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Xixiang Ma

Huazhong University of Science and Technology

Chuhuai Guan

Huazhong University of Science and Technology

Rong Chen

Wuhan Jinyintan Hospital

Yunyun Wang

Huazhong University of Science and Technology

Shenglei Feng

Huazhong University of Science and Technology

rongshuai wang

Department of Forensic Medicine, Tongji Medical College, Huazhong University of Science and Technology

Guoqiang Qu

Hubei Chongxin Judicial Expertise Center

Liling Wang

Huazhong University of Science and Technology

Sijia Zhao

Huazhong University of Science and Technology

Fengli Wang

Huazhong University of Science and Technology

Xiaoli Wang

Huazhong University of Science and Technology

Dingyu Zhang

Wuhan Jinyintan Hospital

Liang Liu

Huazhong University of Science and Technology

Aihua Liao

Huazhong University of Science and Technology <https://orcid.org/0000-0001-8533-8315>

Shuiqiao Yuan (✉ shuiqiaoyuan@hust.edu.cn)

Huazhong University of Science and Technology <https://orcid.org/0000-0003-1460-7682>

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**Pathological and molecular examination findings in postmortem testis biopsies
reveal spermatogenesis damages in COVID-19 patients**

Xixiang Ma^{1a}, Chuhuai Guan^{2a}, Rong Chen^{3a}, Yunyun Wang², Shenglei Feng¹, Rongshuai Wang⁴, Guoqiang Qu⁴, Liling Wang¹, Sijia Zhao¹, Fengli Wang¹, Xiaoli Wang¹, Dingyu Zhang^{5,6}, Liang Liu^{2*}, Aihua Liao^{1*}, Shuiqiao Yuan^{1*}

¹Institute of Reproductive Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China

²Department of Forensic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China

³Department of Pathology, Wuhan Jinyintan Hospital, Wuhan, Hubei 430023, China

⁴Hubei Chongxin Judicial Expertise Center, Wuhan, Hubei 430415, China

⁵Research Center for Translational Medicine, Wuhan Jinyintan Hospital, Wuhan, Hubei 430023, China

⁶Joint Laboratory of Infectious Diseases and Health, Wuhan Institute of Virology and Wuhan Jinyintan Hospital, Chinese Academy of Sciences, Wuhan, Hubei 430023 China

^a Authors contributed equally to the manuscript.

***Corresponding authors**

Liang Liu, M.D., Department of Forensic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China. Phone: +86- 027-83692644; Email: 907505@qq.com

Aihua Liao, M.D., Institute of Reproductive Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China. Phone: +86-15994262573; Email: aihua_liao@sina.com

Shuiqiao Yuan, Ph.D., Institute of Reproductive Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China. +86-15527507840; Email: shuiqiaoyuan@hust.edu.cn

31 **Abstract**

32 Despite widespread interest in the pathophysiology of COVID-19 in respiratory and
33 cardiovascular systems, little is known about the morphologic and molecular changes in the
34 testis of patients with COVID-19 and the effects of SARS-CoV-2 infection on male fertility.
35 We report here on the pathophysiology and molecular feature of testes obtained at autopsy
36 from six men with COVID-19, as compared with those of testes from three men with age-
37 matched, uninfected SARS-CoV-2. Our histopathological results showed that all COVID-19
38 patients had severe spermatogenesis damages compared with controls. Importantly, we
39 detected the nuclear acid of the SARS-CoV-2 virus, viral particles, and SARS-CoV-2 spike
40 S1 protein in COVID-19 patient testes, and we also found ACE2 and TMPRSS2
41 significantly elevated in the testes from COVID-19 patients. Furthermore, we observed a
42 prominent leukocyte infiltration, including CD3⁺ T lymphocytes, CD20⁺ B lymphocytes,
43 CD68⁺ macrophages, HLA-DR⁺ myeloid cells, and CD38⁺ plasma cells in the testes of
44 COVID-19 patients. RNA-Seq analyses further revealed SARS-CoV-2 infection could lead
45 to dysfunction of the genes that regulate the spermatogenesis and inflammation response-
46 related pathways. Collectively, our pathological and molecular examination findings indicate
47 that SARS-CoV-2 could directly attack testicular cells, thereby inducing the damage of
48 testicular immune privilege and spermatogenesis defects.

49

50

51 **Keywords:** COVID-19, spermatogenesis, autopsy, male fertility, SARS-CoV-2

52

53 **Running title:** The SARS-CoV-2 infection leads to spermatogenesis disorder

54

55

56 **Introduction**

57 In late December 2019, a novel coronavirus (severe acute respiratory syndrome
58 coronavirus 2, SARS-CoV-2) has emerged in Wuhan, China and its subsequent rapid
59 international spread pose a global health emergency, which causing pneumonia symptoms
60 (COVID-19) in humans similar to those caused by SARS coronavirus (SARS-CoV). Unlike
61 the mild threaten of SARS-CoV, SARS-CoV-2 having influential infectious and leading to
62 over 10 million people infected, and more than 499,913 patients died of COVID-19 around
63 the world by June 29, 2020 (Coronavirus disease (COVID-19) Situation Report-161, World
64 Health Organization, [https://www.who.int/emergencies/diseases/novel-coronavirus-](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports)
65 [2019/situation-reports](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports)). With the increasing number of people infected with COVID-19
66 across the world, the scientific community began to study the pathophysiology of the SARS-
67 Cov-2 virus to understand a new, emergent, and unknown pathogen and to guide disease
68 transmission, prevention, susceptibility, and treatment. Although an autopsy can provide an
69 opportunity for us to understand the potential key information about the real mechanisms
70 underlying death and organ damage caused by COVID-19 infections(Salerno et al., 2020),
71 there is a lack of well-defined pathophysiology examination of different organs from the
72 patients who died of COVID-19 disease. Since progressive respiratory failure is the primary
73 cause of death in the COVID-19 pandemic, researchers mainly focused on the
74 pathophysiological changes in major organs at autopsy, such as the lung, heart, and Kidney
75 (Ackermann et al., 2020; Fox et al., 2020; Liu et al., 2020; Schaller et al., 2020; Su et al.,
76 2020), whereas ignored the anatomical pathophysiology observation of other organs,
77 especially reproductive organs. Meanwhile, millions of people with SARS-CoV-2 infections
78 have been cured, among them a large number of men who have fertility desire, which
79 raises concerns about whether SARS-CoV-2 could attack the testicular cells and might
80 cause male infertility. Two studies were unable to detect the presence of SARS-CoV-2 in

