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THE ROLE OF TRANSMISSION MICROSCOPY IN ASSESSING MORPHOMETRIC CHANGES IN SPERMATOZOA OF FERTILE AND INFERTILE MEN WHO HAVE HAD COVID-19

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Introduction. As COVID-19-induced disorders wreak havoc on populations worldwide, attention to the rehabilitation of the consequences has focused mainly on vital organ systems (lungs, kidneys, and heart) that are vulnerable to the initial viral attack and contribute to the development of acute pathology associated with this disease. However, we should not lose sight of the fact that COVID-19 affects any cells in the body that express ACE2, including human sperm.
Materials and methods. The results of transmission electron microscopy of the ejaculate of 157 men were analyzed. They were divided into four groups: Group I – 49 patients with idiopathic infertility who had COVID-19, Group II – 42 patients with idiopathic infertility who did not have COVID-19 during the study, Group III – 39 fertile men who had COVID-19, Group IV – 27 fertile men who did not have COVID-19. Objective: To study the role of sperm mitochondrial dysfunction in the development of male infertility associated with COVID-19.
Results. According to transmission microscopy of the ejaculate, men who brought COVID-19 have severe sperm disorders, such as mitochondrial dysfunction and the presence of residual cytoplasm, which were more often recorded in infertile patients, compared to fertile men.
Conclusions. COVID-19 impairs male reproductive potential and may impair male fertility.

K e y w o r d s: *ejaculate, spermatozoa, mitochondrial dysfunction, residual cytoplasm, coronavirus infection; Covid-19; SARS-CoV-2*

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Introduction. Due to the global spread of COVID-19 (Coronavirus Disease 2019) and its detrimental impact on human health, rehabilitation efforts have understandably shifted toward vital organs, such as the lungs, kidneys, and heart. These organs are particularly vulnerable to direct viral involvement and the development of acute complications. However, it is important to recognize that SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2) can infect any cells expressing the angiotensin-converting enzyme receptor (ACE2), including human spermatozoa [1–3].

Examination of testicular samples obtained during autopsy of patients who died from COVID-19 demonstrated that SARS-CoV-2 may induce an infectious-inflammatory process in the testes. Morphological analysis revealed apoptosis of spermatogenic cells, destruction of germ cells, markedly reduced or absent spermatozoa, thickening of the basement membrane, and leukocytic infiltration in all six analyzed samples [4].

Furthermore, patients who have recovered from COVID-19 show decreased serum total testosterone levels. A significant reduction in the testosterone-to-luteinizing hormone ratio (T/LH) has also been reported compared with age-matched healthy men without a history of COVID-19. This T/LH ratio, a marker of testicular endocrine function, may serve as a potential indicator of SARS-CoV-2–induced impairment of male reproductive health [4, 5]. Elucidating the pathogenic mechanisms through which COVID-19 affects semen quality and testicular function, potentially contributing to male infertility remains an important research task.

Molecular studies of coronavirus infection have shown that SARS-CoV-2 enters host cells via binding of its spike proteins to cellular receptors and by spike protein priming through host cell proteases. SARS-CoV-2 attaches to the ACE2 to gain entry into the cell and utilizes the transmembrane serine protease 2 (TMPRSS2) for spike protein priming [6]. ACE2, a negative regulator of

the renin–angiotensin system, is highly expressed in epithelial cells of renal tubules, the epididymal duct, Leydig cells, adrenal glands, and the prostate. TMPRSS2 is abundantly expressed in the kidneys, prostate, seminal vesicles, and epididymis. High expression of both key determinants of SARS-CoV-2 pathogenicity in organs of the genitourinary system suggests that these structures may be susceptible to viral damage [4, 6].

The inflammatory process observed in the testes appears to be autoimmune and abacterial in nature, resulting in structural alterations not only in the testicular tissue but also in spermatozoa [7]. Normal sperm function, including hyperactivation and the acrosome reaction, requires physiologic levels of reactive oxygen species (ROS), primarily generated by mitochondria. Mitochondrial dysfunction in sperm leads to energy deficiency and excessive ROS production, causing oxidative stress [8].

