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Prospective two-arm study of the testicular function in patients with COVID-19

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Abstract

Background: The COVID-19 pandemic has led the international community to conduct extensive research into potential negative effects of the disease on multiple organs and systems in the human body. One of the most discussed areas is potential of the virus to compromise the testicular function. However, the lack of prospective studies on this topic makes it impossible to draw reliable conclusions on whether the disease affects the male reproductive system and, if so, to what extent.

Objectives: The current trial is aimed at investigating the effect of SARS-CoV-2 on the testicular function, hormone levels and determining the extent of impact on spermatogenesis and damage to testicular tissue.

Materials and methods: This prospective study included healthy controls and cases of patients suffering from viral pneumonia based on chest computed tomography (CT) and a positive SARS-CoV-2 throat swab exhibited moderate symptoms (World Health Organization (WHO) classification). Epidemiological, clinical, laboratory and ultrasound data were collected. A semen analysis was performed in cases during their hospital stay and 3 months after the discharge home. We also assessed the testicles obtained during autopsies of patients who died of COVID-19 ($n = 20$).

Results: A total of 88 participants were included (44 controls and 44 cases). Blood testosterone levels were significantly decreased in 27.3% of the cases (12/44). The mean level (7.3±2.7 nmol/L) was lower than that in the healthy controls (13.5±5.2 nmol/L, $p < 0.001$). An increase in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) was also detected compared to the healthy controls ($p = 0.04$ and $p = 0.002$). The semen analysis revealed decreased motility in COVID-19 patients ($p = 0.001$), and a higher number of immobile sperm (during COVID-19: 58.8% and at 3 months 47.4%, $p = 0.005$). All parameters returned to normal at 3 months after discharge. Direct mixed

agglutination reaction (MAR) test at 3 months showed an increase of Ig A ($p = 0.03$). In the majority of autopsies (18/20), structural disorders of the testicular tissue, with signs of damage to germ cells were observed.

Discussion and conclusion: COVID-19 and its management strategies significantly affect male hormone levels and sperm quality at the onset of the disease. Postmortem examination of testicular tissue confirmed inflammation and viral infiltration of the testicles. However, in patients with moderate to severe disease, the studied parameters of the testicular function returned to normal values within 3 months.

KEYWORDS
COVID-19, male fertility, SARS-CoV-2, testes

1 | INTRODUCTION

Research into SARS-CoV-2-associated syndromes and disorders is an active field of research. COVID-19 is associated with inflammatory syndrome targeting a multitude of human organs. One of them was described in the early days of the pandemic by Zou et al. who reported that non-respiratory symptoms may be explained by the binding of the virus to angiotensin convertase Type-2 (ACE-2) in other organs.[1] Previous studies assessed the possible effects of COVID-19 at several organs sites as kidneys, intestines, etc.[2–4] Shen and Wang found ACE-2 expression in testicular cells (namely spermatogonia, Leydig cells, and Sertoli cells) which makes them potential targets for the virus.[5] In fact, there are reports of orchitis and epididymitis in patients diagnosed with COVID-19,[6] which may suggest that SARS-CoV-2 is able to directly damage testicular tissue potentially compromising male fertility and hormone function.

To date, there has not been a focused study of hormonal levels and inflammatory markers in the semen and testicular tissues. It has been suggested that COVID-19 can have a potentially negative effect on male fertility through direct damage to the testes. The systematic reviews by Fathi et al. and Khalil et al. summarized available data which show that the COVID-19 infection often leads to a decrease in testosterone levels and may affect semen quality.[7,8] However, the small size and retrospective nature of the previous studies render any conclusive statements impossible for now. Moreover, a large percentage of the available research separately assessed a limited number of parameters in isolation—such as hormone levels, sperm quality, testicular pathology, without adequate controls.

There is ultimately too little information to draw reliable conclusions regarding the effects of the virus on male reproduction. Published reports are limited by small study groups, lack of the control group, and the absence of follow-up examinations during recovery warranting further research and in-depth exploration of the topic.[9]

The current prospective series is aimed at investigating the effect of SARS-CoV-2 on the testicular function and determining the extent of damage to testicular cells.

2 | MATERIALS AND METHODS

After Institutional Review Board approval and registration (clinicaltrials.gov ID NCT04716179), we prospectively recruited male patients aged 18–65 years. The study included both healthy participants with no history of COVID-19 or vaccination (controls) and those during the acute stage of the disease (pneumonia as identified by chest computed tomography (CT) and confirmed with nucleic acid detection of SARS-CoV-2 from throat swab samples using reverse transcription-polymerase chain reaction, RT-PCR). Only patients with recent disease onset (the onset was defined as the date when symptoms appeared) and who had undergone no prior antiviral therapy were included. At the time of inclusion, all patients experienced moderate to severe symptoms in accordance with the World Health Organization (WHO) classification.[12] It is important to emphasize that while all the patients collected material for semen analysis during hospital stay, the analysis was only conducted after improvement in their general condition. The exclusion criteria were inability to collect semen for analysis, history of congenital anomalies, hypogonadism, testicular dysfunction, infertility, and severe varicocele. All the COVID-19 participants were recruited at a single center (COVID-19 Hospital of Sechenov University, Moscow, Russia). The controls were males with no known disorders of fertility in past medical history. It is necessary to emphasize that all the controls volunteered to join the study, with no specific urological or andrological conditions (the controls received assessment and semen analysis results which motivated them to join). They were recruited separately from the COVID-19 cohort (at the Institute for Urology and Reproductive Health, Sechenov University, Moscow, Russia) and represent a similar geographical cohort. None of the controls had received vaccination or had a history of COVID-19. All those had negative RT-PCR test to SARS-CoV-2 prior to inclusion to the control group. The exclusion criteria were identical to those in the COVID-19 cohort.

