8 and mixed with phycoerythrin (PE) conjugated detection antibodies. These capture beads were incubated with recombinant standards or test samples to form sandwich complexes. The CBA was analyzed at FACSCcanto flow cytometer (BD Bioscience-Pharmingen). Results were anayzed using graphic and tabular formate using CBA analysis software (BD Bioscience-Pharmingen).

Statistical analysis

The investigators were not blinded to allocation during experiments and outcome assessment. Data were analyzed using Prism software (GraphPad8, San Diego, CA). Each singel dot in every graph represents an individual patient. Analyses were performed using Single Linear regression. A p-value of less than 0.05 is considered statistically significant.

Results

Demographics and Characteristics of Recovered COVID-19 Patients.

We enrolled a total of 20 patients between January and April 2021. The average age of the patients was 39 years (**Table 1**) and none of them required hospitalization due to the SARS-CoV-2 infection. The median time between viral infection and collection of semen samples was 3 months. In the semen specimens, all patients tested negative for SARS-CoV-2 RNA. However, before cryopreservation, 5 patients tested positive for the RNA-dependent RNA polymerase (RdRp) in their spermatozoa.

The total sperm count per ejaculate averaged 88 x10^6+/- 70,16. Progressive motility percentage was 26% +/-7,71 and eosin percentage was 78,5% +/-8,42. Out of the 20 patients, 16 had total motile sperm counts and sperm within normal ranges. One patient showed mild necrozoospermia and asthenospermia, two showed mild asthenospermia and one had a mild oligospermia (**Table 1**).

Impact of Testis Inflammatory State on Sperm Vitality.

To investigate the potential impacy of SARS-CoV-2 infection on sperm concentration, motility and vitality, we assessed macroscopic and microscopic parameters of seminal fluid (**Table 1**). Our findings, as shown in **Figure 1,** confirmed that spermatozoa with higher motility percentages exhibited greater vitality scores (p=0,0334;**Figure 1**). Furthermore, we analyzed the presence of inflammatory cytokines and chemokines in the seminal fluid of our cohort (**Figure 2A**) to evaluate the inflammatory state of the testis. Specifically, we observed elevated levels of IL-6, IFN-γ and IL-8. Notably, the concentration of IL-6 positively correlated with concentration of IL-8 (p<0,0001; **Figure 2B**), and the two samples with the highest IL-6 concentration had also exhibited the highest IL-8 level (red circle, **Figure 2A**).

To determine whether high levels of inflammatory cytokines could impact spermatozoa and affect vitality and motility we conducted linear regression analyses. Interestingly, we found that IL-6 and IL-8 level did not significantly influence spermatozoa motility (**Figure 3A**), but both cytokine levels were positively correlated with reduced spermatozoa vitality (IL-6 p=0,0493 and IL-8 p=0.0350; **Figure 3B**). Additionally, we explored the correlations among various inflammatory cytokines and chemokines detected in the seminal plasma. Notably, we observed a positive correlation between GMCSF level and IL-6 (p=0,0097; **Figure 4A**), GMCSF and IFN-γ (p=0,0219;**Figure 4B**), TNF and IFN-γ (p=0,035; **Figure 4C**).

Flow Cytometry Analysis of Semen Samples Recovered from COVID-19 Patients.

In order to gain insight of how the immune cell populations in semen may impact the clereance of SARS-CoV-2, we utilized flow cytometry to characterize the immune cell profile of human semen samples. Due to limited sample recovery after cell isolation, data from 5 out of 16 patients are presented (**Supplementary Table 1**). The scheme presented in **Supplementary Table 2** was used to characterize the $CD45^{+}$ population in monocytes, B cells, memory B cells, cytokine producing NK cells, cytotoxic NK cells, dendritic cells, CD8 central memory T cells, CD8 effector memory T cells, CD8 naïve T cells, CD8 terminal effector T cells, TPEX (exhausted cells progenitors), and exhausted-like T cells (**Figure 5A**). Notably, significant positive correlations were assessed between exhausted-like T cells, CD8 effector memory T cells, CD8 terminal effector T cells and the expression of TMPRSS2 and ACE2 (**Figure 5B**). Furthermore, to elucidate the potential relationship between immune populations and the cytokines released in the seminal fluid, we conducted correlation analyses. Notably, IFN-γ showed positive correlation with monocytes, exhausted-like T cells, CD8 effector memory T cells, and CD8 terminal effector T cells. Positive correlations were also found between cytotoxic NK, dendritic cells, and IL-6. Lastly, a positive correlation was found between IL-1β and monocytes, B cells, and memory B cells (**Supplementary Figure 1, 2, and 3**).

Detection of ACE2 and TMPRSS2 in Plasma and Sperm of Recovered SARS-CoV-2 Patients.

SARS-CoV-2 utilizes the glycoprotein ACE2 to bind to host cells, including those in the testicular tissue. Upon binding to ACE2, the virus enters the cell through endocytosis facilitated by the transmembrane serine protease 2 (TMPRSS2) . In light of this, we aimed to investigate the gene expression of the SARS-CoV-2 receptor ACE2 and the co-receptor TMPRSS2 in spermatozoa and seminal fluid. Among the 20 patients analyzed, ACE2 expression was detected only in one plasmatic fraction (patient 10) and in 3 spermatozoa fraction (patient 1, 13 and 17) **(Figure 6A**). On the other hand, TMPRSS2 gene expression was identified in 10 plasmatic fractions and 14 spermatozoa fractions (**Figure 6B**). Notably, all the spermatozoa that tested positive for ACE2 expression also exhibited TMPRSS2 expression, and there was a positive correlation between ACE2 and TMPRSS2 expression levels (**Figure 6C**).

Safety of Cryopreservation for SARS-CoV-2 Samples.

