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Letter

SARS-CoV-2 infection induces testicular injury in *Rhesus macaque*

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Dear Editor,

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the seventh coronavirus known to infect humans. The COVID-19 pandemic has spread to more than 200 countries, with more than 600 million confirmed cases and over 6.5 million deaths as of September 2022, posing a huge challenge to global public health. Clinical symptoms of COVID-19 have been observed, including fever, cough, fatigue, diarrhea, vomiting, and even neurologic symptoms, and the SARS-CoV-2 has been detected in multiple organs in patients. The receptor-mediated binding and entry is the key stage of viral infection of host cells and humans, and human angiotensin-converting enzyme 2 (ACE2) is the specific receptor mediating the entry of SARS-CoV-2 (Zhou et al., 2020). It has demonstrated that the expression of the ACE2 in the testis of the male reproductive system is high (Li et al., 2020), suggesting that the male reproductive system may be vulnerable to SARS-CoV-2 infection and the ACE2 may regulate testicular function and play a role in sperm production.

Preliminary studies have observed that COVID-19 tends to be more severe in men, with higher morbidity and mortality compared to women (Guan et al., 2020). Autopsy of the deceased due to COVID-19 revealed that the testes exhibit significant seminiferous tubular injury with varying degrees of germ cell reduction and damage, thickened basement membranes, swollen vacuolated Sertoli cells, reduced Leydig cells, and mild lymphocytic inflammation (Yang et al., 2020). Furthermore, assessment of semen quality and changes in hormone levels is also important to understand testicular function. Some researchers found a lower total sperm count and total motility and higher inactive spermatozoa counts in COVID-19 patients (Enikeev et al., 2022). Besides, a retrospective single-center study reported that COVID-19 patients had no statistically significant differences in serum testosterone and follicle-stimulating hormone (FSH) levels but decreased testosterone/luteinizing hormone (LH) and FSH/LH ratios, suggesting hypogonadism (Ma et al., 2021). Moreover, Takahashi et al. found that male patients had higher plasma levels of innate immune cytokines and poorer T cell activation. The impact of these differences on testicular function still needs to be evaluated (Takahashi et al., 2020). Results with respect to the presence of SARS-CoV-2 in semen and testes were contradictory; some researchers detected viral RNA or viral particles in semen or testis samples, while others failed to detect SARS-CoV-2 in these samples (Madjunkov et al., 2020). Therefore, whether SARS-CoV-2 invades and/or damages the male reproductive system has become a question worthy of further research.

Non-human primates (NHPs) serve as the golden standard to study the pathogenesis of SARS-CoV-2 infection in humans due to the phylogenetic, physiological, and immunological similarities between humans and NHPs. *Rhesus macaque* is a useful SARS-CoV-2 infection animal model for studying the pathogenesis of SARS-CoV-2 and to assess the safety and efficacy of novel vaccines and therapeutics (Abdel-Moneim and Abdelwhab, 2020). Most works focus on viral replication in the respiratory tract and viral pneumonia in *R. macaque*, but little is known about the pathological changes of the reproductive system.

Adult macaques aged 6–12 years were inoculated intratracheally with SARS-CoV-2 (IVCAS 6.7512) with 7×10^6 TCID50/animal, and the dynamics of the virus and its effects on pulmonary histopathogenesis were investigated. In our previous study, *R. macaque* was selected to study SARS-CoV-2 infection and to evaluate the effectiveness of several antibodies and vaccines (Shan et al., 2020). Here, the previously collected blood, testes, and epididymal tissues samples of 13 male macaques which infected with SARS-CoV-2 and 1 mock-infected control were used to