81 semen and testis from men who had recovered from COVID-19 (Pan et al., 2020; Song et
82 al., 2020), but in contrast, a recent study has reported that SARS-CoV-2 can be detected in
83 semen of patients with COVID-19 (Li et al., 2020). Therefore, it is currently unknown
84 whether the infection of SARS-CoV-2 will damage spermatogenesis and male fertility or
85 whether SARS-CoV-2 can attack the germ cell into seminiferous tubules.

86 We report here, for the first time to our knowledge, on the pathophysiology and
87 molecular feature of testes obtained at autopsy from six men with a recorded cause of
88 death of COVID-19, as compared with those of testes from three men who died from other
89 diseases and age-matched, uninfected control testes. Our pathological results showed that
90 all six male patients with COVID-19 had severe spermatogenesis damage compared with
91 controls, indicating SARS-CoV-2 infection impairs male germ cell development and
92 spermatogenesis. More importantly, the coronavirus particles were observed in the testis of
93 patients with COVID-19 under an electronic microscope, and the nuclear acid of the SARS-
94 CoV-2 virus was also detectable in COVID-19 patient testis sections, which indicates that
95 SARS-CoV-2 could enter the testicular tubules and attack the testicular cells. At the
96 molecular level, we found that the mRNA and protein levels of ACE2 and TMPRSS2 (the
97 SARS-CoV-2 entry receptor and spike protein priming, respectively) appear to be
98 overexpressed in formalin-fixed paraffin-embedded (FFPE) testis sections from COVID-19
99 patients. We also detected the SARS-CoV-2 spike S1 signals in the seminiferous tubules of
100 patients with COVID-19, which further demonstrate SARS-CoV-2 attack the germ cells via
101 binding its receptors. In addition, we observed a prominent infiltration of leukocytes,
102 including CD3⁺ T lymphocytes, CD68⁺ macrophages, and HLA-DR⁺ myeloid cells in the
103 testes of COVID-19 patients. Most importantly, under normal conditions, CD20⁺ B
104 lymphocytes and CD38⁺ plasma cells are not present in human testes, whereas were
105 observed in testes of COVID-19 patients. RNA-seq analyses further revealed that genes

involved in spermatogenesis and some inflammation response-related pathways in the testis of patients with COVID-19 were deregulated, which provided a possible molecular explanation for the inflammatory immune attack and spermatogenesis damages in testis caused by SARS-CoV-2 infection. Taken together, our pathological and molecular examination findings at autopsy provide the most substantial direct evidence and underlying molecular mechanism for the effects of SARS-CoV-2 infection on spermatogenesis and male fertility and are likely to have important implications for the reproductive treatment of COVID-19 in this male patient population.

114

115 **Results and discussion**

To assess the effects of SARS-CoV-19 infection on spermatogenesis and male germ development, we first performed a PAS-staining assay to examine the testicular morphology in all patients with COVID-19. Numerous degenerated germ cells (GC) could be observed to fall off to the lumen in seminiferous tubules of all six COVID-19 patients (**Fig. 1 and Fig.S1A**). In contrast, the germ cells did not fall off and aligned well in the whole seminiferous tubules of three control patients (**Fig. 1**). Strikingly, in five of six cases, the changes of germ cells lost were massive, with only a few germ cells left and attached the seminiferous tubules (patient-1, 3, 4, 5, and 6). In particular, many seminiferous tubules in the testes of patients 4 and 5 are barely saw the intact germ cells but only present Sertoli cells (SCs), similar to Sertoli cell-only syndrome. In agreement with this morphological observation, the number of DDX4 (a germ cell marker) positive cells was dramatically reduced in all testicular specimens from the COVID-19 groups (**Fig. 2A**). Interestingly, the number of Sertoli cells was comparable in the testes from SARS-CoV-2 infected patients and uninfected patients (**Fig. 2B**), suggesting that SARS-CoV-2 infection may not impair Sertoli cell development. These morphological changes in testes of COVID-19 patients

131 indicate that SARS-CoV-2 infection could impair the male germ cell development and
132 eventually lead to germ cells lost. It is worth noting that the patient-6 was recovered from
133 COVID-19 after nine days of antiviral treatment and died of massive gastrointestinal
134 bleeding caused by liver cirrhosis at home after the discharge of 45 days (**Table S1**). The
135 main reason we included the patient-6 in this study was to observe whether there was any
136 damage in the spermatogenesis of recovered patients with COVID-19 after treatment.
137 Strikingly, the testes of patient-6 also showed severe spermatogenesis damage (**Fig.S1A**),
138 which prompted us to pay more attention to the spermatogenesis and fertility of the
139 recovered patients from SARS-CoV-2 infection. However, we could not rule out the
140 possibility of effects of chronic hepatitis B viral infection and liver cirrhosis on the
141 spermatogenesis in the patient-6 because chronic hepatitis B infection increases the
142 incidence of apoptosis and necrosis in spermatogenic cells(Moretti et al., 2008).