The trial was powered to detect changes in semen analysis (sperm motility, morphology, and count). The sample size calculation was based on the finding that decreased semen quality could be found in almost 50% of otherwise healthy males.[10] According to the available data,

semen quality deteriorated in 20%–30% of patients with COVID-19.[11] Therefore, we suggested that based on non-inferiority sample size calculation, a total of 70 patients should be included in the study for 80% statistical power with the upper limit of a one-sided 95% confidence interval exceeding a > 30% difference in favor of the standard treatment group. Considering a drop-off rate of 20%, 88 study participants were planned to be included (44 patients per cohort).

Epidemiological, clinical (i.e., disease severity and symptoms, glucocorticoid use), and ultrasound data (color Doppler ultrasound of the scrotum) were collected during admission and in 3 months. All ultrasound tests were performed by a single highly-experienced urologist.

2.1 | Laboratory

All the tests, both during hospital stay and 3 months after (semen analysis, hormone levels, inflammatory markers, etc.), were performed in a single EQUAS-certified laboratory (Sechenov University Central Lab, Moscow). For hormone assessment, an immunoassay test instrument (ADVIA Centaur System, Siemens, Germany) was used with the following reference values: testosterone: 8.4–28.7 nmol/l, follicle-stimulating hormone (FSH) 1.4–18.1 mIU/ml, luteinizing hormone (LH): 1.5–9.3 mIU/ml, and prolactin: 45–375 μIU/ml. Coefficients of variations were 8.9% for testosterone, 7.8% for prolactin, 5.8% for LH, and 4.9% for FSH. Hormone levels were assessed at necessary time intervals. The semen sample was analyzed within an hour after collection.

2.2 | Pathology

To conduct a comprehensive trial providing evidence on both functional and structural changes in the testicles of patients with COVID-19, a separate cohort of patients who died from COVID-19 was created. As it was planned to collect the semen samples from those recovering from moderate-to-severe disease, we expected that none of the participants from the main cohort would be included into the pathology cohort. However, to increase homogeneity of the results the inclusion criteria for the pathology cohort were the same as those for the main cohort (in short, positive RT-PCR, no history of infertility or hypogonadism, etc., age up to 65 years). The additional informed consent for the pathology assessment was obtained from relatives of the deceased. The cohort size (20 patients) was calculated based on the previous studies on this topic to represent a valuable addition to available data.[13,14]

Testicles were collected from deceased patients for an autopsy for histological, immunohistochemistry (IHC) tests, and real-time PCR of SARS-CoV-2 RNA from paraffin sections of testicular tissue. For histological and IHC tests, the collected material was fixed in 10% neutral buffered formalin for 72 h. Fixation of the material was performed according to standard protocols. Then the material was embedded in paraffin and serial sections with a thickness of 4–5 μm were made from the resulting paraffin blocks. For histological examination, sections were stained with hematoxylin and eosin.

IHC was performed on dewaxed sections mounted on poly-L-lysine glasses. Dewaxing, antigen unmasking, and IHC reactions were performed according to standard protocols using the Leica Bond Max automated staining system. Rabbit polyclonal antibodies to the spike protein (SARS-CoV-2 Spike Antibody, GeneTex, dilution 1:500), nucleocapsid protein (SARS-CoV-2 Nucleocapsid Antibody, GeneTex, dilution 1:500) of the SARS CoV-2 virus (COVID-19), and ACE-2 (GeneTex, 1:250 dilution) were used as primary antibodies. A streptavidin complex (LSAB KIT; Dako) was used as secondary antibodies and an imaging system. After IHC reaction, cell nuclei were additionally stained with Mayer's hematoxylin. Negative and positive controls were used on the sections provided by GeneTex.

In addition, the obtained histological sections were used for PCR on paraffin sections to detect SARS-CoV-2 virus RNA directly in testicular tissue. For this, real-time RT-PCR was performed and was used to detect viral RNA in samples. Paraffin-fixed tissues were processed with RNeasy FFPE Kit (Qiagen, Hilden, Germany) to isolate total RNA. Kit N2 was used to detect SARS-CoV-2 by RT-PCR. For this purpose, 20 μl of a reaction mixture containing 2 × Master Mix and RT from the QuantiTect probe RT-PCR kit (Qiagen, Hilden, Germany), a set of primers/N2 probes (NIID_2019-nCoV_N_F2 primer, NIID_2019-nCoV_N_ID_R2 primer, and NIID_2019-nCoV_N_P2 probe) and 5 μl of RNA extracted from tissue samples fixed in paraffin were subjected to PCR in a QuantStudio 5 system (Thermo Scientific, USA). For the reaction, conditions of 50°C for 30 min, 95°C for 15 min, and 45 cycles of 95°C for 15 s, and 60°C for 1 min were applied. Ct values were obtained for all RT-PCR positive samples. The Ct values of the samples were compared with the Ct values of the positive and negative control samples. Microphotography was carried out using Olympus b×46 microscope (Japan) with 600× magnification.