Mitochondrial dysfunction is a pathological condition caused by genetic, structural, biochemical, or infectious factors that lead to defects in mitochondrial function, resulting in impaired synthesis of adenosine triphosphate (ATP) and excessive production of reactive oxygen species (ROS) [9, 10].

Recent studies highlight the importance of mitochondrial dysfunction in spermatozoa as a contributing factor to male infertility. Mitochondria play a key role in ensuring sperm functional activity. Their primary function is to provide energy for essential processes such as capacitation, hyperactivation, the acrosome reaction, and penetration of the oocyte, which ultimately determine the fertilizing capacity of male gametes. Under certain pathological conditions, significant alterations occur in the shape and structural organization of mitochondria during spermatogenesis.

Spermatogonia and early spermatocytes contain spherical mitochondria; these organelles subsequently elongate and fragment in haploid round spermatids. During spermiogenesis, most cytoplasm containing mitochondria is eliminated, while the remaining 80–100 mitochondria become tightly arranged in a helical pattern around the midpiece of the developing sperm flagellum. Mitochondria occupy a substantial portion of the total cellular volume (approximately 15–22%) [11, 12].

Our previous studies demonstrated that SARS-CoV-2 negatively affects spermatogenesis, and mitochondrial dysfunction represents one of the mechanisms underlying pathospermia in affected patients.

Aim. To evaluate the morphometric changes in spermatozoa of men who have recovered from COVID-19.

Materials and methods. Mitochondrial dysfunction was assessed using transmission electron microscopy (TEM) of ejaculates from 157 men divided into four groups: Group I (n=49) – idiopathic infertility, recovered from COVID-19; Group II (n=42) – idiopathic infertility, no history of COVID-19; Group III (n=39) – fertile men, recovered from COVID-19; Group IV (n=27) – fertile men, no history of COVID-19. Ejaculate samples were analyzed within a study of reproductive potential in fertile and infertile men, and again 12 months after SARS-CoV-2 infection (n=88), along with a control group (69 patients without COVID-19), to evaluate the negative effects of SARS-CoV-2 on semen parameters.

Semen samples were obtained by masturbation after a recommended abstinence period of 3–4 days and collected in sterile containers. After liquefaction at room temperature, sperm concentration, total and progressive motility, and normal morphology were assessed according to the World Health Organization (WHO) 2010 guidelines. Following routine semen analysis, samples were sent to the laboratory. For electron microscopy, fresh ejaculate samples were fixed in 2.5% glutaraldehyde (AppliChem, Germany) for 24 hours. After fixation, samples were washed three times (15 min each) in phosphate buffer. After buffer processing, specimens were placed in 1% osmium tetroxide (OsO_4) (Acros Organics, Belgium) in phosphate buffer (pH 7.2–7.4) for 1.5 hours, then dehydrated in ascending ethanol concentrations and acetone, embedded in Epon-812 epoxy resin by a flat-embedding method, and polymerized at 60°C.

Epoxy blocks were trimmed using a Leica EM TRIM high-speed milling cutter to obtain semithin sections stained with toluidine blue for locating spermatogonia and spermatozoa. Blocks containing the required structures were sectioned with a diamond knife on an Ultracut-UC6 ultramicrotome (Leica, Germany). Section thickness was 70 nm.

For contrast enhancement, sections were stained with 1.5% uranyl acetate in 70% ethanol and lead citrate. After staining, sections were examined using a Jem 1011 transmission electron microscope (Jeol, Japan) equipped with a digital camera Erlangshen ES500W (Gatan, USA/Canada). Ultrastructural measurements were performed in absolute units (μm and nm). When necessary, morphometric analysis of ultrastructures was carried out using Digital Micrograph software (Gatan, USA).

The study was performed as part of the dissertation project "Assessment of the impact of the novel coronavirus SARS-CoV-2 infection on the reproductive potential of infertile men", approved by the Local Ethics Committee of Rostov State Medical University, Ministry of Health of the Russian Federation.

