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Experimental Research Article

SARS-CoV-2-induced inflammation of testicular tissue mediated cytokine storm and microRNAs involved in apoptosis

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ABSTRACT

Introduction: Coronavirus spread rapidly around the world, posing a major challenge to medical science for controlling and treating its complications. It is well known that this disease can have devastating effects on various body systems, including the male reproductive system. This study aimed to evaluate the impact of COVID-19 on male testicular tissue, by focusing on cytokine storm and microRNAs involved in apoptosis.

Methods: In this case series study, which was conducted on 20 individuals with COVID-19 and healthy cases from March 2021 to May 2021 at the Iranian Legal Medicine Organization, Tehran, Iran. In this study, testicular tissue samples were obtained from the deceased participants approximately 8 to 12 hours after their death to ensure the preservation of tissue integrity and minimize postmortem changes. These samples, each measuring 3×3 cm², were collected. We examined the effects of COVID-19 on the number of immune cells (CD68 and CD3) using immunohistochemically staining. Furthermore, we performed a real-time PCR assay to assess the gene expression of CD68 and CD3 as well as miRNA-32, miRNA-146, miRNA-147, miRNA-148, and miRNA-152 in testicular specimens.

Results: Testicular tissue showed a marked increase in CD68 and CD3 positive cells, markers of macrophages and T-lymphocytes, respectively. Furthermore, our molecular analysis revealed that this viral infection increased the expression of CD68 and CD3 while decreasing that of MiR-146, MiR-148, and MiR-152.

Conclusion: Overall, COVID-19 could negatively affect the function of the male reproductive system through uncontrolled inflammation responses, as indicated by the current study. (www.actabiomedica.it)

Keywords:
COVID-19
MicroRNAs
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Introduction

Coronavirus disease 2019 (COVID-19) is a contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The World Health Organization (WHO) declared a pandemic on March 11, 2020, after the first case of the disease was found in Wuhan, China. As this virus spread rapidly and had a high mortality rate, controlling and finding an effective treatment quickly became a major challenge for medical science. So far, this disease has caused governments worldwide to incur enormous medical costs and has adversely affected people's quality of life (Abdel-Moneim 2021). It is not only possible for this virus to cause respiratory system disorders such as pneumonia and acute respiratory distress syndrome (ARDS), but many studies show that COVID-19 can severely affect the nervous system, cardiovascular system, liver, kidney, and reproductive system (Deshmukh et al., 2021). It has also been reported that the virus can be found in body fluids such as CSF and semen.

In order to enter its target cells, COVID-19 uses the angiotensin-converting enzyme 2 (ACE-2) receptor, which is located on the cell membrane (Moghimi et al., 2021) . According to previous studies, ACE-2 is highly expressed in various human tissues, including the lungs, brain, heart, ovaries, prostate, and testes. In light of these studies, researchers have suggested that ACE-2 receptors in testicular tissue may affect reproductive performance in men (Liu et al., 2020). The expression of ACE-2 receptors in Leydig cells, germ cells, and seminiferous tubules of testicular tissue has been clearly demonstrated in previous research. Many studies have been conducted in this field due to the importance of this issue, which have confirmed significant structural and functional changes in the reproductive organs of men infected with the COVID-19 virus. However, its exact mechanism of action on the reproductive system is unclear (Moghimi et al., 2021).

In recent studies, it has been shown that this virus increases the level of oxidative stress factors and expression of apoptotic-related genes (Bax, Caspase-3), whereas a significant drop was observed in the levels of anti-apoptotic factors (Bcl-2). Testicular tissue also showed an increase in TUNEL-positive cells (Novin et al., 2022). In addition, researchers have reported structural changes in testicular tissue samples from COVID-19-infected patients (Selvaraj et al., 2021). COVID-19 infection of testicular specimens significantly reduced both the length and volume of seminiferous tubules and interstitial tissue, as well as the number of testicular cells (Leydig, Sertoli, Spermatid, and Spermatogonia) (Yang et al., 2020). It is also reported that pro-inflammatory cytokines are upregulated, which affects the integrity of the BTB (blood-tissue barrier), which has devastating effects on germ cells and spermatogenesis. An increase in inflammatory factors can attract peripheral immune cells, such as macrophages and T cells, to the testicular tissue, which can further exacerbate inflammatory conditions (Wang et al., 2020; Zhang et al., 2014).