During the COVID-19 pandemic, cryopreservation techniques were employed by Assistance Reproductive Technology (ART) clinics for precautionary purposes, but without conducting a semen COVID-19 screening due to its unavailability. Therefore, our objective was to investigate whether we could identify SARS-CoV-2 viral RNA in the plasmatic fraction and spermatozoa before and after cryopreservation. We performed viral RNA extraction from both fractions and utilized RT-PCR to detect the nuclear N gene, the Spike S gene and the RdRp gene. Interestingly, we were unable to detect the N and S gene in eithr the plasmatic or spermatozoa fraction. However, RdRp detection was succseful in 5 out of 20 patients before cryopreservation (**Figure**

7). Notably, when the same samples were tested after undergoing slow conventional cryopreservation and vitrification, the RdRp viral gene was no longer detectable. This suggest that both freezing procedures are safe for storing samples collected from individuals exposed to SARS-CoV-2.

Discussion

Viral infections have been associated with male infertility as they can disrupt crucial processes involved in sperm production and function. Testicular inflammation caused by viruses can harm the seminiferous tubules, resulting in reduced sperm count, motility, and morphology. Sexually trasmitted viruses like Zika, HIV, and hepatitis B and C have also been linked to impaired male fertility. Therefore, it is crucial to prioritize the prevention and management of viral infections to safeguard male reproductive health .

Since the outbreak of SARS-CoV-2 in 2002, previous studies have indicated that orchitis, inflammation of the testicles, can disrupt spermatogenesis and germ cell apoptosis, impacting semen quality . In this study, semen samples were collected approximately 3 months after SARS-CoV-2 infection from 20 patients. Interestinlgy, most patients (16 out of 20) exhibited normal ranges of total motile sperm counts and sperm morphology (**Table 1**)[10,29–32].

Histopathological investigations from other SARS-CoV-2 studies have revealed the presence of inflammatory infiltrates primarly in the seminiferous tubules, suggesting the involvement of inflammatory and immunologic reactions in viral-induced testicular damage . Similarly, studies on

human SARS-CoV-2 infection and animal models have associated hypogonadism with increased levels of pro-inflammatory cytokines , particulalry IL-1β, IL-6, and TNF-α, which are significant inflammatory mediators in SARS-CoV-2 pathogenesis. Therefore, the study examined the levels of inflammatory cytokines and chemokines in the seminal plasma fraction of SARS-CoV-2 exposed individuals. Remarkably, high levels of IL-6, IFN-γ and IL-8 were observed (**Figure 2A**). Furthermore, positive correlations were found between IL-6 and IL-8 levels and lower sperm vitality (**Figure 3B**). Additionally, several inflammatory cytokines (IL-6 and IL-8, GMCSF level and IL-6, GMCSF and IFN-γ, TNF and IFN-γ) exhibited positive correlations with each other (**Figure 4A, B, and C**). These results suggest that SARS-CoV-2 increases cytokines level in semen, leading to an inflammatory condition that negatively impacts sperm vitality.

Considering the increased inflammatory cytokine levels in semen and the crucial role of inflammatory and immunologic reactions in viral-mediated testicular damage , the immune cell profile of human semen samples was investigated. As reported in the literature, all the detected immune population (monocytes, NK, and T-cells, except for naïve T cells) play a critical role in clearing long- and short- term viral infection (**Figure 5A**). T cells, in particular, have a significant role in adaptive immunity by recognizing antigenic peptides and differentiating into effector T cells during primary infection. After antigenic clearance, a small fraction of the effector T cells differentiate into memory T cells. However, chronic viral and bacterial infections can lead to altered differentiation, resulting in T-cell exhaustion and impaired immune responses. T-cell exhaustion has been observed in various chronic viral infections and cancer, primarily affecting CD8+ T cells. Interestinlgy, a positive correlation was found between immune cell populations involved in viral clearance (exhausted-like, CD8 effector memory T cells, CD8 terminal effector T cells) and the expression of SARS-CoV-2 receptors TMPRSS2 and ACE2 (**Figure 5B**). Furthermore, several immune populations exhibited positive correlations with IFN-γ, IL-6, and IL-1β (**Supplementary Figure 1, 2 and 3**). Patient 10, the only sample in which ACE2 was detected in the plasmatic fraction, displayed increased immune cell recruitment compared to the other samples (**Table 2**). These findings suggest that the inflammatory condition persists approximately 3 months after SARS-CoV-2 infection, as observed in other studies . Moreover, the higher recruitment of immune cell populations correlates with the detection of ACE2.

Recent data indicate that SARS-CoV-2 exploits ACE2 and TMPRSS2 to enter cells . Genetic aberrations in TMPRSS2 and ACE2 may contribute to the severity of orchitis and fertility outcomes. Mild orchitis can result from SARS-CoV-2, while individuals with germline TMPRSS2 losses may be more susceptible to severe orchitis and have a higher risk of sterility. It has been suggested that ACE2 activates inflammasomes upon SARS-CoV-2 binding, and pro-inflammatory cytokines regulate the expression of TMPRSS2

and ACE2. However, individuals with non-obstructive azoospermia exhibit decreased ACE2 expression in Sertoli cells, indicating that ACE2 defects could enhance the impact of TMPRSS2 hypofunction on infertility predisposition.To investigate whether these receptors were modulated after SARS-CoV-2 infections, mRNA expression in the spermatozoa and seminal fluid fractions was analyzed. Surprisingly, both the ACE2 (1 plasmatic and in 3 spermatozoa) and TMPRSS2 (10 plasmatic and in 14 spermatozoa) were detected in both fractions (**Figure 6A and B**). The presence of SARS-CoV-2 viral RNA was also assessed in the plasmatic and spermatozoa fractions and found that while N or S genes were not detected in either fractions, RdRp was present in 5 out of 20 patients (**Figure 7**). These results indicate that SARS-CoV-2 internalization receptors remain up-regulated and viral RdRp can still be detected 3 months after infection.

