



Histopathology and Ultrastructural Findings of Fatal COVID-19 Infections on Testis

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Purpose: To evaluate the presence and analyze the pathological changes within the testes of patients who died or recovered from severe acute respiratory syndrome coronavirus 2 (COVID-19) complications.

Materials and Methods: Testis tissue was collected from autopsies of COVID-19 positive (n=6) and negative men (n=3). Formalin-fixed paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) and subjected to immunofluorescence for angiotensin-converting enzyme 2 (ACE-2) expression. Fluorescent-labeled tissue slides were imaged on a quantitative pathology scope with various zoom levels allowing for qualitative and quantitative interpretation. Tissue from four COVID-19 positive autopsy cases and a live seroconverted patient was imaged with transmission electron microscopy (TEM).

Results: H&E histomorphology showed three of the six COVID-19 biopsies had normal spermatogenesis while the remaining three had impaired spermatogenesis. TEM showed the COVID-19 virus in testis tissue of one COVID-19 positive autopsy case and the live biopsy, H&E stain on the same autopsy case demonstrated interstitial macrophage and leukocyte infiltration. Immunofluorescent stained slides from six COVID-19 positive men demonstrated a direct association between increased quantitative ACE-2 levels and impairment of spermatogenesis.

Conclusions: The novel COVID-19 has an affinity for ACE-2 receptors. Since ACE-2 receptor expression is high in the testes, we hypothesized that COVID-19 is prevalent in testes tissue of infected patients. This study suggests the male reproductive tract, specifically the testes, may be targets of COVID-19 infection. We found an inverse association between ACE-2 receptor levels and spermatogenesis, suggesting a possible mechanism of how COVID-19 can cause infertility.

Keywords: Autopsy; Coronavirus; COVID-19; Infertility; Testis

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INTRODUCTION

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, COVID-19) first appeared in Wuhan, China in December 2019, and has since become a global pandemic [1]. This novel coronavirus has re-

sulted in over 6 million confirmed cases and more than 370,000 deaths within the first 6 months of spread, confirmed cases as of this writing (August 23, 2020) has reached 23,057,288 globally (<https://covid19.who.int/>). COVID-19 initially appeared exclusively as a respiratory tract infection. As our understanding of the

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virus grew, it became apparent that the virus additionally affects other organs of the human body, such as the liver, kidneys, and gastrointestinal tract. There is a male preponderance for the virus and early studies showed worse disease severity and duration in men compared to women. This preponderance has resulted in an increased incidence of the disease and morbidity rate in men that is double that of women [2]. The 2005 SARS-CoV virus, a respiratory virus part of the same family as the SARS-CoV-2 virus, was also investigated regarding its effects on testes tissue. Xu et al [3] found that all six patients who died of SARS-CoV displayed widespread germ cell destruction with few to no spermatozoon, thickened seminiferous tubule basement membranes, as well as lymphocyte and macrophage infiltration. They suggested orchitis is a complication of SARS-CoV. Previous studies have found that deceased COVID-19 men displayed testicular tissue injury in association with inflammatory infiltrates on light microscopy. COVID-19 was detected *via* reverse transcriptase polymerase chain reaction (RT-PCR); however, transmission electron microscopy (TEM) failed to identify the virus [4]. Other groups have detected COVID-19 in autopsy testes tissue *via* RT-PCR, TEM, and immunohistochemistry [5]. Studies have managed to find COVID-19 in the ejaculate of acutely infected men *via* RT-PCR while others have failed to detect the virus *via* similar methods [6,7]. Although much has been discovered about the novel coronavirus, there remains mixed findings regarding the pathogenesis of the COVID-19 virus in the male reproductive system that need to be researched.

