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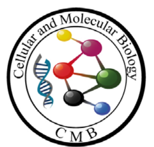

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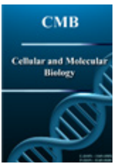

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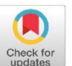

Impact of COVID-19 infection on cyclooxygenase-2 gene expression and mast cell count in testicular tissue of azoospermic men

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Abstract

The COVID-19 pandemic has posed significant threats to human life and health. Numerous studies have shown that men are more vulnerable to this infection, and recent evidence suggests that the presence of angiotensin-converting enzyme 2 (ACE2) receptors in male reproductive tissues may particularly predispose them to viral infection. Therefore, it is crucial to assess the potential impact of COVID-19 infection on male fertility. This study investigates the relationship between COVID-19 and the expression of inflammatory proteins, particularly mast cells and cyclooxygenase-2 (COX-2), in the testicular tissue of azoospermic men undergoing testicular sperm extraction (TESE). The study included 41 TESE candidates who were referred to the Besat Infertility Treatment Center in Kurdistan, Iran. Demographic information, such as age, was recorded for each participant. The subjects were divided into two groups: 20 non-infected and 21 infected with COVID-19. Testicular tissue samples were fixed in formalin and prepared for microscopic examination using toluidine blue staining and immunohistochemistry to assess the distribution and number of mast cells and COX-2 positive cells. Data analysis was performed using SPSS software version 27. The results showed that COX-2 gene expression and the number of mast cells were significantly higher in individuals infected with COVID-19 compared to the non-infected group. This increase in gene expression and mast cell count indicates elevated inflammation in the testicular tissue of COVID-19-infected individuals, which could lead to reduced fertility. This study aligns with previous research highlighting the role of inflammation in testicular tissue damage and decreased fertility.

Keywords: Coronavirus; Mast Cells; COX-2 Expression; Azoospermia.

1. Introduction

With the outbreak of the COVID-19 pandemic, humanity faced unprecedented challenges to health and well-being. While the primary focus has been on the respiratory system, it has become increasingly evident that COVID-19 is a multi-system disease with far-reaching consequences [1]. Beyond the immediate respiratory complications, the virus has been associated with cardiovascular, neurological, and immunological effects, many of which may have long-term implications. As the pandemic evolves, researchers continue to explore the broader systemic impacts of SARS-CoV-2, including its potential effects on reproductive health, particularly in men [2].

One of the critical concerns regarding male reproductive health stems from the discovery that SARS-CoV-2 targets tissues rich in angiotensin-converting enzyme 2 (ACE2) receptors. These receptors are highly expressed in various organs, including the lungs, heart, kidneys, and notably, the testes [2, 3]. The presence of ACE2 receptors in both germinal and non-germinal cells of the seminiferous tubules makes the testicular tissue particularly vulnerable to viral invasion, which can trigger a cascade of inflammatory and immunological responses [3, 4]. Consequently, understanding the pathophysiological changes induced by COVID-19 in testicular tissue has become an important area of investigation [2].

Emerging evidence suggests that SARS-CoV-2 infection can significantly disrupt male reproductive function. Studies have reported that the virus can cause orchitis (inflammation of the testes), damage to the blood-testis barrier, and alterations in the hormonal milieu necessary for normal spermatogenesis [5]. This disruption may result in

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reduced sperm count, motility, and overall quality, raising concerns about the potential for temporary or even permanent infertility in affected men [6]. Autopsy findings from deceased COVID-19 patients have revealed extensive damage to testicular architecture, including the destruction of spermatogenic cells, infiltration of immune cells such as macrophages, and a significant reduction in Leydig and Sertoli cells, which are critical for testosterone production and spermatogenesis [6, 7].