143 To ascertain how the male germ cell loss in the patients with COVID-19, we asked
144 whether the apoptosis and inflammation reaction occurred in the testicular cells of COVID-
145 19 patients. TUNEL assay revealed that the number of apoptotic cells in COVID-19 testes
146 was significantly higher than the control testes (**Fig.S1B-C**), a finding consistent with the
147 phenotype of Zika virus infection in mice(Govero et al., 2016; Ma et al., 2016), raising a
148 possibility of SARS-CoV-2 damage the immune privilege and innate immune homeostasis
149 of the testis. Indeed, immunohistochemical (IHC) staining for various leukocytes revealed a
150 scattered infiltration of CD3⁺ T lymphocytes, CD20⁺ B lymphocytes, CD68⁺ macrophages,
151 and HLA-DR α ⁺ myeloid cells were observed in seminiferous tubules of patients with
152 COVID-19, whereas rarely detected in controls (**Fig. 3A-D**). Besides, we found that CD38⁺
153 cells (a marker of plasma cells) are highly observed in the interstitial compartments of
154 COVID-19 patient testes (**Fig.3E**). Correspondingly, extensive IgG precipitation was mainly
155 found in the interstitial compartments and partly in the seminiferous epithelium of COVID-19

156 patient testes, which is consistent with the effects of SARS infected testes (**Fig.3F**)(Xu et al.,
157 2006). These data indicate that SARS-CoV-2 could directly target testicular cells and
158 thereby cause testicular immune attack, leading to germ cell injury and lost eventually.

159 Furthermore, in the current study, the histological analysis showed exfoliation of the
160 spermatogenic epithelium and reduction of germ cells in all six COVID-19 patient testes,
161 especially the patients 4 and 5 exhibited thickening of the tubular wall and almost Sertoli
162 cell-only symptom (**Fig.1**). These severe damages in the testes are similar to orchitis
163 caused by some viruses, such as HIV and SARS, and leukocyte infiltration is a main
164 pathological feature of these viral orchitides (Liu et al., 2018; Xu et al., 2006). As it has
165 been known, no B cells reside in testes under physiological conditions. Thus, the evidence
166 of a prominent infiltration of leukocytes, specifically the infiltration of B cells and plasma
167 cells, and IgG precipitation observed in the testes of COVID-19 patients indicate that the
168 blood-testis barrier and the immune-privileged environment were damaged in COVID-19
169 patients. Like other viral orchitides(Xu et al., 2006), SARS-CoV-2 might trigger a secondary
170 autoimmune response and contribute to the primary pathogenesis of viral orchitis, which
171 accounts for the testicular damage.

172 To further determine whether the SARS-CoV-2 can attack testicular cells directly, we
173 first detected the nuclear acid of SARS-CoV-2 in formalin-fixed paraffin-embedded (FFPE)
174 testis tissues of COVID-19 patients. Through RT-qPCR-based virus nuclear acid detection,
175 we found that the two testis samples (patient-1 and 3) were positive for SARS-CoV-2
176 nuclear acid (**Table S2**). The detection of the SARS-CoV-2 virus within the testis, a finding
177 consistent with other study(Li et al., 2020), suggests that direct viral effects, as well as the
178 induced damage of the testicular immune privilege, may contribute to the germ cell injury.
179 Since the spike (S) glycoprotein of coronaviruses is known to be essential in the binding of
180 the SARS-CoV-2 to the host cells, we then examined the SARS-CoV-2 spike protein

181 expression in the testes of patients with COVID-19 by immunohistochemistry using SARS-
182 CoV Spike S1 antibody. We found the positive signals of SARS-CoV Spike S1 are present
183 in all COVID-19 patient testicular sections (**Fig.4A**), suggesting that the SARS-CoV-2
184 indeed infected with the testicular cells through its spike glycoprotein binding mechanism. It
185 should note that we detected the SARS-CoV-2 Spike protein in all COVID-19 patient testes,
186 but the nuclear acid of SARS-CoV-2 was only detected in two samples (patient-1 and 3).
187 This difference may be due to the fact that viral RNA was extracted from FFPE samples,
188 which causing the viral RNA is easy to degrade and hard to detect by qPCR assay. Next,
189 we examined the testis sections with electron microscopy to directly see whether the
190 coronavirus particles were present in the testis of patients with COVID-19. We selected
191 three patient testes (Patient-1, 3, and 4) to perform electron microscopy analyses and found
192 that the coronavirus particles can be seen in the interstitial compartment of testes (**Fig. 4B**
193 **and Fig.S1D**). This electron microscopy data provide strong direct evidence for SARS-
194 CoV-2 to enter and attack human testicular tissues. Further studies are required with
195 respect to the detailed information about virus shedding, survival time, and concentration in
196 testis. Although spermatogenesis disruption was probably a common feature in the testes
197 from COVID-19 patients, we observed the different degrees of spermatogenesis damages
198 in patients with COVID-19. This may contribute to the relative number of copies of the virus
199 into the testis and the duration of the virus infection.