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs made from DNA by RNA polymerase II and III. It has been established in numerous studies that these non-coding molecules play a fundamental role in regulating gene expression as well as cell-to-cell communication (Bayraktar et al., 2017). MiRNAs play a role in apoptosis, autophagy, inflammation, cell differentiation, and the cell cycle (Pourhadi et al., 2022). Various pathological conditions, such as viral diseases, cardiovascular disease, diabetes, psoriasis, schizophrenia, and cancer, alter miRNA levels. We aim to evaluate the impact of COVID-19 on male testicular tissue, specifically the expression of miRNA-32, miRNA-146, miRNA-147, miRNA-148, and miRNA-152.

Material and Methods

Study design

The case-control study included a total of 20 participants, divided into 2 groups: cases (n=10) and controls (n=10). The cases were individuals diagnosed with COVID-19 based on computed tomography (CT) imaging and real-time PCR testing. They were matched with healthy controls of the same age range (45-60 years) who did not have a history of reproductive disorders and had tragically passed away due to non-COVID-19-related causes, such as electrocution, carbon monoxide poisoning, or accidental incidents. The control group had no prior history of major illnesses. The study utilized testicular tissue samples obtained from the deceased participants. The collection process was carried out approximately 8 to 12 hours after death to ensure the preservation of tissue integrity and minimize postmortem changes. The samples, each measuring 3 x 3 cm2, were collected from March 2021 to May 2021 at the Iranian Legal Medicine Organization.

TABLE 1: Primers' design.

Gene name	Primers sequences
CD68	F: CCGGAATTCTGCTGGGGCTACTGGCAG R: TGATCTAGAGTCCCCTGGGCTTTTGGCAG
CD3	F: TGC AAG GTT CAC AGT CTT GC R: TTC CAC GAC AGA CAG AAC TC
Mir-32	F: TATTGCACATTACTAAG R: GTGCAGGGTCCGAGGT S: GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACTTGCA
Mir-146	F: TGAGAACTGAATTCCAT R: GTGCAGGGTCCGAGGT S: GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACGGGTT
Mir-147	F: GTGTGTGGAAATGCT R: GTGCAGGGTCCGAGGT S: GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACTCTGC
Mir-148	F: AAAGTTCTGAGACACTC R: GTGCAGGGTCCGAGGT S: GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACCGACT
Mir-152	F: AGGTTCTGTGATACACTC R: GTGCAGGGTCCGAGGT S: TCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACCGACT
U6	F: CGCTTCGGCAGCACATATAC R: AAATATGGAACGCTTCACGA

Immunohistological evaluations

Using immunohistochemistry, testicular tissues were analyzed for the presence of macrophages (CD68+) and T-lymphocytes (CD3+). Firstly, all testicular specimens were fixed in Bouin's solution for 48 hours and then transformed into 10% formalin. After the routine histological process, sections were made using a sliding microtome (Leica RM2125 RTS, Germany) with 5 µm thickness and put on poly-l-lysine slides. In the following step, testicular sections obtained from each block were deparaffinized using fresh xylene, rehydrated in an ethanol gradient with decreasing concentration, and then washed in Tris-buffered saline for a total of 10 minutes. Endogenous peroxidase activity was blocked after antigen retrieval, and all sections were then incubated overnight at 4°C with primary antibodies against CD86 and CD3 antigens. The sections were then washed three times with TBS, followed by a two-hour incubation with secondary antibodies (antibodies details). 3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark) was added after washing. All tissue sections were then counterstained with Mayer's hematoxylin. Tonsil sections were used as positive controls. For the negative control, the tissue sample is processed without the primary antibody but includes all other steps, such as blocking, secondary antibody application, and detection. In the end, the number of positive cells was counted under light microscopy and reported as a numerical density (number/mm^3), while the percentage of positive CD36 and CD8 cells in testis tissues was measured by counting 4 fields in each of 10 sections.