Understanding the safety of cryopreservation techniques such as vitrification or two steps cryopreservation for preserving human semen after SARS-CoV-2 exposure is crucial for clinicians and researchers. Sperm cryopreservation is a crucial technique employed in Assisted Reproductive Technology (ART) programs to preserve male fertility. This study aimed to assess the safety of two commonly utilized methods, namely conventional two-step freezing and vitrification for preserving semen samples from patients who had been exposed to SARS-CoV-2 and required fertility preserrvation.

In conclusion, despite the persistent inflammatory consitions observed in patients recovering from SARS-CoV-2 infection, conventional freezing and vitrification remain safe procedures for preserving male fertility in ART programs. However, it is important to acknowledge certain limitations. The relatively sample size of 20 patients limits the generalizability of our findings to a larger population. The absence of a control group hinders direct comparisons and definitive conclusions regarding the observed effects. Moreover, the potential reversibility of any observed effects on male fertility remains unclear. These limitations sould be taken into consideration when interpreting our results.

Ethic Statement

The study complied with the Declaration of Helsinki and was approved by the institutional ethics committee of Humanitas Research Hospital (Protocol number 230879). Semen samples were obtained after written and informed consent from 20 patients undergoing a seminal fluid evaluation.

Acknowledgments

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Author Contributions

SG designed the study, conceived and performed experiments, analyzed data, wrote the manuscript, supervised the funding of the work, and secured the findings; AMM performed experiments, analyzed the data and wrote the manuscript; AA performed experiments and wrote the manuscript, GD acquired data, analyzed data and contributed to interpretation of the results, EA performed experiments, contributed to interpretation of the results and wrote the manuscript. TS provided expertise and feedback. MR and PELS supervised the study. All authors discussed the results and contributed to the final version of the manuscript.

Data Availability Statment

The raw data supporting the current study have been deposited to Zenodo public repository10.5281/zenodo.8138194

Figures and Tables

Table 1: Semen parameters of SARS-Cov-2 recovered patients.

Individulas, n	20
Age, y	38,55 +/- 8,67
Body mass index kg/m2 (BMI)	25,4 +/- 2,9
Smoker	5/20
Pathologies	6/20
SARS-CoV-2 in semen	5/20
COVID-19-related symptoms	9/20
Duration of symptoms, d	2 +/- 3,84
Hospitalization	N.A.
Intensive care	N.A.
Time between infection and semen collection, m	3 +/- 2,85
Time between positive IgG titer and semen collection, m	4,5 +/- 1,75
Antibiotic treatment	1/20
Cortisone treatment	1/20
Volume, mL	3,5 +/- 1,63
Sperm concentration, n/mL	27 +/- 24,40
pH	8 +/- 0,23
Total no. of sperm per ejaculate	88x106 +/- 70,16
Progressive motility %	26% +/- 7,71
Round cells detected	850.000 +/- 461.005,765
Eosin	78,5% +/- 8,42

Figure 1

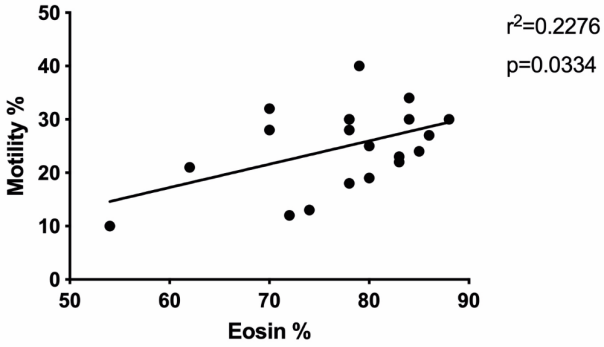

Figure 1: Linear regression showing significant positive correlation between motility and sperm vitality.

Figure 2

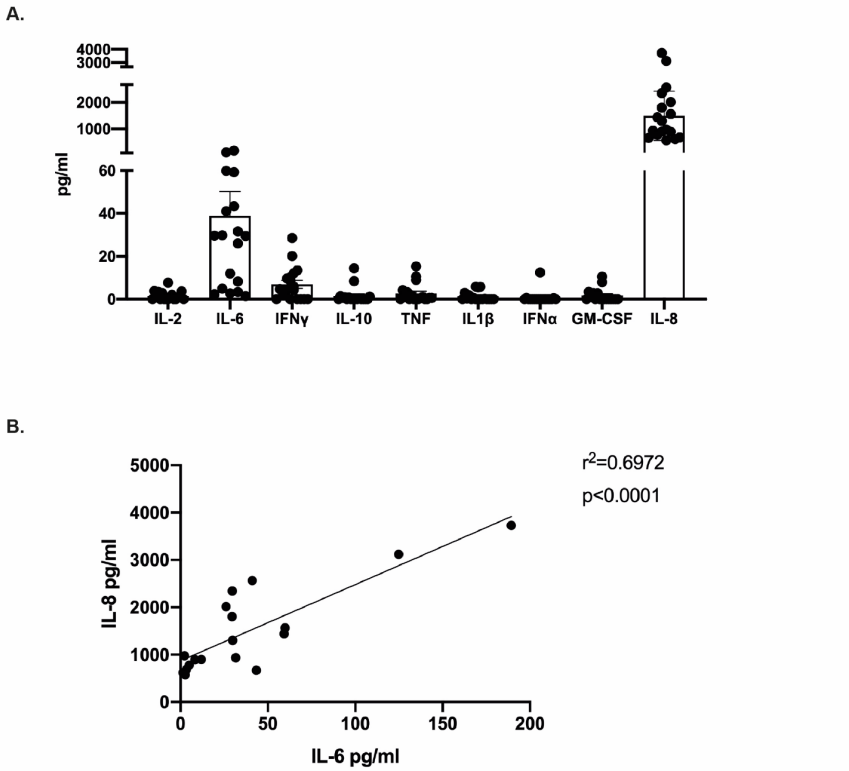

Figure 2: (**A**) Cytokine and chemokine level was analyzed in the seminal plasma of SARS-CoV-2 previously infected patients. (**B**) Linear regression analysis showing significant positive correlation between IL-6 and IL8 seminal concentration.