Pathological studies have shown that the primary target organ of COVID-19 is the lungs. It is believed that this is due to an increased expression of angiotensin-converting enzyme 2 (ACE-2) receptors in lung tissue, of which COVID-19 has a high affinity of binding and subsequent entry [8-10]. Studies have shown the potential risk of COVID-19 impacting and damaging other organs that express ACE-2 receptors, including the heart, kidneys, bladder, oral cavity, esophagus, and ileum [9,11,12]. Interestingly, the ACE-2 receptor is widely expressed in the testes [13]. It has been found that in prior to viral entry *via* ACE-2 the SARS-CoV-2 viral spike proteins must be primed *via* the transmembrane protease, serine 2 (TMPRSS2). Androgens *via* the androgen receptor are the only known transcription promoters for the TMPRSS2 gene [14,15]. Since

both ACE-2 as well as TMPRSS2 have been shown to be expressed in testis tissue, *via* single-cell and single-nucleus RNA-seq studies, we believe the high androgen environment of the testes will allow for viral entry [16]. In addition, multiple studies have reported that the use of renin-angiotensin system inhibitors has neither been shown to confer any protective effects, nor impact testing positive rates or mortality [17-19]. Additionally, it has been shown that viruses, such as human immunodeficiency virus, hepatitis B virus, and mumps, can cross the blood-testis barrier and cause viral orchitis resulting in infertility and cancer [20]. In this study we hypothesized that the SARS-CoV-2 virus can be present in the testis and impact spermatogenesis. We also evaluated the association between ACE-2 receptor levels and impact on spermatogenesis.

MATERIALS AND METHODS

1. Materials

Autopsy specimens of testis were obtained from six consecutive men (cases 1–6) who died of SARS-CoV-2 pneumonia, either alone or in relation to preexisting comorbidities, in Miami, FL, USA. Additionally, one testis biopsy was obtained from a live patient who was previously diagnosed with SARS-CoV-2 and subsequently seroconverted. All seven patients tested positive for COVID-19, *via* nasal swab test, immediately prior to their death, collection, or upon arrival to the medical examiner department. Three COVID-19 negative specimens of testis were taken as controls (cases C1–C3). All COVID-19 negative cases died of accidental causes, such as severe intracerebral hemorrhage following a fall (case C1), suspected overdose (case C2), and aspiration leading to asphyxiation (case C3). All control cases tested negative for SARS-CoV-2.

2. Tissue processing and staining

Both COVID-19 positive as well as negative control cases were immediately fixed with formaldehyde upon receipt per the Miami Dade County Medical Examiner Department protocols. Autopsy and testis tissue collection occurred 24–48 hours after the body was received by the medical examiner. All collected autopsy tissue was fixed in 10% buffered formalin for 48 hours for infection disease control reasons according to Centers for Disease Control and Prevention (CDC) guidelines. Formalin-fixed tissue was paraffin embedded. Samples

were cut to 4-micron sections and stained with hematoxylin and eosin (H&E). After dewaxing and rehydrating, sections were covered with 1× terminal deoxynucleotidyl transferase (TdT) balance buffer before being incubated at room temperature for 15–30 minutes. This was followed by the addition of TdT-labeled reaction mixture with enzyme and incubated at 37°C for 1 hour. Tris-buffered saline (TBS) instead of TdT enzyme was applied for negative control. Sections were rinsed with TBS between incubations at room temperature with stop solution for 5 minutes, 3% H₂O₂ for 5 minutes, and blocking buffer for 10 minutes. Peroxidase streptavidin conjugate was added at room temperature for 30 minutes. Slides were rinsed with TBS again, and incubated for color reaction with 3,3'-Diaminobenzidine (DAB) solution at room temperature for 10–15 minutes. Slides were counterstained with hematoxylin for 30 seconds. Slide evaluation and image analysis were performed under a light microscope at 200× magnification.

For fluorescent microscopy, paraffin sections of the testis were deparaffinized with xylene, hydrated with a series of alcohol solutions, and rinsed one time with Milli-Q water. They were then boiled for 20 minutes in 0.1 M Citrate Buffer in a microwave (800 W) for antigen retrieval. The specimens were cooled for 20 minutes at room temperature, washed four times with phosphate-buffered saline (PBS) (1×) (cat# 14190-144; Gibco, Rockville, MD, USA), and then incubated with 10% Normal Donkey Serum for 60 minutes at room temperature. After rinsing, the slides were incubated with ACE-2 Rabbit mAb (cat# 4355S; Cell Signaling, Danvers, MA, USA) and MERS-CoV Spike Protein S2 Mouse mAb (cat# 40070-MM11; Sino Biological, Wayne, PA, USA). The primary antibodies were diluted in PBS with Tween-20 (1:200) overnight at 4°C. The next day, the sections were washed two times with PBS and then incubated with Alexa Fluor 568 (cat# A10042; Thermo Fisher Scientific, Waltham, MA, USA) Donkey anti-Rabbit Secondary Antibody and Alexa Fluor 568 (cat# A10037; Thermo Fisher Scientific) Donkey anti-Mouse Secondary Antibody. These secondary antibodies were diluted 1:200 in 10% Normal Donkey Serum for 1 hour at 37°C, and then washed in PBS. After a final wash in PBS, the sections were finally dehydrated, fixed, and cover slips were applied with Antifade mounting media (cat# H-1200; Vector Laboratories, Burlingame, CA, USA).