2.3 | Statistical analysis

For statistical analysis, we used SPSS Statistics 23.0 (IBM, USA). The pathology cohort results were assessed using descriptive statistics in Excel 365 (Microsoft, USA). Categorical variables were described in terms of frequency and percentage, and continuous variables were described using mean, standard deviation, median, and interquartile range (IQR) values. When the data were normally distributed, independent *t*-tests or ANOVA were employed to compare the mean of continuous variables. Otherwise, the Mann–Whitney test was used. Categorical variables were compared using Pearson's chi-squared test. Non-categorical variables were compared using Spearman's correlation coefficient. A two-sided *p*-value of 0.05 was considered the threshold for statistical significance.

3 | RESULTS

3.1 | Data on enrollment, general information and medical history of the main group and controls

A total of 250 participants were assessed for eligibility (Figure 1). The main group: after exclusion of ineligible participants, 74 patients joined the main cohort. Thirty patients were unable to provide a

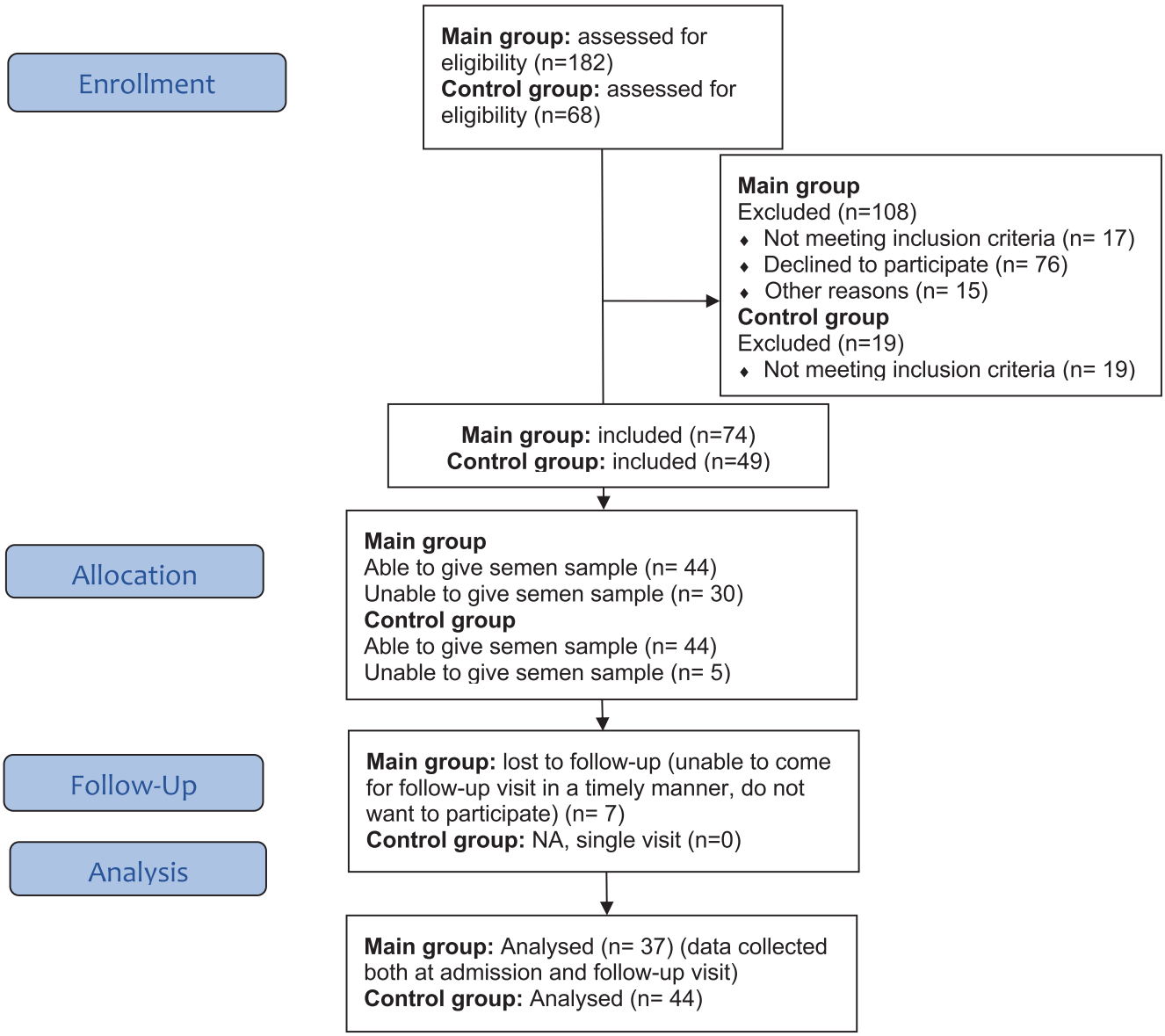

FIGURE 1 Flow diagram of the enrollment of participants to the main (COVID-19 patients) and control (healthy volunteers) groups

semen sample, and 7 patients were lost to follow-up. A total of 37 patients completed all the necessary tests. The controls: sixty-eight patients were initially recruited, and 19 of them were excluded due to findings on initial examination (varicocele, medical history, etc.). Five patients were unable to provide a semen sample. A total of 44 patients in the control group completed all the necessary tests.