Figure 3

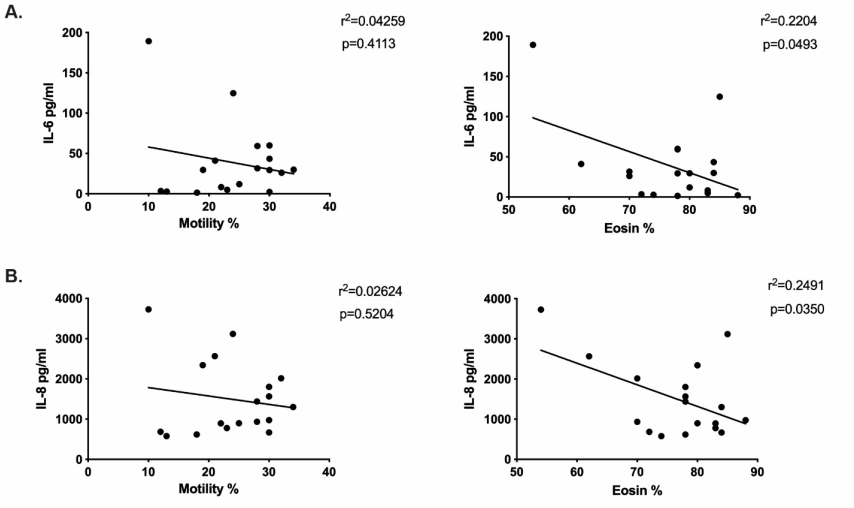

Figure 3: Linear regression showing (**A**) not significant correlation between the IL-6 and IL-8 level and spermatozoa motility. **(B**) Significant positive correlation was identified between IL-6 and IL-8 level and lower sperm vitality.

Figure 4

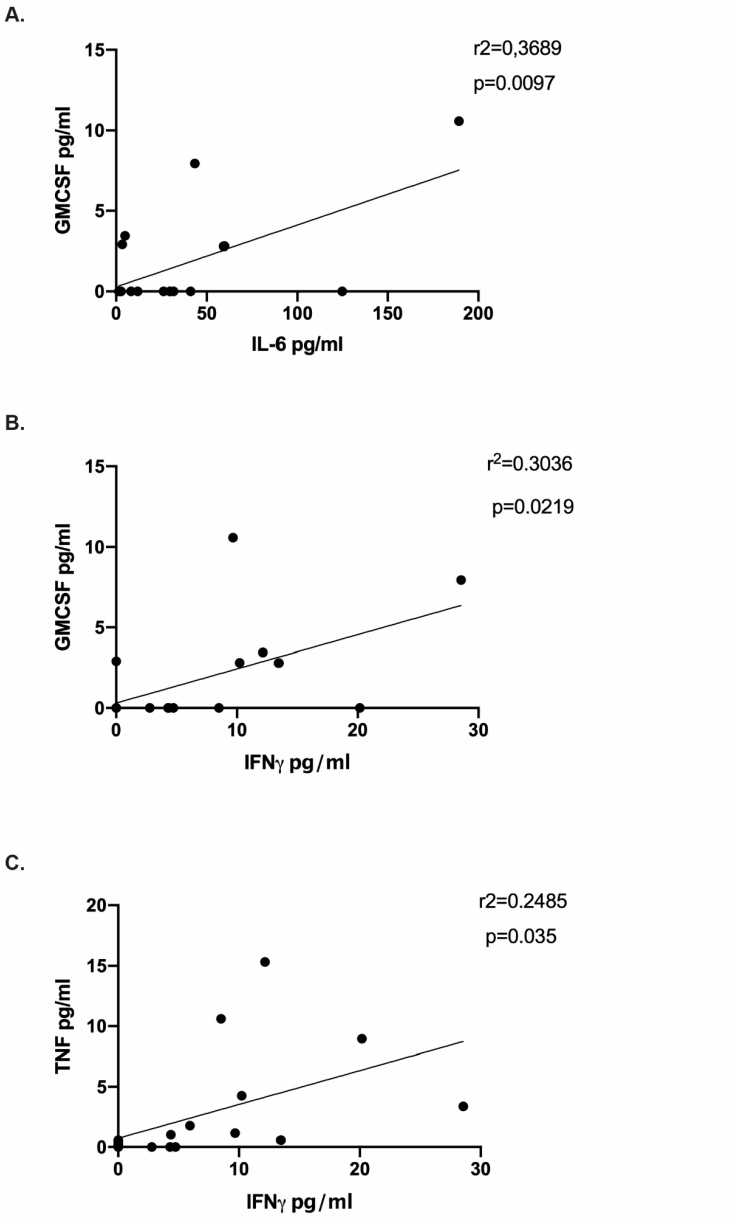

Figure 4: Linear regression showing significant positive correlation between the level of (**A**) GMSF and IL-6, (**B**) GMCSF and IFN-γ and (**C**) TNF and IFN-γ.