200 Since SARS- CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor for
201 entry and the serine protease TMPRSS2 for spike protein priming to attack the
202 cells(Hoffmann et al., 2020; Lan et al., 2020), we next examined both protein and mRNA
203 levels of ACE2 and TMPRSS2 in testes by immunohistochemistry and RT-qPCR analysis,
204 respectively. Immunostainings revealed that although both ACE2 and TMPRSS2 proteins
205 were predominantly expressed in the cytoplasm and membrane of spermatocytes,

206 spermatids, and Sertoli cells in the control testes, the elevated ACE2 and TMPRSS2
207 signals were observed in the seminiferous tubules of all patients with COVID-19 (**Fig. 4C-D**
208 **and Fig. S2A-B**). Interestingly, both ACE2 and TMPRSS2 proteins were not found to
209 express in spermatogonia in our study and one recent report (**Fig.4C-D**)(Yang et al., 2020).
210 In contrast, two previous studies by single-cell transcriptome database analyses revealed
211 that the ACE2 and TMPRSS2 mRNA were predominantly enriched in spermatogonia and
212 Sertoli cells(Pan et al., 2020; Wang and Xu, 2020). Consistent with the
213 immunohistochemistry result, RT-qPCR revealed the mRNA levels of ACE2 and TMPRSS2
214 significantly increased in all COVID-19 patient testes compared to that of control patient
215 testes (**Fig. 4E-F**). Remarkably, both the protein and mRNA levels of ACE2 and TMPRSS2
216 appeared to be a very significant increase in the testes of COVID-19 patients 4 and 5 who
217 had much severe spermatogenesis disruption (**Fig. 1 and Fig. 4E-F**). Given that the fact of
218 ACE2 and TMPRSS2 are integral membrane protein that appears to be the host-cell
219 receptor and spike protein priming for SARS-CoV-2 to enter the cells(Hoffmann et al., 2020;
220 Lan et al., 2020), the increased expression of ACE2 and TMPRSS2 may facilitate infection
221 with COVID-19 and increase the risk of cellular damage by SARS-CoV-2(Fang et al., 2020).
222 In line with this notion, our data showed the significantly higher signal intensity of ACE2-
223 and TMPRSS2-positive cells in the testes from patients with COVID-19 than in those from
224 uninfected controls, which explained the underlying reasons for the SARS-CoV-2 attack
225 testicular cells.

226 To investigate the impact and molecular changes of SARS-CoV-2 infections in testes,
227 we next extracted total RNA from testes of controls (control-1, 2, and 3) and three COVID-
228 19 patients (patient-2, 3, and 4), respectively, and performed RNA-seq to analyze the
229 transcriptome changes. With regard to the coding and lncRNA genes, RNA-seq analyses
230 found 28,801 expressed genes from control and COVID-19 patient testes (**Table S3**), of

231 which 2,645 genes up-regulated and 2,789 genes down-regulated in COVID-19 patients
232 compared with controls (fold of change>2, FDR<0.001) (**Fig. 5A, Fig. S3A, and Table S4**).
233 These results suggest that SARS-CoV-2 infection could trigger the transcriptome dynamic
234 alterations at molecular levels in testes during specific biological processes.

235 To further determine the changes of transcriptome upon SARS-CoV-2 infection in
236 testes, we performed Gene Ontology (GO) term analysis for those differentially regulated
237 genes. Consistent with our virus RNA detection and immunohistochemical results, GO
238 analysis demonstrated that up-regulated transcripts were significantly enriched in the
239 invasion of the virus, such as “viral gene expression” and “viral life cycle” (e.g., RPL13,
240 VTA1, RPS27, WWP1, XPO1, and VAPA) (**Fig.5B**). Importantly, some inflammation-related
241 processes were activated, including “interleukin-6-mediated signaling pathway” and
242 “regulation of B cell proliferation” (e.g., IL6ST, IL2, MEF2C, PTPRC, BCL2, and BCL6)
243 (**Fig.5B**). Additionally, GO enrichment documented that up-regulated genes were
244 significantly enriched in autophagy and response to oxygen levels as well (**Fig.5B**).
245 Consistent with our testis histological results (**Fig.1**), the down-regulated genes significantly
246 enriched in “spermatogenesis” and “reproduction” (**Fig.5C**), which further illustrated the
247 impact of SARS-CoV-2 infection on male fertility. Strikingly, in line with our inflammatory cell
248 immunostaining results (**Fig.3**), we found some genes (e.g., BCR, BCRP1, and BCRP2)
249 that were down-regulated in which involved in some inflammation-related processes and
250 immune response, such as “negative regulation of myeloid leukocyte mediated immunity”,
251 “negative regulation of leukocyte degranulation and migration”, and “neutrophil activation
252 involved in immune response” (**Fig.5C**). We further determined the effect of SARS-CoV-2
253 infection on alternative splicing (AS) events of genes from testes of COVID-19 patients by
254 RNA-seq analyses. Compare to deregulated genes, and we scored only a few significantly
255 altered AS events in testes of COVID-19 patients (224 events with *P*-value<0.05 and

256 $|\Delta\psi|>0.1$) (**Fig.S3B and Table S5**). However, GO analysis revealed the deregulated genes
257 with significant AS events enriched in virus-associated terms, such as “IRES-dependent
258 viral translational initiation” and “viral RNA genome replication” (**Fig.5D**). In addition, some
259 AS genes were enriched in inflammation-related NF-kappaB signaling, “regulation of I-
260 kappaB kinase/NF-kappaB signaling” and “NF-kappaB import into nucleus” (**Fig.5D**). These
261 bioinformatics data suggest that SARS-CoV-2 infection could lead to dysfunction of the
262 genes that regulate the spermatogenesis and inflammation-related pathways, thereby
263 causing inflammatory immune attack in testes and spermatogenesis defects.