3. Microscopic evaluation

Four of the six autopsy patients and the one live testis biopsy patient had tissue that underwent TEM imaging. Samples were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer and 100 mM sucrose, post-fixed overnight in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated through a series of graded ethanols, and embedded in a mixture of EM-bed/Araldite (Electron Microscopy Sciences, Hatfield, PA, USA). A total of 1 µm thick sections were stained with Richardson's stain for observation under a light microscope. A total of 100 nm sections were cut on a Leica Ultracut-R ultramicrotome (Leica, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. The grids were viewed at 80 kV in a JEOL JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) and images captured by an AMT BioSprint digital camera (AMT, Woburn, MA, USA).

4. Histomorphological evaluation

The testicular tissue was assessed similar to a fertility biopsy assessment. We additionally recorded the presence of inflammation and its compartmentalization within the testis. When evaluating sperm on histology the term normal spermatogenesis was used only when there was full spermatogenesis in in most of the tubules. If full spermatogenesis was not visualized in all the tubules, then the term abnormal spermatogenesis was applied and histological findings were described according to McLachlan et al [21].

5. Statistical analysis

The difference between mean values of COVID-19 and control groups was analyzed by Student t-test (Graphpad Prism 8 Software; Graphpad Software, San Diego, CA, USA), the level of significance was determined to be $p < 0.05$. All slides were assessed in a double-blinded fashion.

6. Ethics statement

The study protocol was approved by the Institutional Review Board (IRB) of the University of Miami Miller School of Medicine (IRB No. 20150740). Informed consent was waived by the IRB.

RESULTS

1. Clinical demographics

The COVID-19-positive autopsy patients' ages ranged from 20 to 87 years old (n=6, mean=56 years old). The COVID-19-negative patients' ages ranged from 28 to 77 years old (n=3, mean=57 years). The average length of time from the first positive COVID-19 test to death was 11 days, (range=2–36 days), with one case tested postmortem (Table 1). The age of the live patient with

antibody seroconversion post-COVID-19 infection was 28 years old.

2. Pathological findings

We found that three of the six COVID-19 positive men who underwent autopsy had normal spermatogenesis, while the remaining 3 men had various abnormalities of spermatogenesis. In addition to the above, COVID-19 positive case 4 was found to have lymphocytic and macrophage infiltration. All other COVID-19

Table 1. Clinical features of 6 deceased patients with COVID-19 and 3 deceased control patients

Variable	Status	Age (y)	Race	Confirmed disease duration (d)	Hospital stay (d)	Comorbidity	Symptom	Cause of death
Case C1	Control	77	White	NA	3	T2DM, CVD, HTN, deafness, Alzheimer disease	Respiratory failure, vomiting, altered mental status	Subarachnoid hemorrhage
Case C2	Control	28	Hispanic	NA	1	SUD	Unresponsive	Suspected overdose
Case C3	Control	68	White	NA	1	Atrial fibrillation, GERD	SOB, unresponsive	Asphyxiation
Case 1	COVID-19	20	White	4	0	T1DM, SUD	Unknown	COVID pneumonia
Case 2	COVID-19	87	Hispanic	Unknown	0	T2DM, CVD	Unresponsive	COVID pneumonia
Case 3	COVID-19	51	Black	3	4	HTN, CHF, CKD	Cardiac failure, Seizures	COVID pneumonia
Case 4	COVID-19	45	Black	6	6	Tracheotomy, obesity, HTN, bilateral leg amputation, paraplegia	SOB	COVID pneumonia
Case 5	COVID-19	55	White	9	1	CKD, CLD	SOB	COVID pneumonia
Case 6	COVID-19	80	White	44	1	HTN, CVD, hydrocephalus	Unknown	COVID pneumonia

Clinical features of the six severe acute respiratory syndrome coronavirus 2 (COVID-19) positive autopsy cases as well as the three COVID-19 negative autopsy cases selected as controls. All comorbidities were diagnosed prior to hospitalization and/or death.