Figure 5

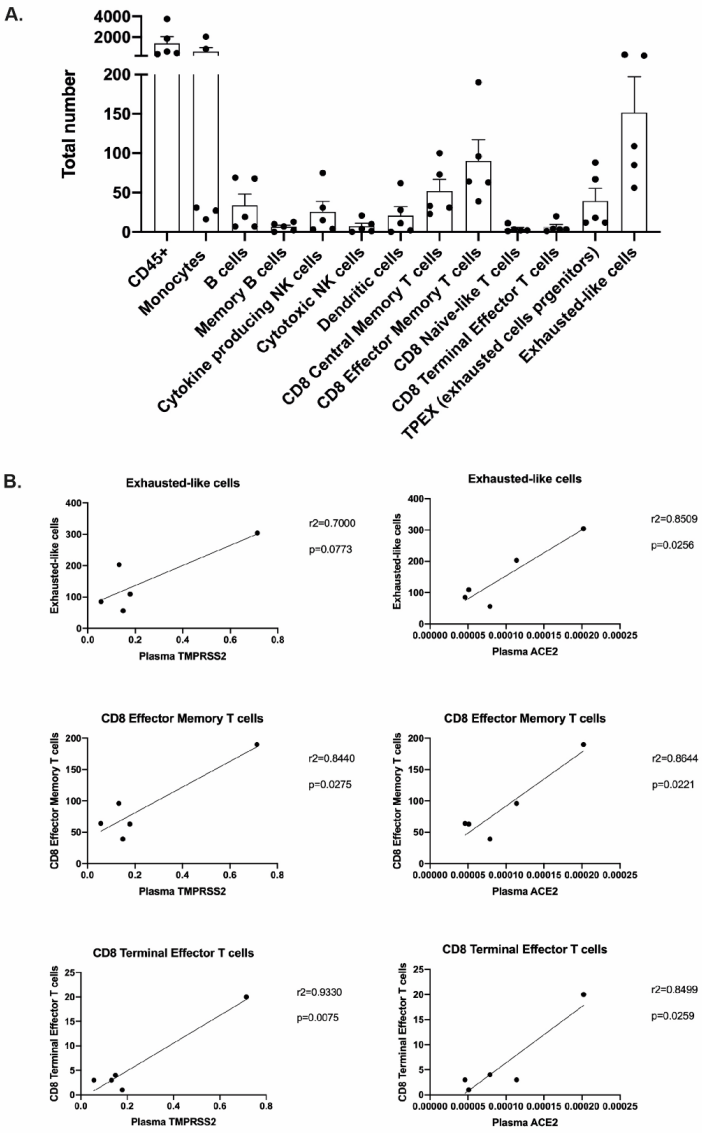

Figure 5: Flow cytometry analysis were performed on semen samples of a total 20 patients.
Graph shows the total number of samples with detectable immune population. Each dots represent a human specimen (**A**). Linear regression showing positive correlation between exhausted-like cells, CD8 effector memory T cells and CD8 terminal effector cells and the level of TMPRSS2 and ACE2 (**B**).

Figure 6

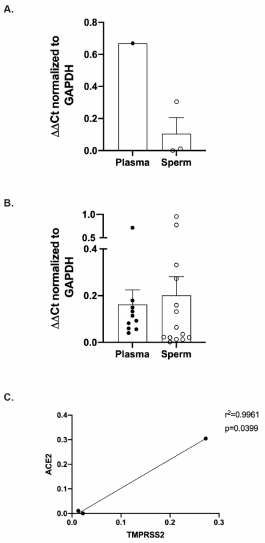

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Figure 6: Figure 6: Evaluation of the (**A**) ACE2 and (**B**) TPMRSS2 gene expression level in both plasmatic fraction (full symbols) and spermatozoa (open symbols). Graphs show only samples with detectable mRNA level. (**C**) Linear regression showing positive correlation between ACE2 and TMPRSS2 level in the spermatozoa fraction.

Figure 7

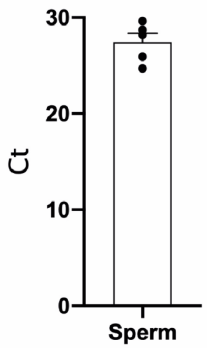
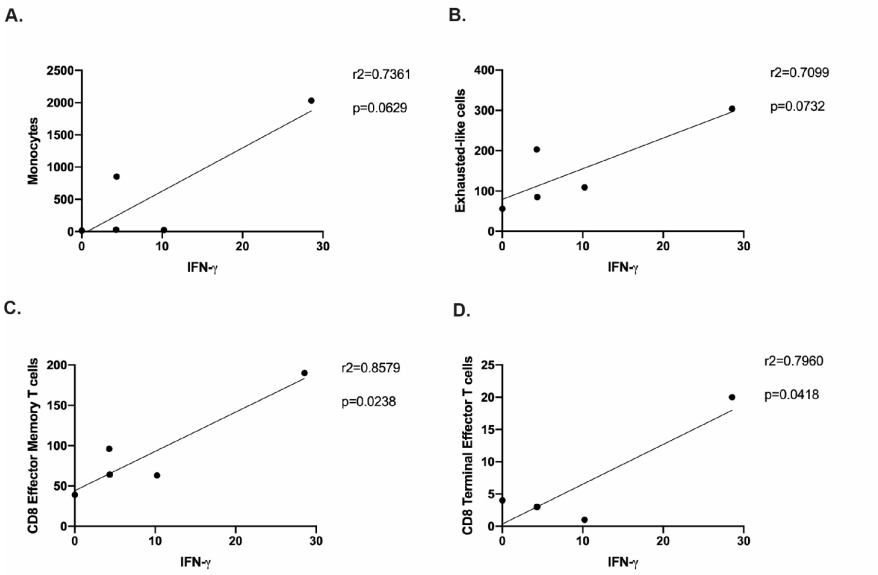


Figure 7: Evaluation of the viral RdRp RNA in spermatozoa samples before cryopreservation (full symbols). Graphs show only samples with detectable mRNA level. Ct= cycle threshold.