264 Furthermore, since previous clinical reports indicate that SARS-CoV-2 infection can
265 cause inflammatory cytokine storm syndrome in COVID-19 patients(Gao et al., 2020;
266 Huang et al., 2020), we asked whether the inflammatory cytokines significantly elevated in
267 the testes of COVID-19 patients. Through analyses of our RNA-seq data, we found that 32
268 Inflammatory cytokines were up-regulated considerably with P -value<0.05 and fold of
269 change>2 in COVID-19 patient testes. There were 10 cytokines (CMTM6, FAM3C, INHBA,
270 IL33, TNFSF10, NAMPT, CMTM4, CCL28, IL2, and TIMP1) significantly up-regulated in
271 testes of COVID-19 patients when adjusting FDR<0.001 (**Fig. 5E**). Taken together, our data
272 indicate that the testis, despite its immune-privileged status, cannot be isolated from the
273 whole immune system upon infection of SARS-CoV-2, thereby inducing immune cells can
274 directly or indirectly target the testicular cells to promote spermatogenesis damage. The
275 RNA-Seq data further support the observation in the testes of SARS-CoV-2 infected
276 patients that inflammatory cytokines can activate the autoimmune response (**Fig.3**),
277 destroying the seminiferous epithelium (**Fig.1**), which is similar with SARS infected men
278 that were showing autoimmune orchitis(Xu et al., 2006). Besides, it should be noted that
279 high levels of cytokines following viral infection, illness, or injury can cause deterioration in

280 spermatogenesis and steroidogenesis(Hedger and Meinhardt, 2003), thus adversely
281 affecting fertility, as all COVID-19 patients had other underlying diseases (**Table S1**).

282 Nevertheless, this study has limitations, including the small number of cases from a
283 single-center, and the patients were older people, which may not represent the fecundity of
284 men in the golden age of childbearing. For example, the patients with COVID-19 were
285 examined in this study are all aging men and had comorbidities, which may compromise
286 spermatogenesis and male germ cell development(Almeida et al., 2017; Shiraishi and
287 Matsuyama, 2018). Nonetheless, our analysis suggests that this possibility is unlikely,
288 particularly since the degree of germ cell apoptosis in the patients with COVID-19 increased
289 significantly, whereas in the control patients (also aging men), it remained stable at a
290 significantly lower level. Although our sample was small, 5 out of 6 cases showed a very
291 severely disrupted spermatogenesis feature in COVID-19 patients (**Fig.1 and Fig.S1A**),
292 which indicates that, to no small extent, SARS-CoV-2 infection does damage the human
293 reproductive system, especially spermatogenesis and male fertility.

294 Collectively, our findings provide direct evidence and underlying molecular mechanism
295 of SARS-CoV-2 for entry testis and attack the germ cells, which enhances the
296 understanding of COVID-19 pandemic in spermatogenesis and male fertility. Our findings
297 also suggest that researches should be undertaken to find ways to mitigate the risk of
298 testicular cell damage to protect male fertility during the COVID-19 course and treatment.
299 However, the relationship of these findings to the clinical course of COVID-19 and the in-
300 depth mechanism of SARS-CoV-2 infect testicular cells requires further study to elucidate.
301 Further research is still urgently needed for a comprehensive understanding of COVID-19
302 on male reproductive health, including effects on the viral sex transmission and testicular
303 endocrine function.

304

305 **Materials and methods**

306 **Patient selection and brief clinical summary**

307 We analyzed autopsy specimens from five male patients who died from respiratory failure
308 caused by SARS-CoV-2 infection and compared them with testes from three uninfected
309 control males who died from other diseases (control-1 died with hemorrhagic shock,
310 control-2 died with coronary disease, and control-3 died with traffic accident). The COVID-
311 19 patients were aged 51-83 with mean ($\pm$ SD) ages of 69 ± 11.5 years, and the uninfected
312 control patients were aged 71-80 with mean ages of 76 ± 4.7 years. All patients with COVID-
313 19 had at least one common underlying disease, the most common of which were
314 hypertension (detailed patient characteristics in **Table S1**). All the COVID-19 patients were
315 confirmed SARS-CoV-2 positive by RT-qPCR and bilateral ground-glass opacities by Chest
316 X-rays according to the New Coronavirus Pneumonia Prevention and Control Program (7th
317 edition). Treatment in the ICU initially included antiviral drugs, antibiotics, glucocorticoid,
318 and antihypertensive drugs for all patients. The study was approved by and conducted
319 according to requirements of the ethics committees at Wuhan Jinyintan Hospital and Tongji
320 Medical College, Huazhong University of Science and Technology. Written informed
321 consent was obtained from the next of kin of each enrolled case.

322

323 **Histological analysis**

324 Testes were obtained at autopsy and fixed in a 10% formaldehyde solution. Then the
325 samples were processed to paraffin blocks according to the standard procedure. 5 μ m
326 sections were cut and stained with periodic acid-Schiff (PAS) after being dewaxed and
327 rehydrated.