NA: not applicable, T2DM: type 2 diabetes meletus, CVD: cardiovascular disease, HTN: hypertension, SUD: substance use disorder, GERD: gastro-esophageal reflux disease, T1DM: type 1 diabetes meletus, CHF: congestive heart failure, CKD: chronic kidney disease, CLD: chronic liver disease, SOB: shortness of breath.

Table 2. The evaluation of spermatogenesis and testicular pathology in the testes of 6 deceased patients with COVID-19 and 3 deceased control patients

Variable	Status	Leukocyte infiltration	Normal teste	Hypo-spermatogenesis	Early maturation arrest	Late maturation arrest	Sertoli cell only	Sclerosis
Case C1	Control	Negative	-	-	-	100%	-	-
Case C2	Control	Negative	-	10%	-	90%	-	-
Case C3	Control	Negative	-	-	-	5%	-	95%
Case 1	COVID-19	Negative	100%	-	-	-	-	-
Case 2	COVID-19	Negative	100%	-	-	-	-	-
Case 3	COVID-19	Negative	95%	-	-	-	-	5%
Case 4	COVID-19	Positive	-	20%	-	-	40%	40%
Case 5	COVID-19	Negative	-	-	20%	-	60%	20%
Case 6	COVID-19	Negative	-	-	-	-	90%	10%

Histomorphological analysis of spermatogenesis in the six severe acute respiratory syndrome coronavirus 2 (COVID-19) positive autopsy cases as well as the three COVID-19 negative control autopsy cases.

-: not applicable .

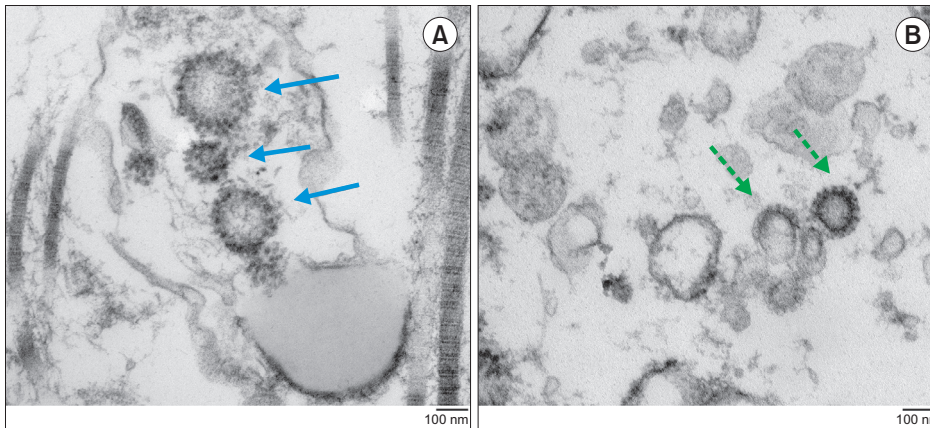


Fig. 1. Ultrastructure features of testes from live seroconverted severe acute respiratory syndrome coronavirus 2 (COVID-19) patient and patient who died due to COVID-19 pneumonia. (A) Coronavirus-like spiky viral particles (blue arrows) in the seminiferous tubules of a live patient who had previously contracted the COVID-19 virus and subsequently seroconverted. (B) Coronavirus-like spiky viral particles (green dotted arrows) in the seminiferous tubules postmortems of a patient who had been acutely infected with the COVID-19 virus.