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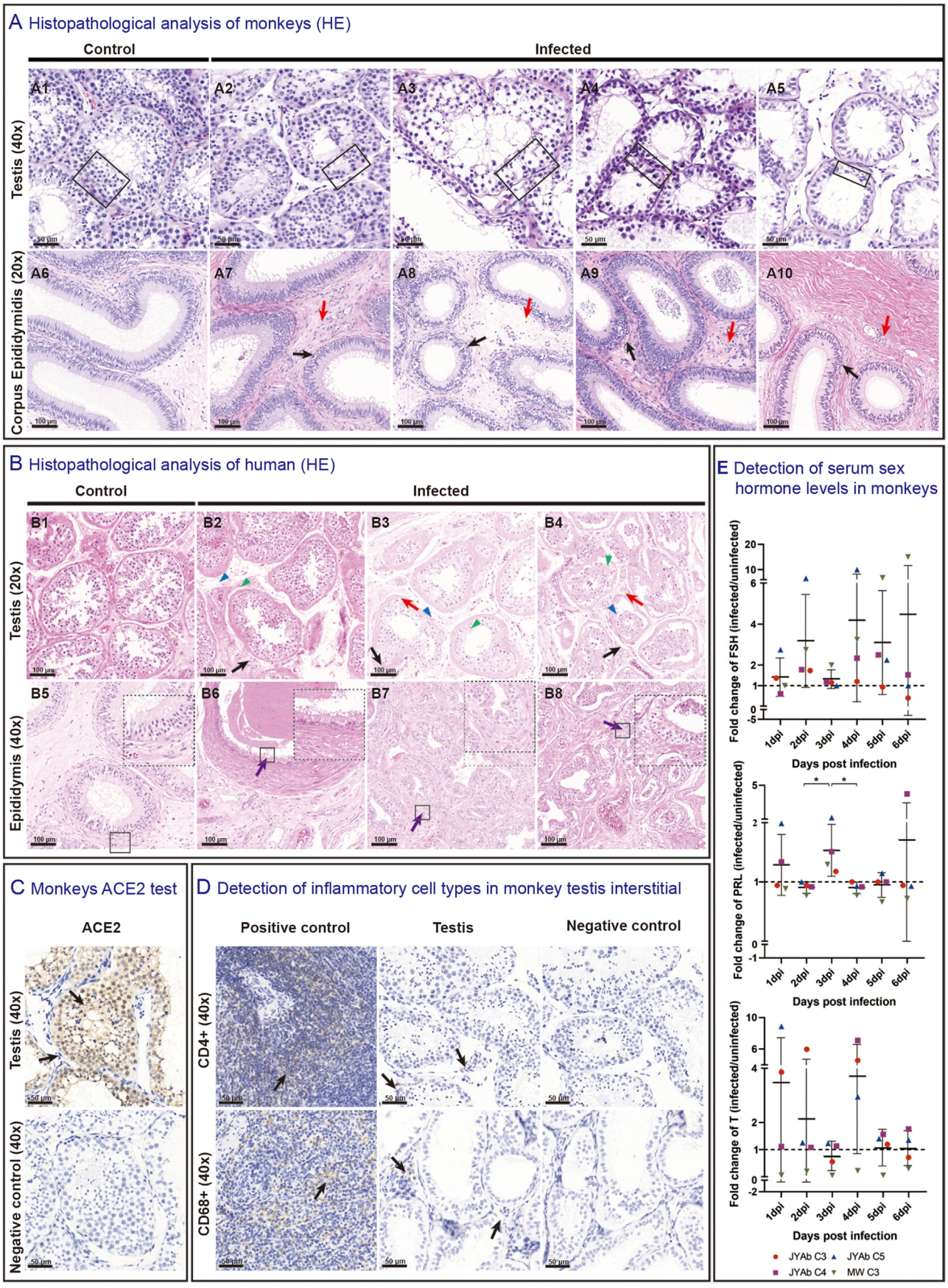

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evaluate the effects of viral infection on the male reproductive system. The testicular and epididymal tissues were dissected for RNA extraction and fixation with 10% formalin. Fixed biopsies were processed according to standard dehydration and paraffin wax infiltration protocols and subsequently embedded in paraffin blocks. The paraffin-embedded testicular and epididymal tissues were used for subsequent observation of pathological changes and detection of SARS-CoV-2 infection and inflammatory cell types. Serum samples were used to detect the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P), testosterone (T), prolactin hormone (PRL) in *R. macaque* before and after SARS-CoV-2 infection.

Consistent with most reported studies, the hematoxylin-eosin (H&E) staining analysis showed that testicular tissues had different degrees of damage, including four mild, three moderate, five severe, and one very severe (Fig. 1A, A2 to A5, Supplementary Table S1 and Figure S1). In mild injury (Fig. 1. A2), the number of epithelial layers was normal and the grades of spermatogenic cells were present, but the arrangement was slightly disordered, and the exfoliated germ cells were observed in the lumens of some seminiferous tubules. In moderate injury (Fig. 1A3), the number of epithelial layers was significantly reduced, but the grades of spermatogenic cells and the interstitial loose edema were still present. In severe injury (Fig. 1A4), seminiferous epithelial layers were dominated by a one-to two-layer structure, spermatogenesis stagnated in the primary spermatocyte stage, and sperm cells and sperm were not observed. In very severe injury (Fig. 1A5), all levels of spermatocytes were absent and the seminiferous epithelial layers were only a single-layer structure, dominated by Sertoli cells and edematous spermatogonia. However, the damage in all of the samples from corpus epididymidis was relatively mild (Fig. 1A, A6 to A10). In addition, we did not directly detect viral RNA in testicular tissues by RT-qPCR (Supplementary Table S2) in spite of positive test results for the lungs, throat swabs, and nose swabs (Shan et al., 2020). Moreover, although a broad distribution of ACE2 could be observed in the male reproductive system samples, positive signals could not be detected by N protein specific immunofluorescent assay and immunohistochemical assays (Fig. 1C and Supplementary Figure S2).