Molecular assessment

Real-time PCR was used to assess CD86 and CD3 genes, as well as miRNA-32, miRNA-146, miRNA-147, miRNA-148, and miRNA-152 expression in testicular samples. Following the manufacturer's protocol, the whole RNA was isolated from testicular samples with the RNX-Plus kit (SinaClon). DNase I (Roche, Basel, Switzerland) was applied to remove likely genomic DNA after RNA extraction. In order to synthesize cDNA from isolated RNA, a commercial kit (Fermentas, Lithuania) was used at 42 °C for 60 minutes according to the manufacturer's instructions. Additionally, miRNAs were evaluated using isolated RNA. cDNA was synthesized by the TaqMan microRNA Reverse Transcription kit following the manufacturer's protocol. To confirm their specificity, we used the NCBI BLAST tool (http://

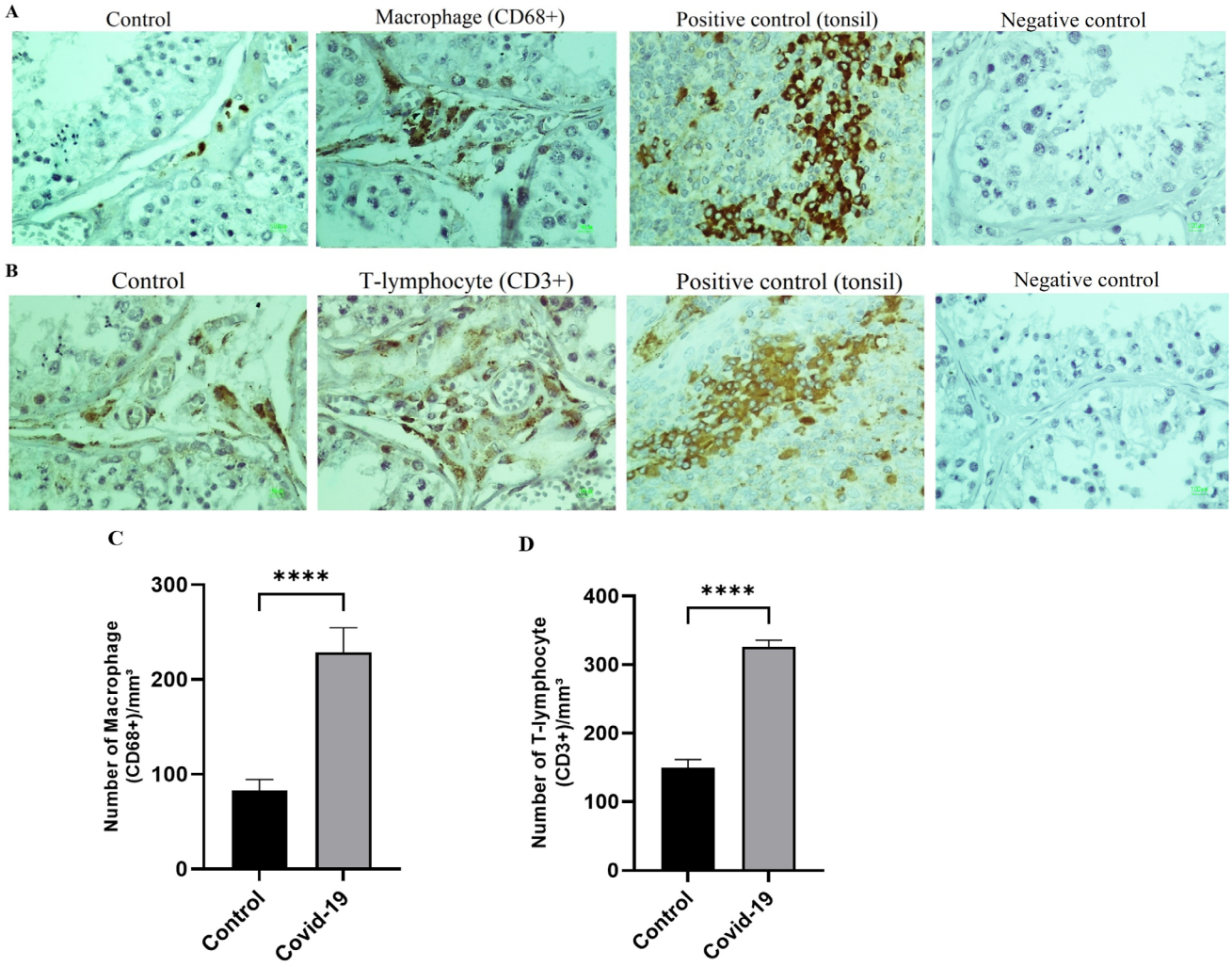

FIGURE 1. Micrographs show testicular IHC staining against CD68 and CD3 markers (A-B). The number of macro-phages and T-lymphocyte increased in COVID-19 patients (C-D). (****P<0.0001). (Scale bar: 10µm). Mean±SD.

www.ncbi.nlm.nih.gov/BLAST) to produce primer sets (forward and reverse) based on NCBI sequence databases (Table 1). The relative expression of genes was quantified using real-time PCR (TaqMan) using the Quanti-Tect SYBR Green RT-PCR kit (Takara Bio Inc., Japan).