The mean age of patients in the main group was 46.7±9.9 years. It was significantly higher than the age of the controls: 30.7±9.8. The majority of the patients (40/44, 91%) experienced moderate COVID-19 according to the WHO classification, and 5/44 (9%) patients experienced severe COVID-19. None of the patients had ongoing lung ventilation; however, most of them had previously been on oxygen support (91%). The mean length of fever episodes was 4.7±1.6 (range, 3–8) days. Prior to inclusion to the study, SpO_2 was assessed. Mean level was 93.8% (range, 86–98%).

Most common comorbidities included chronic obstructive pulmonary disease (3/44, 7%), diabetes mellitus (2/44, 5%), and congestive heart failure (1/44, 2%). Mean IIEF-5 was 20.8±5.2. None of them had a history of hypogonadism or testicular dysfunction, and all patients were sexually active at the time of COVID-19 onset. None of the patients reported prior treatment for erectile dysfunction, infertility, or ejaculatory disorders. All the patients were stable and were receiving standard treatment (dexamethasone, enoxaparin sodium injections). Patients with severe COVID-19 received additional therapy with tocilizumab.

3.2 | Reproductive hormones

Blood tests revealed that the testosterone level was below normal (local reference values, 8.4–28.7 nmol/L) in 27.3% of COVID-19

TABLE 1 Age, erectile function, and hormonal levels of patients with COVID-19 at hospital admission and at 3 months after compared to controls

Parameter	Patients with COVID-19		Controls ($n = 44$)
	At admission ($n = 44$)	At 3 months ($n = 37$)	
Age, years	46.7±9.9 (26–62)[b]		30.7±9.8 (19–66)[b]
IIEF-5, score (range)	20.7±5.3 (12–25)	23.1±4.3 (11–25)	20.8±5.2 (13–25)
p-Value	0.73		
Testosterone, nmol/L (range)	**7.3±2.7 ***(3.8–13.3)**[b]	**13.7±4.5 ***(4.5–22.3)**	13.5±5.2 ***(5.2–29.6)[b]
p-Value	**0.004**		
Prolactin, μIU/ml, median (IQR)	267.5 (214–348)	144.5 (106.8–182.8)	138.0 (106–166)
p-Value	0.90		
Luteinizing hormone, mIU/L, median (IQR)	**3.2 (2.6–4.2)**[b]	**2.7 (2.0–4.5)**	3.0 (2–3.6)[b]
p-Value	**>0.001**		
Follicle-stimulating hormone, mIU/L, median (IQR)	5 (3.3–7.1)[b]	4.65 (3.2–7.7)[a]	3.1 (2.2–4.6)[a,b]
p-Value	0.33		

Notes: Presented as mean ± SD, median (IQR), or *N* (%) where appropriate.
In-bold: statistically significant difference (at admission vs. 3 months) ($p < 0.05$).
[a]Statistically significant difference (3 months vs. controls) ($p < 0.05$).
[b]Statistically significant difference (at admission vs. controls) ($p < 0.05$).

patients (12/44). The mean level was 7.3±2.7 nmol/L which was significantly lower than that in the healthy volunteers (13.5±5.2 nmol/L, $p < 0.001$). At 3 months after discharge, the level returned to normal (13.7±4.5 nmol/L) and did not differ from that of healthy volunteers. A small increase in LH was defined as—3.5±1.85 mIU/L versus 2.8±1.1 mIU/L in healthy participants ($p = 0.04$) and in FSH—5.6±3.32 mIU/L versus 3.6±1.9 mIU/L ($p = 0.002$) (both within normal reference values). Both parameters returned to normal at 3 months after discharge and do not differ from those in healthy controls (Table 1). No statistically significant changes in prolactin were found.

Additional Spearman correlation analysis was carried out to assess testosterone change (from admission to 3 months after) depending on patients' condition. No significant correlations were found with patient age ($r = -0.08$, $p = 0.6$), concomitant diseases (congestive heart failure (CHF), chronic obstructive pulmonary disease (COPD), diabetes mellitus, $r = 0.1$, $p = 0.2$), disease severity ($r = -0.19$, $p = 0.3$), or duration of fever ($r = -0.05$, $p = 0.7$).