328

329 **TUNEL staining**

330 Apoptotic cells were detected by TUNEL assay using TUNEL Apoptosis Detection Kit
331 (C1098, Beyotime Biotechnology, Inc., Beijing, China) according to the manufacturer's
332 protocols. In brief, after dewaxing and rehydration, the testicular sections were incubated
333 for 15 min in 20 mg/mL Proteinase K (in PBS) solution. Then, the sections were covered
334 with 3% H₂O₂ and incubated at room temperature for 15 min, followed by a TdT-labeled
335 reaction mixture with an enzyme, and avoid light incubation at 37°C for 1h. The sections
336 were rinsed with PBS between incubations at room temperature with a stop solution for 10
337 min. Finally, Streptavidin-HRP was added at room temperature for 30 min. After another
338 rinse with PBS, the sections were incubated for color reaction with DAB solution at room
339 temperature for 5-10 min. Dehydrate using graded ethanol, vitrification by dimethylbenzene.
340 Images were obtained with an Axio Scope A1 microscope (Zeiss, Germany).

341

342 **Immunohistochemistry**

343 After being dewaxed and rehydrated, the 5µm thick sections were microwaved in 0.01 M
344 sodium citrate buffer (pH=6.0) for antigen retrieval, then cooled down to room temperature.
345 The sections were washed in PBS three times (10min per time), and endogenous
346 peroxidase activity was blocked with 3% H₂O₂ in PBS. The sections were blocked in
347 blocking solution (containing 3% normal goat serum and 3% fetal bovine serum in 1%
348 bovine serum albumin) for 1 h and were incubated with primary antibodies, including
349 primary antibodies against ACE2 (dilution 5 µg/mL; AF933; R&D Systems), TMPRSS2
350 (dilution 1:1000; ab92323; Abcam), DDX4 (dilution 1:200; ab13840; Abcam), SOX9 (dilution
351 1:1000; EPR14335-78; Abcam), CD3 (dilution 1:200; 17617-1-AP; Proteintech), CD20
352 (dilution 1:10000; 60271-1-Ig; Proteintech), CD68 (dilution 1:1000; 28058-1-AP;
353 Proteintech), HLA-DRA (dilution 1:300; 17221-1-AP; Proteintech), CD38 (dilution 1:200;
354 25284-1-AP; Proteintech), human IgG (dilution 1:10000; 10284-1-AP; Proteintech), SARS-

355 CoV-2 Spike antibody (dilution 1:200; 40150-T62-COV2; Sino Biological) in a humidified
356 box overnight at 4°C. After three times washes in PBS, sections were then incubated with
357 peroxidase-labeled secondary antibody (dilution 1:1000; Rabbit Anti-Goat IgG, A21030;
358 Goat Anti-Mouse IgG, A21010; Goat Anti-Rabbit IgG, A21020; Abbkine) for 1h at room
359 temperature and counterstained with hematoxylin. Images were photographed with an Axio
360 Scope A1 microscope (Zeiss, Germany).

361

362 **RNA isolation and RT-qPCR for SARS-CoV-2 virus**

363 Total RNA was extracted from deparaffinized formalin-fixed paraffin-embedded (FFPE)
364 sections using RNA RNeasy FFPE Kit (Qiagen, Germany) according to the manufacturer's
365 protocols. Virus quantification in the FFPR sample was performed using the COVID-19
366 (ASRS-CoV-2) Nucleic Acid Test Kit (Easy Diagnosis Biomedicine Co., Ltd, Wuhan, China)
367 with primers/probes for RNaseP and ORF1ab genes amplification following the Kit
368 instruction. Two-step real-time PCR was run on the StepOnePlus™ Software system
369 (ThermoFisher, USA). PCR running conditions were as follows: 50°C for 15min, 95°C for
370 the 30s, 40 cycles at 95°C for 3s, 60°C for 40s. The amplification curve was exponential,
371 ORF1ab with CT value less than 38, and RNaseP with CT value less than 35 considered as
372 positive.

373

374 **RNA library preparation and sequencing**

375 Total RNAs were extracted from deparaffinized FFPE sections of controls (control-1, 2, and
376 3) and three COVID-19 patients (patient-2, 3, and 4) using RNA RNeasy FFPE Kit (Qiagen,
377 Germany) according to manufacturer's protocol. The RNA concentration was verified using
378 a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc). RNA finally quantified
379 by Qubit3.0 with Qubit™ RNA Broad Range Assay kit (Life Technologies, Q10210).

380 For RNA library preparation, 2µg of total RNA was used from each sample to prepare
381 RNA sequencing library using Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Cat.
382 MRZG12324, Illumina) and KC-Digital™ Stranded mRNA Library Prep Kit for Illumina®
383 (Cat. DR08502, Wuhan Seqhealth Co., Ltd. China) following the manufacturer's instruction.
384 The kit eliminates duplication bias in PCR and sequencing steps, by using a unique
385 molecular identifier (UMI) of 8 random bases to label the pre-amplified cDNA molecules.
386 The library products corresponding to 200-500 bps were enriched, quantified, and finally
387 sequenced on NovaSeq 6000 sequencer (Illumina) with PE150 model.

388

389 **RNA-Seq data analysis**

390 Trimmomatic (version 0.36) was used to remove adaptor sequences, and low quality reads
391 from the sequencing data. Clean Reads were further treated with in-house scripts to
392 eliminate duplication bias introduced in library preparation and sequencing. De-duplicated
393 Reads were mapped to the reference genome of Homo sapiens from
394 ftp://ftp.ensembl.org/pub/release-87/fastq/homo_sapiens/dna/ using STRA software
395 (version 2.5.3a) with default parameters. Reads mapped to the exon regions of each gene
396 were counted by featureCounts (Subread-1.5.1; Bioconductor), and then RPKM was
397 calculated. Genes differentially expressed between groups was performed by the edgeR
398 package (version 3.12.1). The differential expressed genes were set with the threshold of
399 $FDR < 0.001$ and $\text{fold change} \geq 2$. Alternative splicing events were detected by using rMATS
400 (version 3.2.5) with an FDR value cutoff of 0.05 and an absolute value of $\Delta\psi$ of 0.05. Gene
401 ontology (GO) analyses for differentially expressed genes were implemented by KOBAS
402 software (version: 2.1.1) with a *P*-value cutoff of 0.05 to judge statistically significant
403 enrichment.