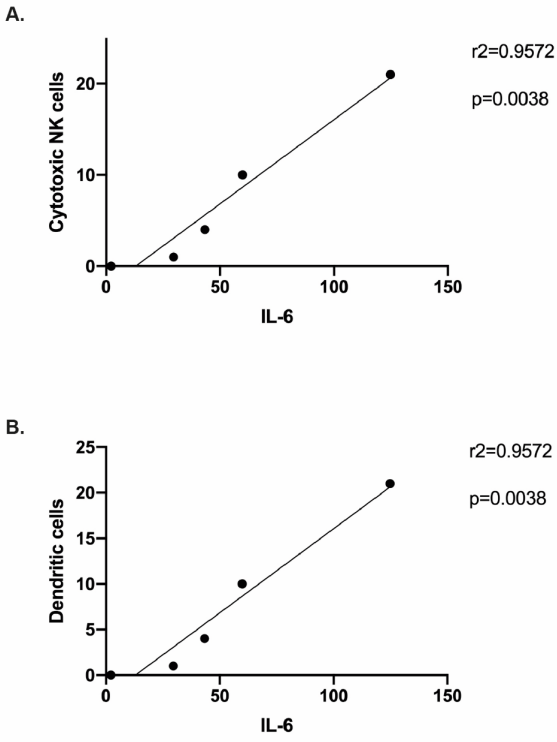
Supplementary Figures and Tables

Supplementary Figure 1



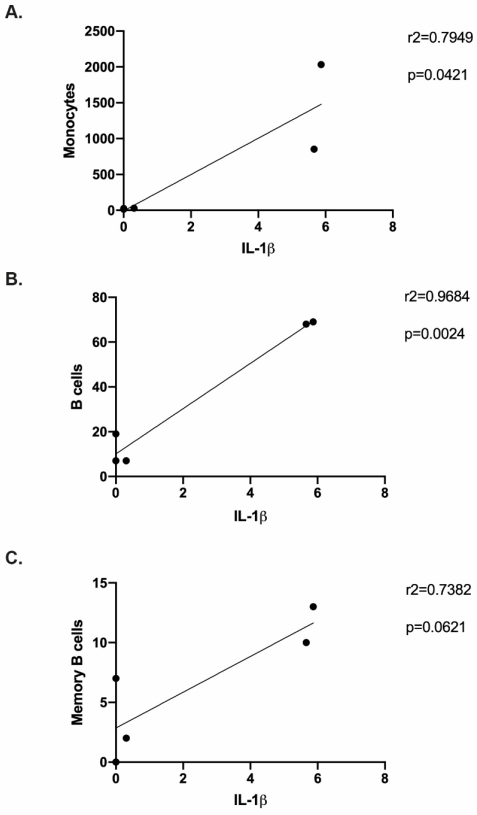
Supplementary Figure 1: Linear regression showing positive correlation between monocytes (**A**), exhausted-like cells (**B**), CD8 effector memory T ce**lls** (**C**)**,** CD8 terminal effector cells (**D**) and IFN-γ level.

Supplementary Figure 2



Supplementary Figure 2: Linear regression showing positive correlation between cytotoxic NK (**A**), Dendritic cells (**B**) and IL-6 level.

Supplementary Figure 3



Supplementary Figure: 3 Linear regression showing positive correlation between Monocytes (**A**), B cells (**B**), Memory B cells (**C**) and IL-1β.

Supplementary Table 1: Patients specimens and total number of live CD45+ population are shown. Only 5 out of 16 samples were analyzed were due to low recovery after cell isolation (red).

Patients	Tot Number of Live CD45+
1	242
2	364
3	74
4	112
5	115
6	468
7	167
8	168
9	616
10	3746
11	212
12	94
15	40
16	109
17	33
18	1850

Supplementary Table 2: Gating strategy used for immune cell phenotyping.

Markers	Cell population
$CD14^{+}$	Total monocytes
$CD14^{-}/CD3^{-}/CD19^{+}$	Total B cells
$CD14^{-}/CD3^{-}/CD19^{+}/CD27^{+}$	Memory B cells
$CD14^{-}/CD3^{-}/CD19^{-}/CD56^{br}$	Cytokine producing NK cells
$CD14^{-}/CD3^{-}/CD19^{-}/CD56^{dim}/CD16^{+}$	Cytotoxic NK cells
$CD14^{-}/CD3^{-}/CD19^{-}/CD56^{-}/Cd16^{-}/CD11c^{+}/HLA\text{-}DR^{+}$	Dendritic Cells
$CD14^{-}/CD3^{+}$	CD3
$CD14^{-}/CD3^{+}/CD4^{+}$	CD4
$CD14^{-}/CD3^{+}/CD4^{+}/CCR7^{+}/CD45RO^{+}$	CD4 Central Memory
$CD14^{-}/CD3^{+}/CD4^{+}/CCR7^{-}/CD45RO^{+}$	CD4 Effector Memory
$CD14^{-}/CD3^{+}/CD4^{+}/CCR7^{+}/CD45RO^{-}$	CD4 Naive
$CD14^{-}/CD3^{+}/CD4^{+}/CD25^{hi}/CD127^{low}$	CD4 Treg
$CD14^{-}/CD3^{+}/CD8^{+}$	CD8
$CD14^{-}/CD3^{+}/CD8^{+}/CD95^{+}/CD27^{+}/CCR7^{+}/CD127^{+}/PD1^{+}$	TPEX (exhausted cell progenitors)
$CD14^{-}/CD3^{+}/CD8^{+}/CD95^{+}/CD127^{+}/PD1^{+}$	Exhausted-like cells
$CD14^{-}/CD3^{+}/CD8^{+}/CCR7^{+}/CD45RO^{+}$	CD8 Central Memory
$CD14^{-}/CD3^{+}/CD8^{+}/CCR7^{-}/CD45RO^{+}$	CD8 Effector Memory
$CD14^{-}/CD3^{+}/CD8^{+}/CCR7^{+}/CD45RO^{-}$	CD8 Naive
$CD14^{-}/CD3^{+}/CD8^{+}/CCR7^{-}/CD45RO^{-}$	CD8 Terminal Effector