In addition, a postmortem examination of the testes from four deceased COVID-19 patients has been performed to observe histopathological changes by H&E staining (Fig. 1B and Supplementary Figure S3). The results showed that the testis and epididymis tissues from COVID-19 patients exhibited different degrees of injury from mild to severe in testicular tissues (Fig. 1B, B2 to B4), like reduced spermatogenic cells and spermatogenic epithelial layer, significant thickening of the basement membrane (Fig. 1B, Blue triangle and Supplementary Figure S3), lymphocyte infiltration, interstitial edema (Fig. 1B, Green triangle), and vascular congestion. Edema and necrosis of epididymis epithelium was observed in Epididymis (Fig. 1B, Purple arrow). Consistent with previous reports (Yang et al., 2020), COVID-19-related testicular injury was observed.

Of note, a number of viruses found in human and NHP testes can infect macrophages or testicular cells, such as Zika virus, Ebola virus, Marburg viruses, and mumps virus (Le Tortorec et al., 2020). Currently, it remains unclear whether SARS-CoV-2 can infect testicular cells. Testicular homeostasis disruption and inflammatory insult may be the main reasons of the pathological changes. Literature has shown that macrophages in testicular interstitium can secrete tumor necrosis factor, interleukin, and other cytokines, which participate in local regulation of paracrine and autocrine testicular function. In addition, these cells and the inflammatory cytokines they produce may also activate the testis' autoimmune response to form autoantibodies (Xu et al., 2006). To this end, we further confirmed that the mononuclear cell infiltrate within testes was mainly composed of $CD4^+$ T cells, whose proportion varied from 2% to 18% in the stroma, and $CD68^+$ macrophages, whose proportion varied from 3% to 8% in the stroma (Fig. 1D), which can lead to immune-mediated tissue injury (Kotb et al., 2005).

Abnormal levels of sex hormones and declining sperm quality were observed in patients during and after recovery from SARS-CoV-2. Therefore, we selected serum samples to test FSH, PRL, T, P, and E2 levels to analyze changes in sex hormones before and after SARS-CoV-2 infection. Compared to the pre-infection stage, changes were observed in the levels of sex hormones after infection (Fig. 1E and Supplementary Figure S4). Statistical analysis showed that PRL increased significantly on the third day, while P and E2 increased significantly on the fourth day. In addition, there was no significant statistical difference for FSH and T, this may be caused by the individual differences and limited sample size. But we can still observe that FSH levels showed a rising trend during the late infection stage, which may indicate that after infection, the hypothalamus–pituitary gland–gonads axis was affected. And T showed a characteristic trend of firstly rising and then falling, which is different from previous studies (Tian and Zhou, 2021). During the early infection stage, we speculated that T rise maybe involved into the process of the upregulation of ACE2 and TMPRSS2 caused by the activation of the androgen receptor (AR) (Qiao et al., 2020). The results showed that the serum levels of some sex hormones in *R. macaque* also changed significantly after infection with SARS-CoV-2.

Overall, we report a preliminary NHP-based study to determine the testicular pathophysiology of SARS-CoV-2 infections. Based on our results, unlike the direct clinical symptoms of the respiratory system due to SARS-CoV-2 infection and amplification, infection-associated inflammatory insult and sex hormone fluctuations may account for the testicular pathophysiology. However, it remains to be confirmed whether the data obtained from *R. macaque* could reflect the clinical and pathological features in COVID-19 patients, and the effects of SARS-CoV-2 infection on the male reproductive function need to be further investigated systematically. In addition, the possible long-term transgenerational effects of SARS-CoV-2 should be evaluated and monitored to provide guidance for the development of therapeutics assisted reproductive technologies.

Footnotes

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Fig. 1. Pathological analysis and changes of hormone levels in *Rhesus macaque* and human. **A** Histopathology of testes and epididymides of *R. macaque*. The testicular spermatogenic tubules show increasing degrees of testicular damage from A2 to A5, with decreased spermatogenic cells and seminiferous epithelial layers. A few cells were necrotic in the base of the epididymal epithelium (black arrow) and a few lymphocytes infiltrated in the stroma (red arrow). **B** Histopathology of testes and epididymides of human. In human testicular tissue, spermatogenic cells and spermatogenic epithelium decreased to varying degrees. B2 to B4 is from mild to severe. Blue triangle: thickened basement membrane; Green triangle: interstitial edema; Black arrow: edema of Spermatogonia or spermatocyte cells; Red arrow: Leydig cells edema; Purple arrow: epididymal epithelial cell damage. **C** Detection and localization of ACE2 in *R. macaque* (Shown by the black arrow). **D** Location of testicular interstitial inflammatory cell types (Shown by the black arrow). **E** Changes in follicle-stimulating hormone (FSH), prolactin (PRL), and testosterone (T) levels at 1–6 days compared with 0 days. Macaque identification No: JYAb C3/C4/C5 and MW C3 (macaque infected with SARS-CoV-2 from the same batch). *: $P < 0.05$.

All the data generated during the current study are included in the manuscript and supplementary material.

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