404

405 **Statistical analysis**

406 All comparisons of numeric variables (including those in the gene-expression analysis)
407 were conducted with Student's *t*-test family-wise error rates. Original *P* values are reported
408 only for the tests that met the criteria for false discovery rates. The results were presented
409 as mean $\pm$ SD, and significance was assumed for $P < 0.05$. All the analyses were done
410 using SPSS, version 20 (IBM).

411

412 **Online supplemental material**

413 Fig. S1 shows the representative PAS-staining images of Patient-6 testes (A), TUNEL
414 assays for testicular sections of control patients and COVID-19 patients (B-C), and electron
415 microscopy image of the testis from Patient-3 (D). Fig. S2 presents the ACE2 and
416 TMPRSS2 immunohistochemical staining images in the testicular sections of all control
417 patients and COVID-19 patients. Fig.S3 shows the differential gene expression and
418 alternative splicing events in the testes of COVID-19 patients. Table S1 contains detailed
419 information on patient clinical characteristics and medical history in the study. Table S2 lists
420 the results of SARS-CoV-2 nuclear acid detection in FFPE testis samples of COVID-19
421 patients by RT-qPCR. Table S3 lists the total coding and lncRNA genes identified from the
422 testes of control and COVID-19 patients. Table S4 contains the up- and down-regulated
423 genes identified from testes of COVID-19 patients. Table S5 lists the alternative splicing
424 events identified from deregulated genes.

425

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436 Author contributions: SY and AL conceived and designed the experiments; LL, CG,
437 RC, YW, RG, GQ, and DZ conducted autopsy; XM, SF, LW, SZ, FW, and XW carried out
438 the testicular pathology experiments and contributed to data interpretation; XM and SF
439 performed the immunohistochemical experiments and RNA extraction; SY wrote the
440 manuscript. All authors have read and approved the final manuscript.

441
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512 **Figure legends**

513

514 **Figure 1. Spermatogenesis damages were observed in COVID-19 patients.** Histological
515 analyses of the testicular sections from COVID-19 patients (patient-1, 2, 3, 4, and 5) and
516 uninfected control patients (control-1, 2, and 3) showing numerous degenerated germ cells
517 fell off to the lumen in seminiferous tubules of all five COVID-19 patients, whereas normal
518 spermatogenesis was observed in control patients. Spg, Spermatogonia; Spc,
519 Spermatocytes; Sd, Spermatids; Spz, Spermatozoa; Sc, Sertoli cells. Arrows indicate
520 degenerated germ cells. Scale bar=100µm.

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Figure 2. Germ cells but not Sertoli cells lost in the testes of COVID-19 patients. (A) Immunohistochemical staining of DDX4 (a germ cell marker) in the testicular sections of control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5) is shown. Scale bar=100µm. **(B)** Scatter plot showing the quantification of DDX4-positive cell numbers per tubule in (A). ***, $P < 0.001$ by Student's t -test. **(C)** Immunohistochemical staining of SOX9 (a Sertoli cell marker) in the testicular sections of control patient (control-3) and COVID-19 patients (patient-2, 3, and 4) is shown. Scale bar=100µm. **(D)** Scatter plot showing the quantification of SOX9-positive cell numbers per tubule in (C). *ns.*, not significant.

Figure 3. COVID-19 patient testes exhibit a prominent infiltration of various inflammatory cells. (A) Representative CD3 immunohistochemical staining images in the testicular sections of control and COVID-19 patients (patient-2, 3, and 4) are shown. Red arrows indicate CD3⁺ T lymphocytes. Scale bar=100μm. (B) Representative CD20 immunohistochemical staining images in the testicular sections of control and COVID-19

548 patients (patient-2, 3, and 4) are shown. Red arrows indicate CD20⁺ B lymphocytes. Scale
549 bar=100µm. (C) Representative CD68 immunohistochemical staining images in the
550 testicular sections of control and COVID-19 patients (patient-2, 3, and 4) are shown. Red
551 arrows indicate CD68⁺ macrophages. Scale bar=100µm. (D) Representative HLA-DR α
552 immunohistochemical staining images in the testicular sections of control and COVID-19
553 patients (patient-2, 3, and 4) are shown. Red arrows indicate HLA-DRA⁺ myeloid cells.
554 Scale bar=100µm. (E) Representative CD38 immunohistochemical staining images in the
555 testicular sections of control and COVID-19 patients (patient-1, 2, and 4) are shown. Red
556 arrows indicate CD38⁺ cells. Scale bar=100µm. (F) Representative human IgG
557 immunohistochemical staining images in the testicular sections of control and COVID-19
558 patients (patient-1, 2, and 4) are shown. Red arrows indicate IgG⁺ cells. Scale bar=100µm.