Results. According to transmission electron microscopy of ejaculates performed 12 months after SARS-CoV-2 infection, pathological changes in spermatozoa characterized by the presence of residual cytoplasm were identified in 15 patients (30.6%) from Group I and 5 patients (12.8%) from Group III. Residual cytoplasm (excessive residual cytoplasm) is a defect marked by an excessive number of cytoplasmic residues located in the neck region of the spermatozoon. No such abnormalities were detected in Groups II or IV. At baseline, residual cytoplasm was not observed in any patient from any group.

In addition, 12 months after SARS-CoV-2 infection, mitochondrial dysfunction in spermatozoa was detected in patients from Groups I and III, whereas initially, this abnormality had not been observed in any participant ($p < 0.005$) (*Table*).

Among infertile patients who recovered from SARS-CoV-2, mitochondrial dysfunction occurred significantly more frequently than among fertile men. Pathological mitochondrial alterations were characterized by dystrophic structural changes, including heterogeneity and fine granularity of the mitochondrial matrix, as well as mitochondrial swelling with matrix rarefaction (*Fig. 1*).

Moreover, in most cases, mitochondrial swelling was accompanied by fragmentation and destruction of cristae, often combined with signs of vacuolization (*Fig. 2*). In all patients with mitochondrial dysfunction, cytoplasmic

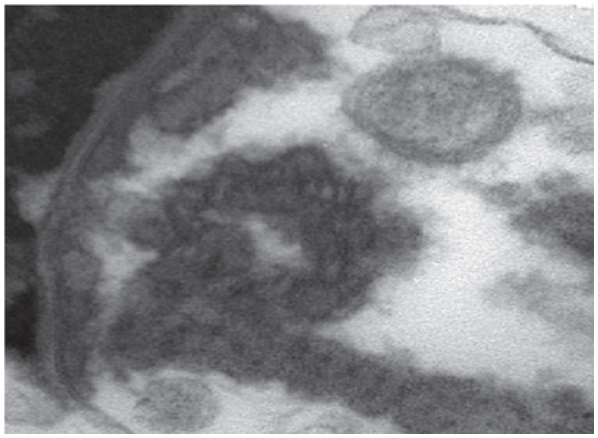


Figure 1. Transmission electron microscopy of ejaculate from a patient in Group I. Abnormally shaped sperm head, residual cytoplasm. Swollen midpiece mitochondria lacking cristae. Electron micrograph, ×30,000.

vacuolization was also observed, varying in size and localization. Under high-resolution interference-contrast microscopy, these vacuoles appeared as superficial pocket-like indentations.

Notably, all patients with mitochondrial insufficiency also exhibited additional severe sperm abnormalities, including abnormal sperm head morphology, deformation of the subacrosomal plate, expansion of the subacrosomal space, and the presence of immature chromatin (*Fig. 3*). Microscopic semen analysis revealed that all patients with mitochondrial dysfunction exhibited oligoasthenoteratozoospermia (OAT syndrome).

Discussion. Currently, there is no conclusive evidence to assert that COVID-19 has a long-term negative effect on key semen parameters such as sperm count, motility, morphology, or sperm DNA integrity [13]. However, in our study we found that COVID-19 may exert sustained detrimental effects on major aspects of male fertility, particularly sperm motility and morphology. Due to the potential consequences of upregulated angiotensin II signaling at various stages of the sperm pathway, the pathogenic impact of SARS-CoV-2 may stimulate the intrinsic apoptotic pathway, leading to sperm ageing and cellular death. Furthermore, prolonged viral influence may result in acrosomal abnormalities, subsequently reducing sperm motility.