Bibliography

Figure 1

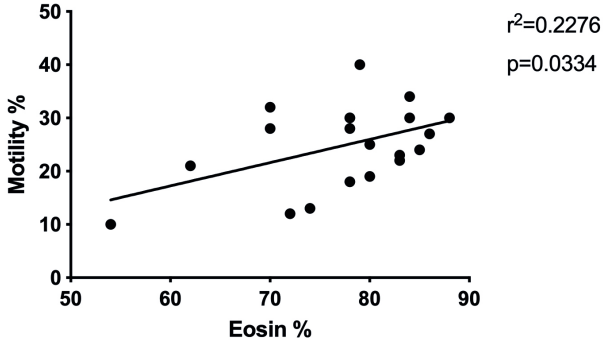


Figure 1: Linear regression showing significant positive correlation between motility and sperm vitality.

Figure 2

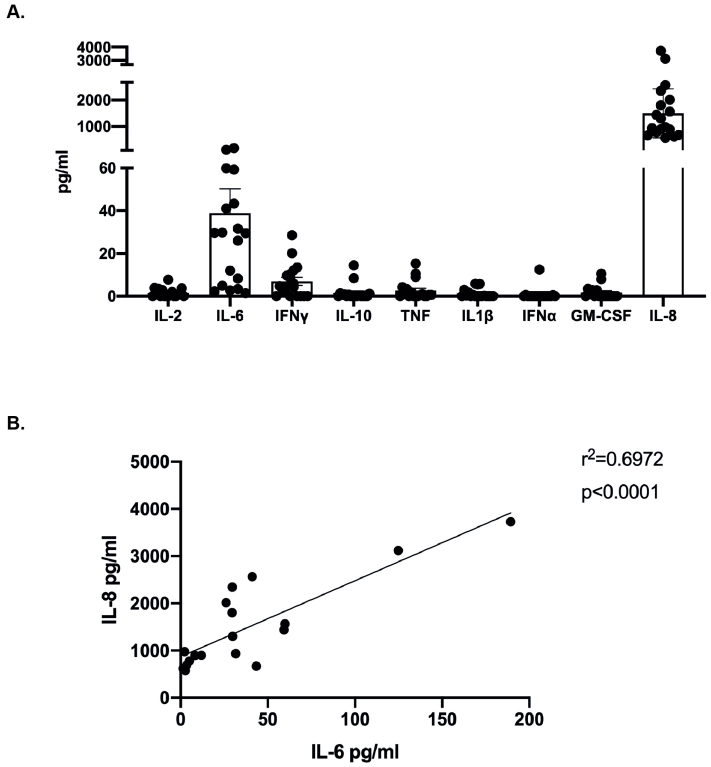


Figure 2: (**A**) Cytokine and chemokine level was analyzed in the seminal plasma of SARS-CoV-2 previously infected patients. (**B**) Linear regression analysis showing significant positive correlation between IL-6 and IL8 seminal concentration.

Figure 3

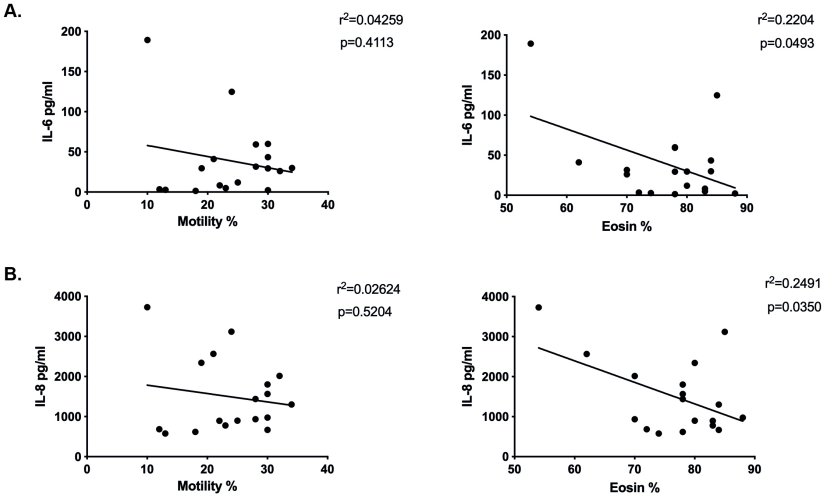
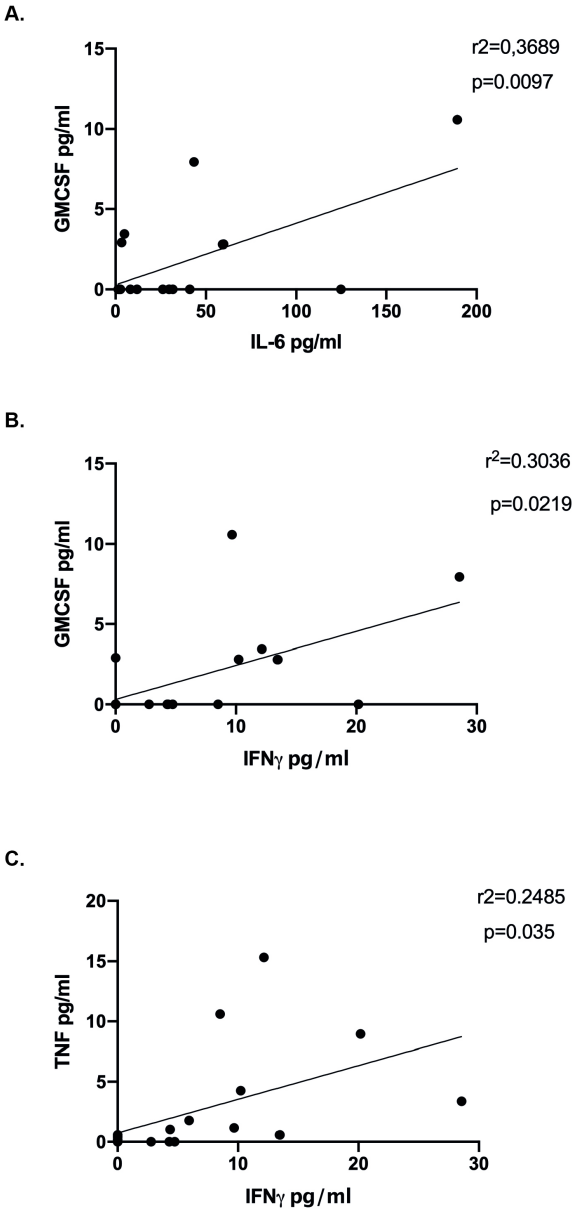