Statistical analyses

Independent samples data were analyzed using SPSS software (version 23). The normal distribution of quantitative data was analyzed using Kolmogorov–Smirnov test. Paired Student's t-tests was used for statistical analysis. Statistical significance was considered at a p-value ≤ 0.05. Data are presented as mean ± standard deviation (SD).

Results

Demographic Characteristics:

Age: The participants' age range fell between 45 and 60 years.

Gender: All participants were men, and all of them were married. Unfortunately, the following information was not available for all participants:

Income Level: Information on income levels was not collected for all participants, leading to incomplete data on their economic status.

Ethnic Origin: Ethnicity details were not available for all participants, making it challenging to provide a comprehensive analysis of the ethnic composition of the sample.

Resources: This study utilized various resources for data collection and analysis.

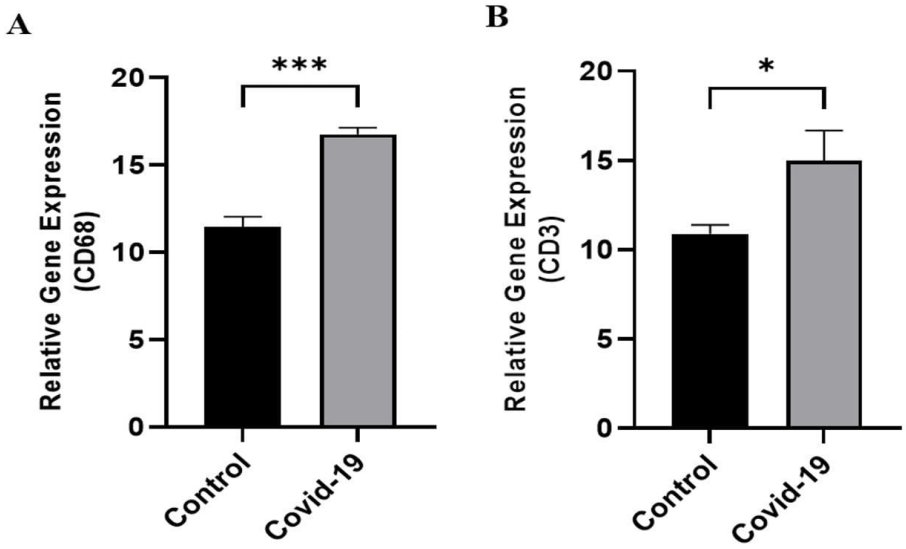

FIGURE 2. Graphs illustrate the impact of COVID-19 on CD68 and CD3 gene expression. COVID-19 up-regulate CD68 and CD3 expression in testicular samples. ($^{***}P<0.0001$, $^{*}P<0.05$). Mean±SD.

Medical Records: The participants' medical histories, death certificates, and autopsy reports were carefully reviewed to identify underlying health conditions and causes of death.

Informed Consent: Before performing postmortem examinations and obtaining tissue samples, explicit informed consent was obtained from the families of the deceased.

COVID-19 increased the number of CD68 and CD3 cells in the testicular tissue

We carried out an immunohistochemical assay to evaluate the number of macrophages and T-lymphocytes. We carried out an immunohistochemical assay to evaluate the number of macrophages and T-lymphocytes. Our stereological assessment revealed a marked increase in the number of CD68-positive (Macrophage marker) as well as CD3-positive cells (T-lymphocyte marker) in COVID-19 testicular samples compared to control samples ($^{****}P<0.0001$) (Fig. 1 A-D).

COVID-19 elevated the level of CD68 and CD3 gene expression

At the molecular level, we measured the amount of CD68 and CD3 mRNAs expression by the polymerase chain reaction (PCR) method. Our data revealed that COVID-19 caused a considerable elevation in CD68 and CD3 in the testicular specimens ($^{***}P<0.001$, $^{*}P<0.05$, respectively) (Fig. 2 A-B).