Delaying the management of infertility associated with SARS-CoV-2 infection may ultimately result in an inability to conceive, which is an especially relevant concern in infertile men, who demonstrated more severe sperm morphological abnormalities compared with fertile men. In our previous studies, in men who had recovered

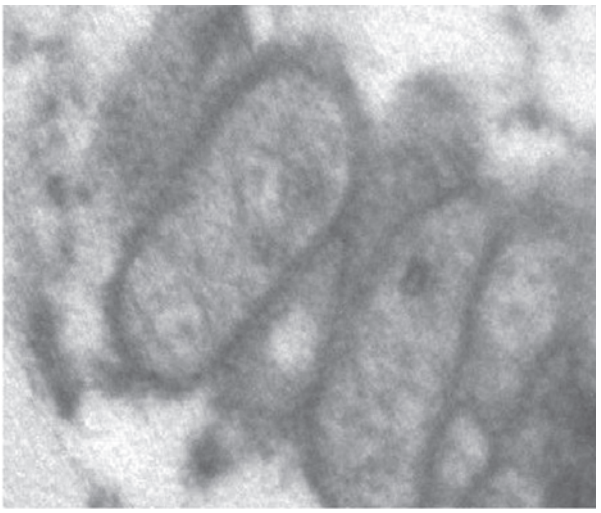


Figure 2. Transmission electron microscopy of ejaculate from a patient in Group I. Fragmentation-related destruction of spermatozoa. Pathological features of mitochondria. Heterogeneous and finely granular mitochondrial matrix with cristae disruption and signs of vacuolization. Electron micrograph, ×50,000.

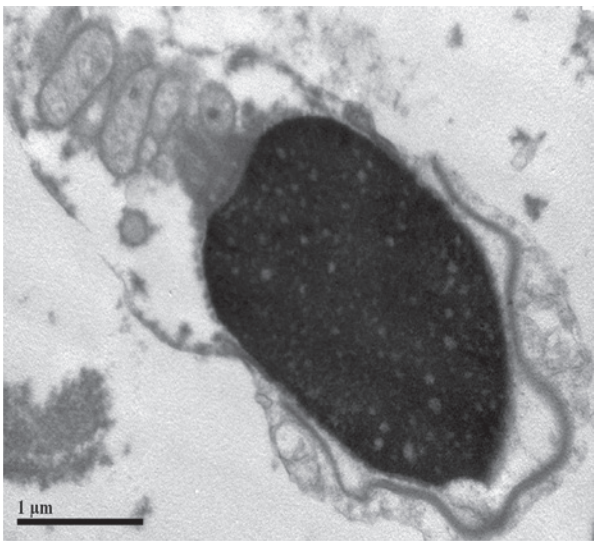


Figure 3. Transmission electron microscopy of ejaculate from a patient in Group I. Severe sperm abnormalities. Abnormally shaped sperm head, mature chromatin, acrosome with expanded subacrosomal space. Swollen midpiece mitochondria lacking cristae. Electron micrograph, ×30,000.

Table

Mitochondrial abnormalities in spermatozoa one year after SARS-CoV-2 infection

Group	Abs.	%
I	16	32.6%
II	–	–
III	6	15.3%
IV	–	–

from SARS-CoV-2 infection, we observed marked sperm abnormalities, including abnormal sperm head morphology, deformation of the subacrosomal plate, expansion of the subacrosomal space, and the presence of immature chromatin, which were significantly more prevalent in infertile patients than in fertile men [14].

Further analysis of newly obtained data revealed not only acrosomal and axonemal abnormalities but also profound cytoplasmic defects, most notably mitochondrial dysfunction and residual cytoplasm. Both abnormalities occurred more frequently in infertile patients compared with fertile men following COVID-19 infection.

Naturally, we acknowledge the need for further research to evaluate fertility-related complications after COVID-19 and to optimize assisted reproductive technology strategies for patients recovering from this novel infection.

Conclusions. COVID-19 impairs male reproductive potential and may negatively affect male fertility. Mitochondrial dysfunction represents one of the most significant morphological abnormalities in men who have recovered from COVID-19, occurring predominantly in infertile patients. Clinicians involved in male reproductive health should perform andrological evaluation in both "currently infected" and "recovered" male patients following COVID-19.

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