Figure 3: Linear regression showing (**A**) not significant correlation between the IL-6 and IL-8 level and spermatozoa motility. **(B**) Significant positive correlation was identified between IL-6 and IL-8 level and lower sperm vitality.

Figure 4



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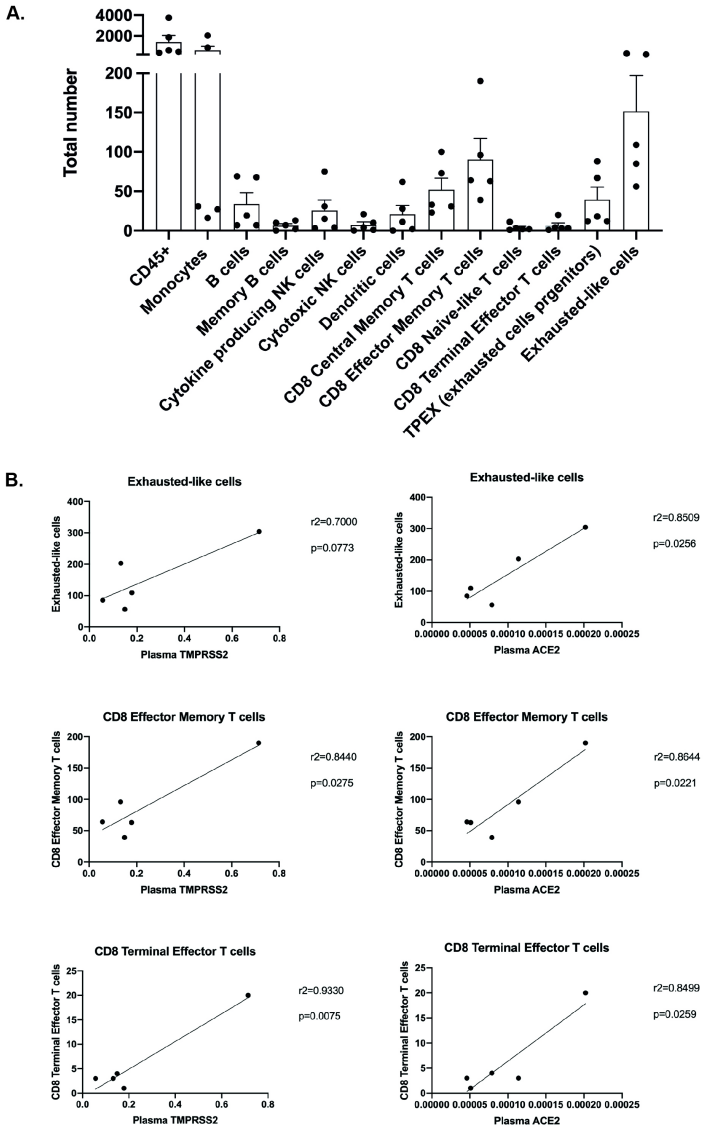

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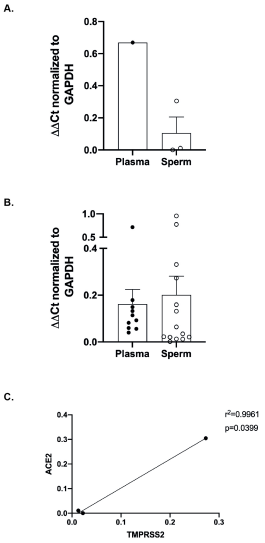

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Figure 7

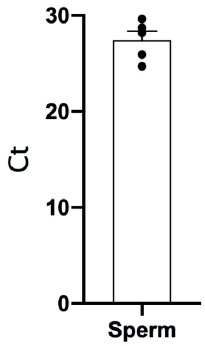

Figure 7: Evaluation of the viral RdRp RNA in spermatozoa samples before cryopreservation (full symbols). Graphs show only samples with detectable mRNA level. Ct= cycle threshold.

Individulas, n	20
Age, y	38,55 +/- 8,67
Body mass index kg/m2 (BMI)	25,4 +/- 2,9
Smoker	5/20
Pathologies	6/20
SARS-CoV-2 in semen	5/20
COVID-19-related symptoms	9/20
Duration of symptoms, d	2 +/- 3,84
Hospitalization	N.A.
Intensive care	N.A.
Time between infection and semen collection, m	3 +/- 2,85
Time between positive IgG titer and semen collection, m	4,5 +/- 1,75
Antibiotic treatment	1/20
Cortisone treatment	1/20
Volume, mL	3,5 +/- 1,63
Sperm concentration, n/mL	27 +/- 24,40
pH	8 +/- 0,23
Total no. of sperm per ejaculate	88x106 +/- 70,16
Progressive motility %	26% +/- 7,71
Round cells detected	850.000 +/- 461.005,765
Eosin	78,5% +/- 8,42