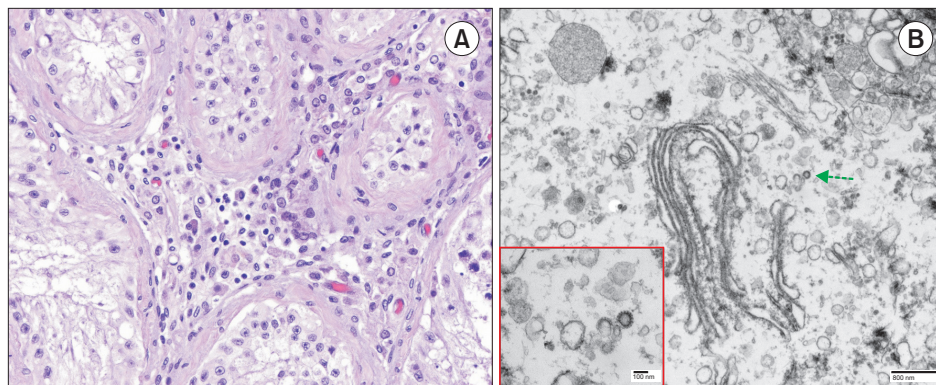


Fig. 2. Histological and ultrastructural features of testes under 40× magnification from postmortems of patient demonstrating inflammation and the severe acute respiratory syndrome coronavirus 2 (COVID-19) viral particle. (A) H&E stained sections showing hyalinization and thickening of the basement membrane of the seminiferous tubules with lymphocyte infiltration. (B) Coronavirus-like particles (green dotted arrow) with distinctive spikes seen in the cytoplasm of the interstitial cells of the testes, magnified image in lower left corner.

cases displayed no signs of inflammation. Table 2 presents pathological results of the 9 testes examined post-mortem. Under TEM, we observed spiky viral particles in one of the four COVID-19-positive autopsy patients (case 4) and the one live testis biopsy patient that we believed to be that of COVID-19 (Fig. 1). This autopsy patient had associated findings of macrophage and lymphocyte infiltration on H&E histomorphological analysis (Fig. 2).

ACE-2 receptor expression was quantified through immunofluorescence. In COVID-19-positive patients with normal spermatogenesis, ACE-2 receptor expression was noted to be significantly decreased when compared to COVID-19-positive patients with impaired spermatogenesis ($p < 0.05$) (Fig. 3-5). The COVID-19-positive patients with impaired spermatogenesis and higher levels of ACE-2 receptor expression demonstrated a mixture of findings including Sertoli cell only pathology, hypospermatogenesis, early maturation arrest,

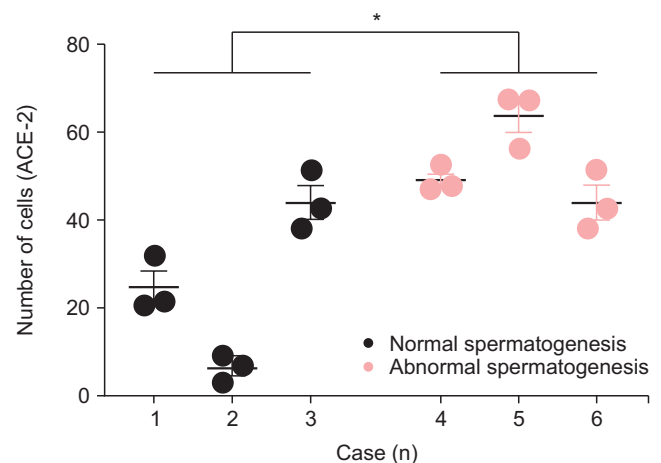


Fig. 3. Expression of angiotensin-converting enzyme 2 (ACE-2) in human testicular cells using ACE-2 Rabbit antibody. Scatter plot quantitating the number of cells expressing ACE-2 (x-axis). *It denotes a significant difference ($p \leq 0.05$) between cases of normal and abnormal spermatogenesis.