COVID-19 dropped the level of Mir-146, Mir-148, and Mir-152

The gene expression level of Mir-32, Mir-146, Mir-147, Mir-148, and Mir-152 was examined by the Real-time PCR method. Our PCR data showed a marked increase in the level of Mir-146 ($^{**}P<0.01$), Mir-148 ($^{*}P<0.05$), and Mir-152 ($^{**}P<0.01$) in COVID-19 patient samples compared to the control group. Furthermore, we observed a reduction in Mir-32 and elevation in Mir-147 gene expression in the COVID-19 group in comparison with the control group, but it was not statistically significant (Fig. 3A- E).

Discussion

In the present investigation, our focus was to explore the impact of the SARS-CoV-2 virus on the testicular tissue of individuals afflicted with COVID-19. Through extensive PCR analysis, our study revealed noteworthy upregulation of CD68 and CD3 genes and proteins, which are indicative of the increased presence of T lymphocytes and macrophages, within the testicular tissue of COVID-19 patients as compared to the healthy control group. Furthermore, our research uncovered intriguing alterations in gene expression within testicular samples affected by COVID-19. Specifically, we identified significant changes in the expression of Mir-146,

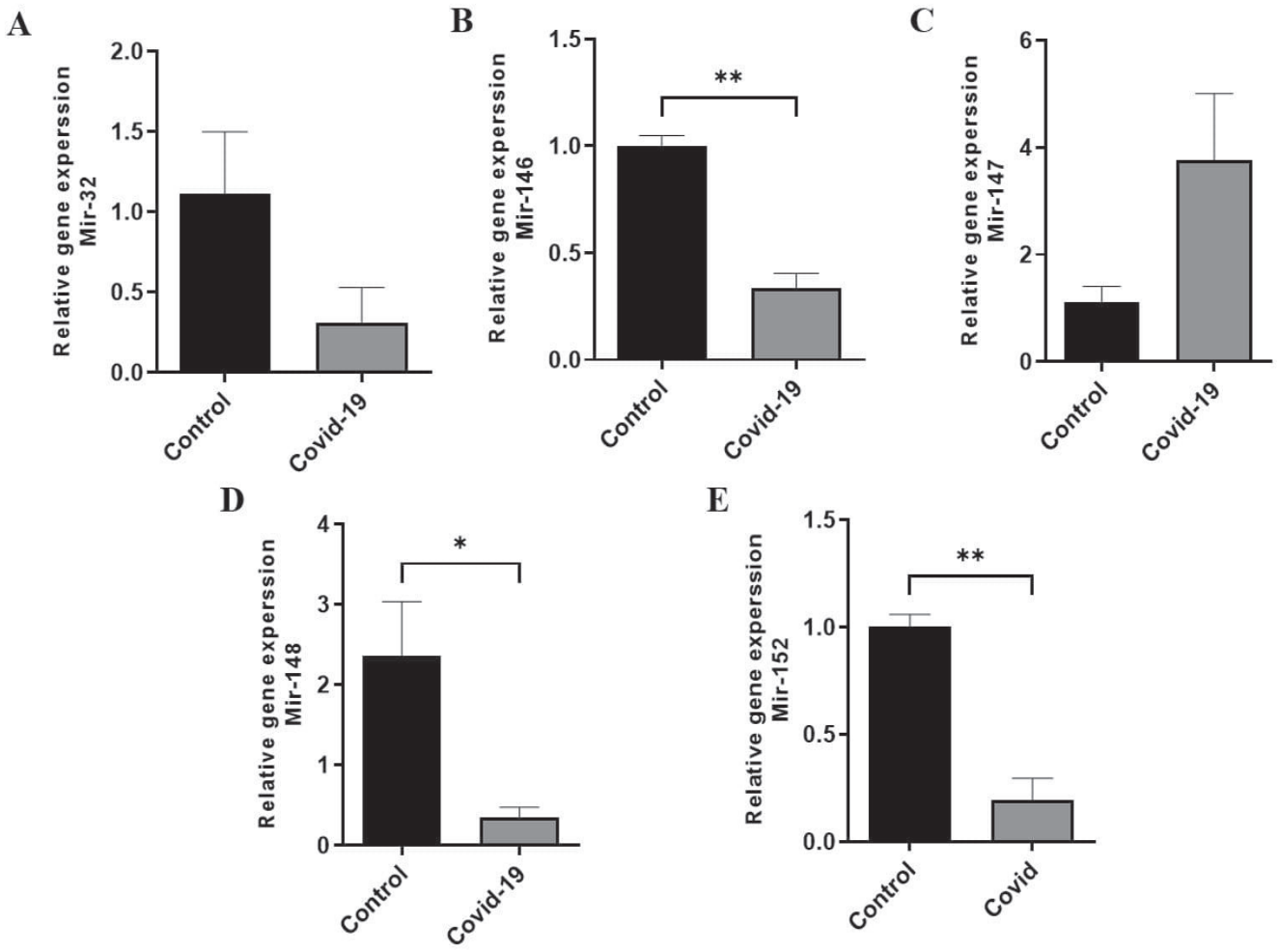

FIGURE 3. These graphs show the impact of COVID-19 on the level Mir-32, Mir-146, Mir-147, Mir-148, and Mir-152 gene expression. COVID-19 decrease the level of Mir-146, Mir-148, and Mir-152 ($^{*}P<0.05$, $^{**}P<0.01$). Mean±SD.

Mir-148, and Mir-152 genes.

Nowadays, there is a multitude of studies that demonstrate COVID-19 could impact almost all systems of the human body, such as the nervous system, cardiovascular system, gastrointestinal system, and respiratory system, as well as the reproductive system (Deshmukh et al., 2021). Therefore, many studies have been conducted to examine the effects of this virus on the male reproductive system, the results of which confirm the adverse effects of COVID-19 on this system. But despite many studies in this field, the exact mechanism of action of COVID-19 is still not known correctly (Deshmukh et al., 2021). Nevertheless, some researchers have reported that COVID-19 may affect the male reproductive system through direct effects on sperm via interaction with ACE-2 receptors, enhanced immune cell response, and changes in hormone levels(Moghimi et al., 2021; Selvaraj et al., 2021). In this study, we investigated the effects of this virus on the testicular tissue of patients with COVID-19. The PCR results of this study showed increased expression of the CD68 and CD3 (T lymphocytes and macrophages) genes in the testicular tissue of people with a SARS-CoV-2 virus infection compared to the healthy group, which is consistent with our histopathological findings. In addition, we found that COVID-19 infection could affect Mir-146, Mir-148, and Mir-152 gene expression in testicular samples.