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Figure 4. SARS-CoV-2 can invade testis and attack testicular cells. (A) Representative SARS-CoV-2 Spike protein immunohistochemical staining images in the testicular sections of control (control- 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5) are shown. Black arrows indicate SARS-CoV-2 Spike S1 positive cells. Scale bar=100µm. (B) Electron microscopy of the testis from Patient-1, showing coronavirus particles suggestive of viral infection (viral particles highlighted by red arrows). (C) Representative ACE2 immunohistochemical staining images in the testicular sections of control (control- 3) and COVID-19 patient (patient-4) are shown. Black arrows indicate round spermatids; Black arrowheads indicate pachytene spermatocytes; Red arrows indicate Sertoli cells; Red arrowheads indicate spermatogonia. Scale bar=100µm. (D) Representative TMPRSS2 immunohistochemical staining images in the testicular sections of control (control- 3) and COVID-19 patient (patient-4) are shown. Black arrows indicate round spermatids; Black arrowheads indicate pachytene spermatocytes; Red arrows indicate Sertoli cells; Red arrowheads indicate spermatogonia. Scale bar=100µm. (E) RT-qPCR analyses of the relative ACE2 mRNA levels in control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5). (F) RT-qPCR analyses of the relative TMPRSS2 mRNA levels in control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5).

Fig.S2. Immunohistochemical stainings of ACE2 and TMPRSS2 in the testicular sections are shown. (A) Representative ACE2 immunohistochemical (IHC) staining images in the testicular sections of all control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5) are shown. Scale bars=100 μ m. **(B)** Representative TMPRSS2 immunohistochemical (IHC) staining images in the testicular sections of all control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5) are shown. Scale bars=100 μ m.

Fig.S3. RNA-seq analyses show differential gene expression and alternative splicing events in the testes of COVID-19 patients. (A) Heat-map of genes significantly deregulated (FDR<0.001 and fold change > 2) in testes of COVID-19 patients compared to those controls. (B) Volcano plot showing alternative splicing events of the deregulated genes in testes of COVID-19 patients compared to those controls. Five types of alternative splicing events are shown with different colors.

Table S1. The detection of nuclear acid of SARS-CoV-2 virus in FFPE testis samples of COVID-19 patients by RT-qPCR.

Table S2. Patient clinical characteristics and medical history.

Table S3. The total coding and lncRNA genes are identified from the testes of control and COVID-19 patients determined by RNA-seq analyses.

636 **Table S4.** The up- and down-regulated genes are identified from testes of COVID-19
637 patients determined by RNA-seq analyses.

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639 **Table S5.** 251 alternative splicing events are identified from deregulated genes determined
640 by RNA-seq.

Figures

Figure 1

Spermatogenesis damages were observed in COVID-19 patients. Histological analyses of the testicular sections from COVID-19 patients (patient-1, 2, 3, 4, and 5) and uninfected control patients (control-1, 2, and 3) showing numerous degenerated germ cells fell off to the lumen in seminiferous tubules of all five COVID-19 patients, whereas normal spermatogenesis was observed in control patients. Spg, Spermatogonia; Spc, Spermatocytes; Sd, Spermatids; Spz, Spermatozoa; Sc, Sertoli cells. Arrows indicate degenerated germ cells. Scale bar=100µm.

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Figure 4

SARS-CoV-2 can invade testis and attack testicular 573 cells. (A) Representative SARS-CoV-2 Spike protein immunohistochemical staining images in the testicular sections of control (control-3) and COVID-19 patients (patient-1, 2, 3, 4, and 5) are shown. Black arrows indicate SARS-CoV-2 Spike S1 positive cells. Scale bar=100µm. (B) Electron microscopy of the testis from Patient-1, showing coronavirus particles suggestive of viral infection (viral particles highlighted by red arrows). (C) Representative ACE2 immunohistochemical staining images in the testicular sections of control (control-3) and COVID-19 patient (patient-4) are shown. Black arrows indicate round spermatids; Black arrowheads indicate pachytene spermatocytes; Red arrows indicate Sertoli cells; Red arrowheads indicate spermatogonia. Scale bar=100µm. (D) Representative TMPRSS2 immunohistochemical staining images in the testicular

sections of control (control- 3) and COVID-19 patient (patient-4) are shown. Black arrows indicate round spermatids; Black arrowheads indicate pachytene spermatocytes; Red arrows indicate Sertoli cells; Red arrowheads indicate spermatogonia. Scale bar=100µm. (E) RT-qPCR analyses of the relative ACE2 mRNA levels in control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5). (F) RT-qPCR analyses of the relative TMPRSS2 mRNA levels in control patients (control-1, 2, and 3) and COVID-19 patients (patient-1, 2, 3, 4, and 5).

Figure 5

CoV-2 infection alters expression patterns of genes in testes of patients with COVID-19. (A) Volcano plot showing the deregulated genes in testes of COVID-19 patients compared to those controls. 2645 genes and 2789 genes were up regulated and down-regulated in COVID-19 patient testes, respectively. Significantly deregulated genes have an FDR of < 0.001 and fold change of > 2. Three biological replicates were indicated. (B-D) Gene ontology analyses of up-regulated genes (B), down regulated genes (C), and alternative splicing changed genes (D) that identified in testes of COVID-19 patients. Genes enriched in the viral life cycle (B), spermatogenesis (C) indicated fly-out according to the degree of differences. (E) Heat-map is showing the expression of the inflammatory cytokines in testes of COVID-19 patients using P value<0.05 as the cut-off. Genes significantly up-regulated or down-regulated using FDR<0.001 labeled with asterisks.

Supplementary Files

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- [TableS3RPKMofALLGeneswithPvalue.xlsx](#)
- [TableS4Geneexprdiffwithanno.xlsx](#)
- [TableS5Junctioneventrecords.xlsx](#)
- [TableS1Patientclinical.docx](#)
- [TableS2Virusdetection.docx](#)
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