3.3 | Semen parameters

The semen analysis revealed increased sperm concentration ($p = 0.003$) with similar total sperm count ($p = 0.05$) and semen volume ($p = 0.5$). We still found decreased motility in patients with COVID-19 ($p = 0.001$), and higher number of immobile sperm (during COVID-19: 58.8% and at 3 months 47.4%, $p = 0.005$), as well as lower percent of vital sperm (47% vs. 72%, $p = 0.001$), compared to healthy volunteers. Also, a lower percent of normal morphology ($p = 0.01$) was found in those with COVID-19. All these parameters returned to normal at 3 months after discharge, no statistically significant differences were observed compared to healthy controls. No increase in leucocyte count in sperm was seen despite the frequency of erythrocytes in sperm being higher ($p = 0.04$) than after discharge and in controls (found in 18% vs. 4.5% vs. 4.5% of patients) (Table 2). Direct mixed agglutination reaction (MAR) tests for IgA and IgG showed no changes during hospital admission (4.6%±6.2% and 2.7%±3.8% vs. 5.2%±9.3% and 4.3%±9.9% in healthy volunteers, $p = 0.7$ and $p = 0.3$). However, both tests showed an increase at 3 months, compared to the results during hospital admission ($p = 0.01$ and $p = 0.03$), direct MAR tests for IgA at 3 months were also increased compared to controls ($p = 0.03$). It should be noted, that despite statistically significant difference, test results were above local reference values only in one patient (2%) at 3 months.

3.4 | Ultrasound and color Doppler imaging of the scrotum

All the patients underwent ultrasound and color Doppler of the scrotum while in the hospital (to exclude from analysis those with significant varicocele) and at 3 months after discharge. No specific changes were observed during the hospital stay or at 3 months after discharge. No changes in blood flow on color Doppler were found (Table 3). Among other findings spermatocele were encountered in 6/44 (14%) of patients at admission and in 3/37 (8%) at 3 months, testis hypotrophy was encountered in 4/44 patients both at admission and after discharge. No signs of orchitis were seen in patients with COVID-19.

TABLE 2 Semen analysis results of patients with COVID-19 at hospital admission and at 3 months after discharge compared to controls

	Patients with COVID-19		
Parameter	**At admission ($n = 44$)**	**At 3 months ($n = 37$)**	**Controls ($n = 44$)**
Semen volume, ml	2.6 ± 1.71 (0.5–9)	2.2± 1.3 (0.5–6)	2.8 ± 1.5 (0.8–9)
p-Value	0.13		
pH, score	7.8±0.5 (7–8.5)	7.9±0.5 (7.2–9)	7.7±0.4 (7.2–8.5)
p-Value	0.42		
Agglutination, *N* (%)[c]	17 (20.5)	11 (22.7)	9 (22.7)
p-Value	0.13		
Total sperm count, 10*6/ ejaculate	**349.2±363.7 (4–1475)**	**222.4±222.4 (6–1050)**	223.5±205.1 (10.8–840)
p-Value	**0.04**		
Sperm concentration, 10*6ml	128.9±98.15 (20–325) [b]	107.9±98.1 (2–424)	76.3±53.1 (3–210)[b]
p-Value	0.28		
Vitality (%)	47.0+24.5 (0–87)[b]	62.8±20.9 (27–89)	72.5±15.2 (44–91)[b]
p-Value	0.63		
Total motility, %	**38.8±23.8 (0–84)** [b]	**52.6±14.5 (22–77)**	53.6±13.9 (12–78) [b]
p-Value	**0.006**		
Progressive motility, %	**32.8±21.7 (0–82)** [b]	**44.3±14.7 (17–69)**	44.9±14.3 (11–69) [b]
p-Value	**0.04**		
Rapid progressive motility, %	18.4±16.3 (0–65)[b]	25.9±14.7 (0–55)	26.9±11.7 (4–56)[b]
p-Value	0.91		
Slow progressive motility, %	14.5±9.8 (0–41)[b]	18.3±8.3 (10–47)	18.8±9.5 (4–39)[b]
p-Value	0.06		
Non-progressive motility, %	5.9±4.9 (0–17)	8.3±6.7 (1–24)	8.9±9.2 (1–36)
p-Value	0.05		
No motility, %	**58.8±24.8 (0–100)**[b]	**47.4±14.6 (23–78)**	45.1±14.5 (11–88)[b]
p-Value	**0.005**		
Normal morphology, %	10.9±7.8 (0–39)[b]	12.8±8.29 (1–36)	17.3±14.7 (0–87)[b]
p-Value	0.52		
Direct MAR-test, IgA, %	**4.6±6.2 (0–21)**	**12.1±13.6 (0–51)**[a]	5.2±9.3 (0–44)[a]
p-Value	**0.01**		
Direct MAR-test, IgG, %	**2.7±3.8 (0–15)**	**6.3±7.6 (0–30)**	4.3±9.9 (0–58)
p-Value	**0.03**		
Leucocytes, 10*6/ml	0.3±0.2	0.4±0.5	0.3±0.4
p-Value	0.39		
Erythrocytes, *N* (%)	**8 (18.2)**	**2 (4.5)**	2 (4.5)
p-Value	**0.04**		

Notes: Presented as mean ± SD or *N* (%) where appropriate.
In-bold: statistically significant difference (at admission vs. 3 months) ($p < 0.05$).
[a]Statistically significant difference (3 months vs. controls) ($p < 0.05$).
[b]Statistically significant difference (at admission vs. controls) ($p < 0.05$).
[c]Only scant agglutination observed.