Based on previous studies, the ACE-2 receptor has been found in many body systems, including the nervous system, respiratory system, cardiovascular system, and reproductive system(Ardestani Zadeh and Arab 2021). Especially, in the reproductive system, it is well documented that the ACE-2 receptor is highly expressed in testicular tissue cells (Leydig, Sertoli, in-

terstitial, and germ cells) (Divani et al., 2020). Hence, researchers have suggested that COVID-19 could have a direct impact on the reproductive system through the interaction with ACE-2 (8). It is also proposed that interaction between COVID-19 and ACE-2 receptors on testicular cells may initiate the inflammatory process which finally leads to Leydig and Sertoli cells dysfunction(Abdel-Moneim 2021).

Similar to other viral infections, such as the Influenza virus, the Mumps virus, and HIV, in patients infected with COVID-19, inflammatory signaling pathways play an important role in the pathogenesis of COVID-19 infection, which can result in multiorgan failure. It has also been established that pathological conditions in testicular tissue can cause infiltration of peripheral immune cells (lymphocytes and macrophages) (Renu et al., 2020). The study by *Maleki et al. 2021* showed that high levels of TGF-β, IL-6, IL-8, IFN-α, TNF-α, IL-1β, and IFN-γ were present in the seminal plasma of COVID-19 patients (Hedger 2011). In addition, they reported that this viral infection causes an impairment in the levels of oxidative and antioxidant factors in seminal samples (34). Several studies have found that inflammatory processes in pathological conditions can initially have positive effects. On the contrary, the lack of control of this process and the excessive progression of inflammation can cause irreparable complications(Maleki and Tartibian 2021). In this regard, another investigation showed an increased level of pro-inflammatory cytokines such as TNF-α, IL-1β , and IL-6 in the testicular tissue of COVID-19 patients in comparison with healthy people. Researchers suggested that significant increases in the production of inflammatory factors have negative effects on BTB integrity(Dong et al., 2021).