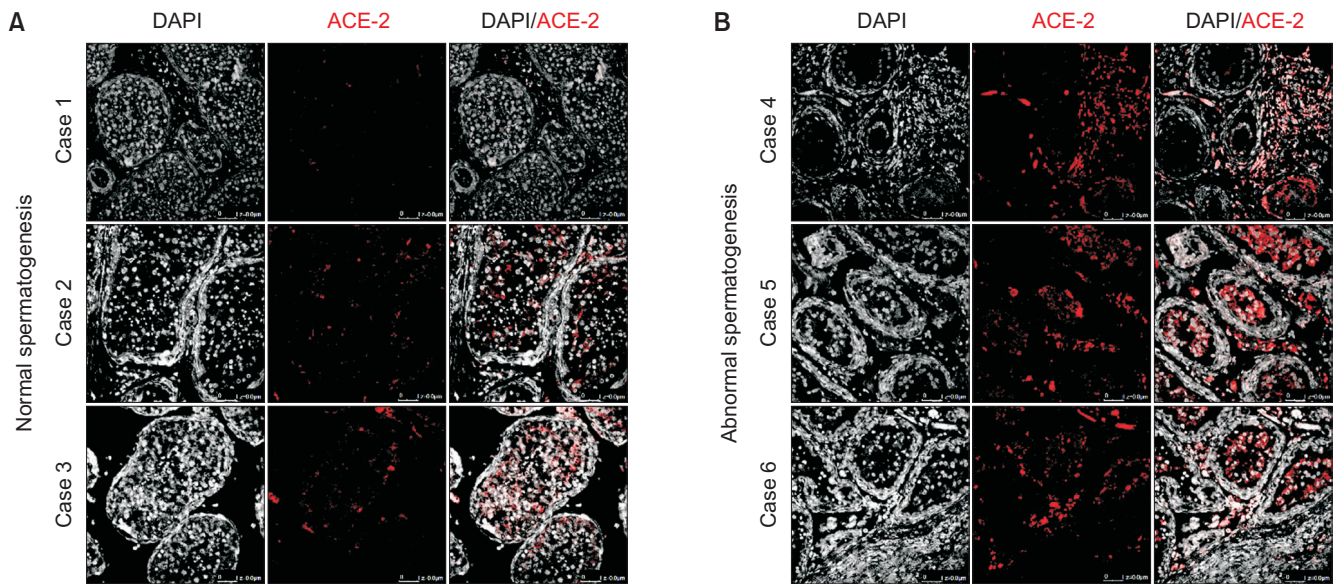


Fig. 4. Immunofluorescence of human testicular cells showing expression of angiotensin-converting enzyme 2 (ACE-2) and DAPI (4',6-diamidino-2-phenylindole), grouped according to normal vs abnormal spermatogenesis. (A) Severe acute respiratory syndrome coronavirus 2 (COVID-19) positive cases with normal spermatogenesis on histomorphology had weaker ACE-2 staining of testes tissue. (B) COVID-19 positive cases with abnormal spermatogenesis on histomorphology had stronger ACE-2 staining of testes tissue.