3.5 | Pathology findings

Mean age in the autopsy cohort was 50.5±10.6 (35–65). All of the patients were admitted to hospital due to significant hypoxemia, and all received oxygen support. All of them experienced severe fever. Despite treatment (previously mentioned + tocilizumab), all the patients were transferred to emergency room (ER) for invasive lung ventilation within a dozen days (range, 5–12) from admission. None of them had any record of hypogonadism or infertility in the medical history.

TABLE 3 Ultrasound, color Doppler imaging of the scrotum, and additional findings in patients with COVID-19 at hospital admission and at 3 months after discharge compared to controls

		Patients with COVID-19		
Parameter		**At admission ($n = 44$)**	**At 3 months ($n = 37$)**	**Controls ($n = 44$)**
Testis volume, ml	Right	12.5 ± 4.2	16.2± 5.9	16.8 ± 5.3
	p-Value	0.32		
	Left	14.4 ± 4.8	15.2± 4.7	15.6 ± 4.4
	p-Value	0.58		
Vein diameter in Valsalva maneuver, mm	Right	1.7±0.6	1.7±0.9	2.0±0.4
	p-Value	0.59		
	Left	2.3±1.2	1.8±0.9[a]	2.1±0.9[a]
	p-Value	0.09		
Retrograde flow, *N* (%)	right	6 (13.6)	7 (15.9)	8 (18.2)
	p-Value	0.36		
	Left	5 (11.3)	5 (11.3)	7 (15.9)
	p-Value	0.47		
Other ultrasound findings				
Hydrocele, *N* (%)		None	None	1
p-Value		–		
Spermatocele, *N* (%)		6 (14%)	3 (8%)	8 (22%)
p-Value		0.24		
Testis hypotrophy, *N* (%)		4 (9%)	4 (9%)	1 (3%)
p-Value		0.643		
Orchitis, *N* (%)		None	None	None
p-Value		–		

Notes: Presented as mean ± SD or *N* (%) where appropriate.
In-bold: statistically significant difference (at admission vs. 3 months) ($p < 0.05$).
[a]Statistically significant difference (3 months vs. controls) ($p < 0.05$).
[b]Statistically significant difference (at admission vs. controls) ($p < 0.05$).

The pathology assessment of patients who died from COVID-19 revealed impaired histologic structure in 18 out of 20 patients. The RT-PCR test for SARS-CoV-2 was positive in all (20) patients. At the same time, a decrease in the number of male germ cells, frequent detachments from the basal membrane that fill the lumen (conglomeration) were found in the convoluted seminiferous tubules; sperms were partially absent in the lumen of some seminiferous tubules. Sertoli cells persisted throughout the basal membrane, which showed signs of thickening and loosening (Figure 2).

Hypospermatogenesis was diagnosed (5 ± 1 points) by means of testicular biopsy score count (Johnsen score). In the majority of sections (18/20), thickening of the interstitial component and pronounced edema were noted. In all samples, inflammatory infiltration in intertubular spaces was found: individual plasma cells, mast cells and lymphocytes, as well as single neutrophils. In addition, there were signs of thrombophilic manifestations—intravascular thrombosis and endotheliitis (Figure 2). The lumens of the blood vessels and capillaries were full-blooded with signs of thrombus formation in some places; the inner membrane also showed marked metachromasia. Immunohistochemistry revealed ATC2 in cell membranes (Figure 3), nucleocapsid protein (Figure 4), and spike protein (Figure 5) in the nuclei and cytoplasm of spermatogenic epithelial cells as well as in the Leydig cells and endotheliocytes of the peritubular blood vessels.

4 | DISCUSSION

The current study showed that male patients with moderate to severe COVID-19 experience decrease in the blood testosterone level, with small increase in LH and FSH. A quarter of the patients became hypogonadal during the COVID-19 infection, but all recovered at 3 months. No association with treatment, age, or disease severity was noted. A slight decline in sperm quality was also noticed: a lower percent of normal morphology and lower motility in all patients. These changes are also only transient, and all patients had their values return to baseline at 3 months after discharge.

Compared to healthy volunteers only three parameters differed at 3 months after hospital discharge. We expect that the difference in the

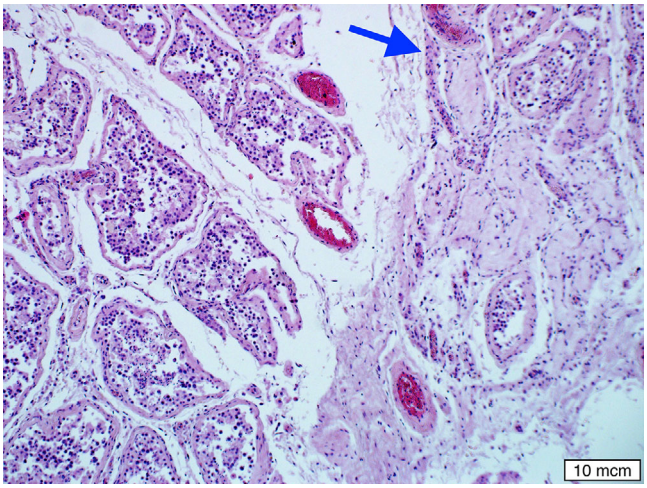

FIGURE 2 Focal desquamation of germ cells in the tubules with preserved spermatogenesis, stromal edema, sclerosis and lymph and macrophage infiltration, thrombosis of small arteries and individual Leydig cells, H&E staining