In this regard, the results of *Peirouvi et al*. 2021 also showed a significant increase in the number of macrophages and pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β in the testes of patients infected with COVID-19. They suggested that inflammatory conditions could impact testicular cells (Leydig, Sertoli, and germ cells) and disrupt their normal functions (Olaniyan et al., 2020). Interestingly, it was reported that macrophages have vital roles in normal Leydig cell development in non-inflammatory situations. In addition, increased activation of immune cells in the testes results in a marked elevation of ROS, such as hydrogen peroxide production, which could carry on with apoptosis induction in testicular cells (Peirouvi et al., 2021). Consistent with the mentioned studies, our data showed that COVID-19 increased mRNA levels of markers CD68 and CD3. In addition, the immunocytochemical results of this study indicated an increased presence of macrophages and lymphocytes in the testicular tissue.

Nowadays, it has been suggested that multiple mechanisms are involved in the pathogenesis of COVID-19 infection in reproductive system dysfunction, such as cell apoptosis, autophagy, ROS production, and inflammation(Hales 2002). A bulk of studies described that microRNAs, small and non-coding RNAs, have pivotal roles in the regulation of the mentioned processes (Sun 2020). Previous studies have shown a miR-146a deficit in some inflammatory diseases, such as brain, heart, skin, lungs, and autoimmune diseases (Donyavi et al., 2021). MiR-146a is accepted as a strong regulator of immune cell activities. It is hypothesized that this factor could suppress excessive inflammation response through the inhibition of various TLR-signaling pathway factors, such as interferon regulatory factor 5 (IRF5), tumor receptor-associated factor 6 (TRAF6), and IL-1 receptor-activated kinase 1 (IRAK1). A recent study demonstrated that miR-148 functions as a key regulator of the inflammatory response by modulating macrophage activity during infection through the NF-κB signaling pathway, ultimately leading to a marked reduction in the progression of the inflammatory process. Moreover, several studies indicated a significant change in the level of Mir-152 gene expression in some inflammatory diseases (Roganović 2021). For instance, it is well-documented that inflammatory response is one of the most important factors in the pathogenesis of spinal cord injury (Ma et al., 2020; Vafaei-Nezhad et al., 2021). In this regard, Zhang et al, 2012 showed that MiR-152 could reduce the inflammatory response by modulating the c-Jun N-terminal kinase (JNK) pathway, thereby enhancing functional recovery.Additionally, upregulation of this microRNA has the potential for suppression of inflammatory cytokines (Khatmi et al., 2022).

In line with the above studies, which demonstrated a decrease in the level of MiR-146, MiR-148, and MiR-152 in inflammatory disorders, our results also showed a significant decrease in the gene expression level of these factors in the testicular tissue of COVID-19 patients. According to our data, we propose that a decrease in these inflammatory regulators' microRNA could cause a

progressive inflammatory response. These uncontrolled inflammatory reactions may result in male reproductive system dysfunction.

Conclusion

To sum up, the results of current studies have revealed that the male reproductive system, especially the testes, is severely affected by COVID-19 infection. Although there is a bulk of research to find the exact mechanism of this virus on testicular function, it is not well understood. Our data revealed that this viral infection causes the down-regulation of some microRNAs, which control the inflammatory process. Besides, our results demonstrated that immune cells were also increased in testicular tissue. Hence, controlling the inflammation in this infection can have beneficial effects on male reproductive functions.

Authors Contribution

MA. A and MH. H designed this study, conducted the stereological study, and drafted the manuscript. A. S helped draft the manuscript and carried out the molecular test. P. G and A. H helped design the study, contributed the statistical analysis, and wrote the manuscript draft. S. E, S. VN, and R. S helped draft the manuscript and performed the statistical analysis. GR. M provided the clinical sample and clinical data. S. A and A. A carried out the TUNEL assay. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that they have no competing interests.

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Ethic Committee

The Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran approved this study. (Approval code: IR.SBMU.MSP.REC.1401.127). Postmortem investigations were conducted with meticulous attention to the medical history of the cases, including death certificates and autopsy reports. These examinations were carried out only after obtaining informed consent from the bereaved family members. Following the demise of the individuals, postmortem procedures were meticulously performed, allowing the collection of relevant tissue samples for further analysis.

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