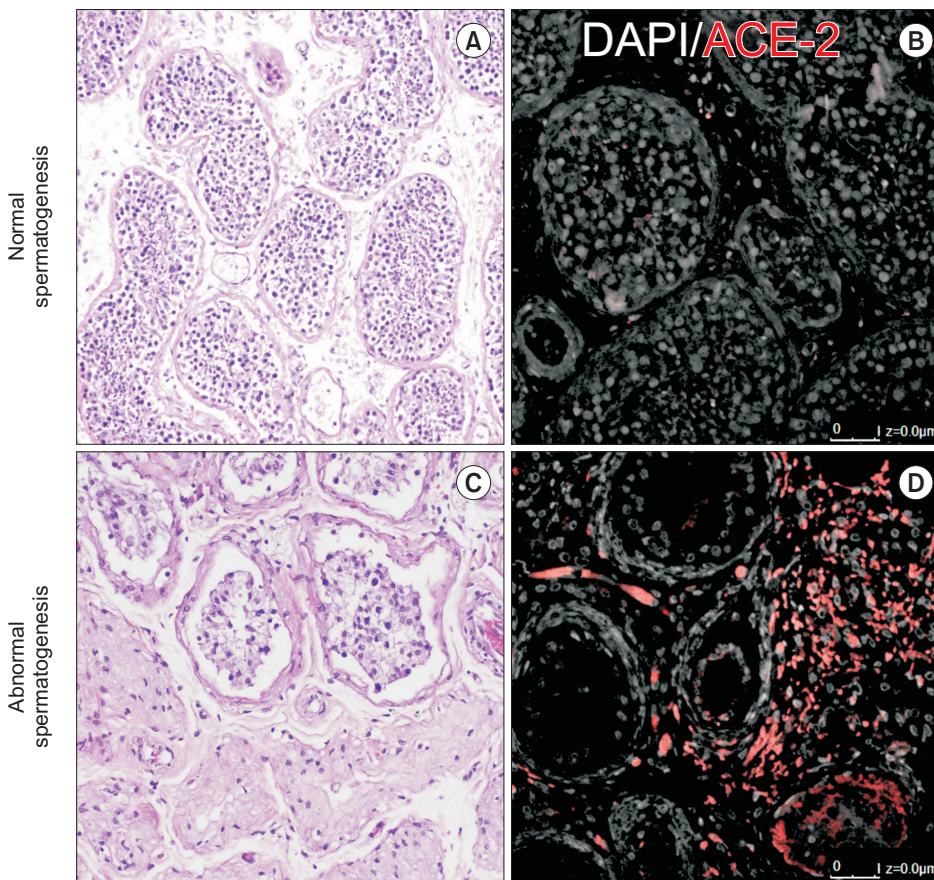


Fig. 5. Spectrum of pathologic abnormalities in spermatogenesis of testes from postmortem patients with severe acute respiratory syndrome coronavirus 2 (COVID-19) and the associated angiotensin-converting enzyme 2 (ACE-2) immunofluorescence. (A, B) H&E stained slide (20× magnification) showing normal spermatogenesis and decreased immunofluorescent expression of ACE-2. (C, D) H&E stained slide (20× magnification) showing hypospermatogenesis and occasional sclerosis with high immunofluorescent expression of ACE-2.

and sclerosis of the seminiferous tubules. Whereas the patients with decreased ACE-2 receptor expression displayed between 95% to 100% normal spermatogenesis.

DISCUSSION

The SARS-CoV-2 virus is known to affect the lungs, heart, kidneys, and liver; however, little is known about the pathogenesis of the virus in the testes. In our study we used various imaging studies to better understand the changes in testicular tissue in COVID-19-positive men. We observed what we believed to be SARS-CoV-2 viral particles in the testis tissue under TEM (Fig. 1). Two of the five samples that underwent TEM displayed spiked viral particles that ranged in size from 0.083–0.0898 μm consistent with previously published literature on SARS-CoV-2 [22,23]. Of those two samples one was from a patient who was previously COVID-19 positive but had subsequently seroconverted prior to testis biopsy collection. Suggesting that the presence of COVID-19 in the testes is not simply due to hematogenous spread during active infection. Additionally, when all six COVID-19-positive samples were subjected to immunofluorescence it was found that the density of ACE-2 receptors were inversely proportional to the level of spermatogenesis seen on pathological examination (Fig. 3). Our study suggests the possibility that men with higher ACE-2 receptor levels who become infected with the COVID-19 virus may be at greater risk for impaired spermatogenesis. Damage to cells involved in spermatogenesis by the COVID-19 virus could be the reason why men with greater ACE-2 receptor expression were found to have impaired spermatogenesis, while men with poor ACE-2 receptor expression had normal spermatogenesis. Of note, we found that all three COVID-19 negative control cases displayed impaired spermatogenesis. We believe this is due to age in the cases of C1 and C3. In the case of C2, this younger individual was found after a suspected drug overdose, and it is possible that the delay between time of death to time of fixation could have impacted the quality of tissue and level of spermatogenesis.

Other groups have studied the presence and impact of COVID-19 on testicular tissue. Bian [5] showed that SARS-CoV-19 can be detected post-mortem *via* RT-PCR, immunohistochemistry, and TEM in many tissues, including testis, however testis tissue collection was *via* percutaneous, minimally invasive autopsy. Similar to

our study, Bian [5] found that COVID-19 positive men showed various degrees of spermatogenesis destruction and testicular tissue injury. One recent study investigated the effects of the COVID-19 virus on testis tissue and found that the virus causes tissue damage and causes lymphocytic infiltration. RT-PCR was able to detect the virus in one of 12 cases; however, TEM did not show the presence of the virus or its impact on normal spermatogenesis [4]. Pan et al [7] found that 19% of men presented with scrotal discomfort suggestive of viral orchitis around the time of their confirmatory COVID-19 testing. Measurement, *via* RT-PCR, of SARS-CoV-2 in the semen of 34 adult Chinese men recovering from COVID-19 did not detect the virus in ejaculate. Additionally, the authors found low ACE-2 receptor expression in convalescing COVID-19-positive men and no evidence of ACE-2 and TMPRSS2 receptor co-expression in the testes tissue [7]. Li et al [6] found that 6 out of 38 men who were COVID-19-positive had identifiable virus in their semen *via* RT-PCR. A greater proportion of positive semen samples were in acutely infected men (26.7%) as compared to men recovering from COVID-19 (8.7%). Thus far there is no data indicating fertility would be impacted by the virus [6]. Additionally, the association of increased cytokine levels seen in COVID-19 infection may be similar to the increased cytokine levels caused by mumps orchitis [24,25]. These studies with opposing results reveal the unknown nature of COVID-19 on the testes and semen. The implications are important as there may be concerns for infertility and/or sexual transmission in infected patients.