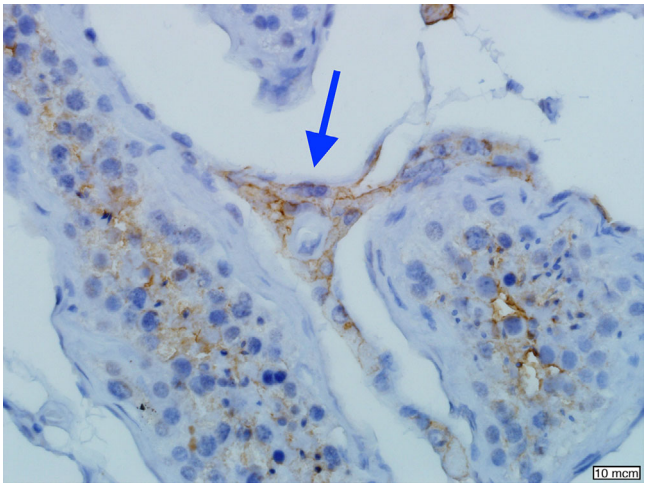

FIGURE 3 Expression of angiotensin convertase in cytoplasmic membranes of germ cells of the tubules, Leydig cells (arrow), immunoperoxidase reaction

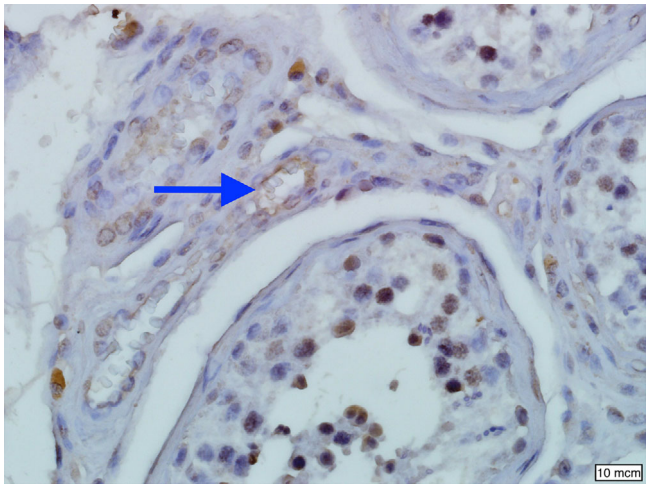

FIGURE 4 Nucleocapsid protein of the virus in the nuclei and cytoplasm of germ cells of tubules, individual Leydig cells, and vascular endothelium (arrow)

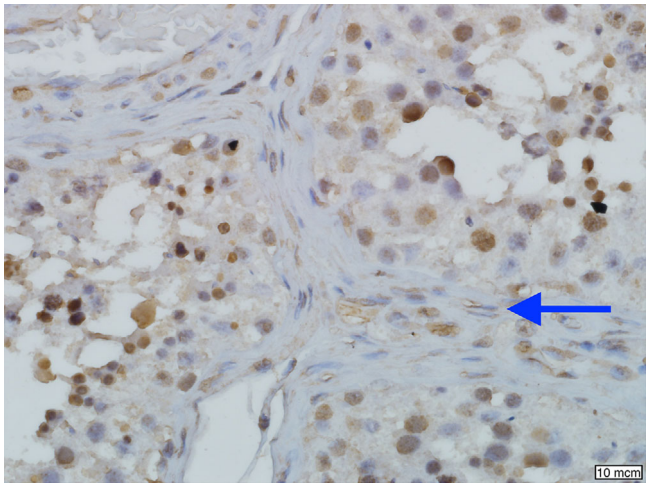

FIGURE 5 SARS-CoV-2 spike protein in the nuclei and cytoplasm of germ cells of tubules, individual Leydig cells (arrow) and vascular endothelium; immunoperoxidase reaction

vein diameter is due to the small group size and significant operator dependency of Doppler ultrasound. The FSH level increase was significant yet was within local reference values in all the patients. A specific interest is an increase in direct MAR test for IgA, being statistically significant in most patients it was within reference values, with an increase above them in a single patient. No other influencing factors for this increase were detected. Such finding supports the presence of inflammatory response in testes of those with moderate-to-severe COVID-19.[11]

As for pathology findings, the examination of the testicles of patients who died from a severe form of COVID-19 showed that the majority of them had structural disorders of the testicular tissue, with signs of damage to germ cells, Leydig cells, and microvascular endothelium, in combination with microthrombi. The presence of stromal edema in association with lymphatic cell elements, as well as the detection of SARS-CoV-2 viral proteins in the nuclei and cytoplasm of spermatogenic epithelial cells, Leydig cells and blood vessels endotheliocytes might confirm the viral nature of the lesion. Clinical data confirm the presence of the described changes which seems to be reversible in cases of both moderate and moderate-to-severe disease.