The possibility that COVID-19 damages the testes and impacts fertility, either through an inflammatory orchitis or some other process, warrants gonadal function evaluation in men infected with COVID-19, or who have recovered from COVID-19, and desire fertility [26]. Sperm banking should be offered; however, preliminary assessment and molecular confirmation of the absence of COVID-19 in seminal fluid in asymptomatic men should be performed, as viruses stored in liquid nitrogen have been shown to retain their pathogenic properties [27,28]. Prior studies have shown this preliminary evaluation to aid in the safety of sperm cryopreservation [29]. Still, larger cohorts of current or previously COVID-19 infected men are needed to confirm the safety of cryopreservation and assisted reproduction techniques in this population.

In this study we attempted to address the question of the presence of SARS-CoV-2 virus in the testes and the possible concerns it may present. Our study is unique in that we incorporated histomorphology to look for the presence of orchitis and impacts to spermatogenesis, similar to Xu et al [3] when they studied the SARS-CoV virus in 2006. To date, we are the first US study to show the presence of the SARS-CoV-2 virus in testis tissue *via* TEM. We are also the first to associate the level of ACE-2 receptor expression to changes in spermatogenesis in COVID-19-positive men. The strengths of this study are the diverse representation of men across many races and ages, as well as the ability to show the SARS-CoV-2 virus in testes tissue. In addition, finding identical spiked viral particles in a biopsy of the seminiferous tubules of a living patient who was previous COVID-19 positive; however, seroconverted four weeks later helps to preclude the idea that the presence of the SARS-CoV-2 virus in testis tissue of acutely infected men was due to hematogenous spread and not productive infection.

This study is limited by the small sample size and inability to assess the long-term consequences of the SARS-CoV-2 virus on spermatogenesis. Further studies are needed to strengthen the findings of this study in light of conflicting results from previous published studies. To confirm the TEM findings, we attempted to detect the presence of amplified DNA on PCR; however, we were unable to perform this due to lack of sufficient tissue and cDNA. Next steps involve sequencing genomic DNA from the testes of acutely infected COVID-19 patients to show that SARS-CoV-2 sequences have entered the genome of the cells. Possible follow-up studies include comparing COVID-19 positive men to controls with other (non-COVID) causes of acute respiratory distress syndrome, allowing for the definition of COVID-19 specific findings *versus* those due to having a general systemic illness. Future studies are required with respect to identifying any impact on testis function after chronic infection or resolution of COVID-19 in men.

CONCLUSIONS

The presence of SARS-CoV-2 viral particles in the testicular tissue fills a fundamental gap in knowledge of the affected organs and possible sequelae of COVID-19 in men. The findings of this study could be

the first step in discovering impacts to fertility or the possibility of sexual transmission of the virus. On the basis of these preliminary findings, we believe that COVID-19 can penetrate the blood-testis barrier and enter the testis in some men. Presence of the virus can still be identified in the testis after patients have seroconverted. ACE-2 receptor density in testis tissue may be a factor influencing the extent of damage to cells responsible for spermatogenesis, with higher ACE-2 expression possibly leading to poorer spermatogenesis. However, further experiments are needed to validate this association. The relationship between possible viral particles on TEM and leukocyte infiltration suggests the COVID-19 virus may enter the testis and potentially cause orchitis. Further studies need to be undertaken to better understand the effects of this virus on reproductive organs.

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Conflict of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: JKA, RR. Data curation: EI, HA. Formal analysis: HA. Funding acquisition: RR. Investigation: JKA, KYC, KK, HA. Methodology: JKA, EI, RR. Project administration: RR. Resources: KSD, OAI, ONK, HA. Supervision: RR. Validation: HA. Visualization: JKA. Writing – original draft: JKA. Writing – review & editing: KYC, HA, RR.

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