Previous studies on the topic revealed similar results. Postmortem needle and open biopsies of the testicles performed within an hour after death from COVID-19 revealed that testicular tissues were free of SARS-CoV-2 in 10 of 11 cases (91%).[13] At the same time, sperm tests in COVID-19 patients showed low ejaculate volume, sperm motility, and sperm count.[11] Previously, it has been shown that the COVID-19 effect on vasculature (i.e., microthrombi) is a possible contributor to the COVID-19-related impaired fertility.[14,15] Similar findings were observed in our study when the pathology assessment of

those who died from COVID-19 revealed prominent microthrombosis and inflammatory reaction. However, examination of the testes with Doppler ultrasound revealed no abnormalities, which could be due to its inability to detect minor blood flow changes. However, all changes were transient with both hormone levels and semen analysis returning to normal levels at 3 months after discharge from the hospital.

Holtmann et al. found that SARS-CoV-2 RNA could not be detected in the semen of recovered and acute COVID-19–positive men.[11] Our pathology assessment revealed viral proteins in the testes of patient who died from COVID-19. Peirouvi et al. suggested a possible explanation for this and how the virus may affect spermatogenesis.[16] Other potential explanations include long-term fever, overproduction of reactive oxygen species, and leukocyte infiltration in the testes.[7,17,18] However, Li et al. showed that sperm concentration in patients with COVID-19 was decreased in 39.1% of patients,[19] and leukocytosis was found in almost 60% of patients. Holtmann et al. confirmed the findings and suggested that moderate COVID-19 infection may impair semen quality.[11] However, no significant decrease in their cohort was noted. Our findings demonstrate that the semen quality of those with COVID-19 differs from healthy volunteers and from those suffering from COVID-19 at 3 months. Whereas the greater sperm count could be due to the absence of sexual intercourse during COVID-19 infection, the lower motility, vitality, and inferior morphology are likely due to active generalized inflammation during the disease onset. However, we would like to see further confirmation of this in other cohorts.

Hallak et al. in their systematic review found that most of the COVID-19 studies on fertility made a reference to the drop in semen quality, but it is unknown how long this dysfunction lasted for.[20] Another point highlighted in the article is transmission of SARS-CoV-2 through semen. Our study did not aim at detecting viral presence in semen. However, it should be noted that we were able to identify the viral presence with RT-PCR in all the pathology samples obtained. Despite the previous results suggesting prominent changes even after discharge, which were similar to our findings (percentage of progressive motility, and normal sperm morphology after COVID-19 and decline in the testosterone level 350.1 ± 115.5 vs. 289.8 ± 103.3 ng/dl, $p = 0.009$),[21] we found that the adverse effects of COVID-19 on the sperm quality last no longer than 3 months. However, the above-mentioned effects are still a matter for discussion but the effect of corticosteroids on male hypogonadism has been reported previously.[22]

The general decrease in the semen analysis results observed in our trial is similar to those of previous epidemiological studies.[23] Sperm quality is decreasing among certain (ethnic) groups around the world. This trend appears to be particularly prevalent in big cities.[23] When we designed this study, we expected that the use of reference values alone would not allow us to conclude whether the abnormal results are due to COVID-19, or a decrease found in the population of a specific region. Therefore, we expected the reference cohort to shed light on the real-life sperm quality in the local population. Moreover, we observed that the majority of the hormone and semen analysis results did not differ at 3-month follow-up in the COVID-19 cohort from those of healthy participants. However, these results should be used carefully especially when applying them to other parts of the world.

Limitations. Among the study limitations is the small number of participants and the absence of data on those in whom both the sperm test and pathology results are available. Yet we believe that these findings will shed a little light on the fertility of patients who survived COVID-19. Our study in turn provides substantial data on those who recovered from COVID-19 and despite a relatively small number of participants was powered to detect possible changes in sperm tests.

5 | CONCLUSIONS

COVID-19 and its management significantly affect male hormone levels and sperm quality during the disease onset. Postmortem examination of testicular tissue confirmed inflammation and viral infiltration of the testicles. However, in patients with moderate to severe disease, the negative effect of SARS-CoV-2 on the testicular function vanishes within 3 months.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR CONTRIBUTIONS

Dmitry Enikeev, Mark Taratkin, Andrey Morozov,: conception. Mark Taratkin, Andrey Morozov, Vladislav Petov, Leonid Spivak,, Evgenia Kogan, Nikolay Zharkov, Gregory Demyashkin: interpretation. Marina Geladze, Aichurok Mambetova, Dmitry Fiev, Timur Ganzha, Svetlana Kharlamova, Irina Shchedrina, Oleg Mestnikov, Dmitry Korolev : collection of the data.Anastasia Shpikina, Mark Taratkin, Andrey Morozov: writing. Mark Taratkin, Vladislav Petov: statistical analysis. Shahrokh F. Shariat, Evgenia Kogan: editing. Petr Glybochko - management, funding.

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