Another important aspect of the inflammatory response in testicular tissue is the upregulation of cyclooxygenase-2 (COX-2), a key enzyme involved in the synthesis of pro-inflammatory prostaglandins. Increased COX-2 expression has been associated with impaired sperm function and quality, suggesting a potential link between COVID-19-induced inflammation and male infertility [5, 8]. Additionally, mast cells, which play a crucial role in the immune response, have been shown to infiltrate testicular tissue during inflammation, further contributing to tissue damage and functional impairment [7].

Despite these findings, significant gaps remain in our understanding of how COVID-19 impacts male fertility, particularly in specific patient populations such as azoospermic men. Azoospermia, a condition characterized by the absence of sperm in the ejaculate, can have various etiologies, including genetic, hormonal, and obstructive factors [6]. However, the impact of systemic infections like COVID-19 on the testicular environment and spermatogenesis in azoospermic men has not been thoroughly investigated.

Given the critical role of the testes in male fertility and the potential long-term consequences of COVID-19 on reproductive health, it is essential to elucidate the mechanisms underlying testicular inflammation and damage in affected individuals. Understanding these mechanisms could pave the way for targeted interventions aimed at preserving fertility in men recovering from COVID-19 [9].

Therefore, the present study investigates the relationship between COVID-19 and the expression of inflammatory proteins, particularly mast cells and COX-2, in the testicular tissue of azoospermic men who underwent testicular sperm extraction (TESE). By examining the inflammatory landscape of testicular tissue in this unique patient population, this study aims to shed light on the potential reproductive consequences of COVID-19 and contribute to the development of strategies for fertility preservation in affected men.

2. Materials and Methods

2.1. Participants and Data Collection

This retrospective cohort study, conducted in 2023, involved men who visited the infertility treatment center at Besat Hospital in Kurdistan, Iran. Participants eligible for testicular sperm extraction (TESE) were selected based on the following inclusion criteria: age between 30 and 50 years, no history of surgery, no use of medications during the study, azoospermia status, no family history of infertility, and no underlying diseases. Patients with incomplete or inaccurate records were excluded from the study.

Participants were divided into two groups: 20 non-COVID-19 individuals and 21 COVID-19-positive individuals. COVID-19 status was self-reported by participants. While larger sample sizes increase the precision and power of cohort studies in detecting small differences between groups, the population of azoospermic men is relatively small. Therefore, due to cost, time, and logistical constraints, a larger sample size was not feasible.

All study procedures adhered to the ethical guidelines of the Medical Ethics Committee of Kurdistan University of Medical Sciences. Participant confidentiality was maintained throughout the study, and findings were accurately reported. The study was approved by the Ethics Committee of Kurdistan University of Medical Sciences under code IR.MUK.REC.1402.128.

2.2. Histological Studies

Samples for TESE diagnosis were obtained, fixed in formalin, and stained with Toluidine Blue. Testicular tissue samples were collected during biopsy surgery by a urologist. After opening the scrotum and tunica albuginea, a portion of the tunica vaginalis was selected for sampling from the seminiferous tubules, where sperm production is most likely. Tissue samples were isolated in a sterile environment using sperm culture medium (Pro Gena Teb, Iran) under an inverted microscope.

Some of the collected tissue was transferred to formalin for fixation to prepare it for immunohistochemical and histological analyses prior to isolating testicular germ cells. After fixation, the tissues were processed as follows:

1. The tissue was immersed in 70% ethanol for 1 hour, followed by an additional 2 hours in 70% ethanol.
2. Sequential immersions in 80% ethanol (2 hours), 90% ethanol (1.5 hours), and 100% ethanol (1.5 hours) were conducted, concluding with another 2-hour immersion in 100% ethanol.
3. The tissue was cleared in xylene twice for 2 hours each.
4. Paraffin embedding was performed in two steps, each lasting 2 hours at 58°C.

The paraffin-embedded tissues were placed into molds. To ensure proper embedding, melted paraffin was poured into the molds, and the tissue was transferred so that the cut surface faced downward. The mold was cooled on a cold plate to solidify the paraffin, holding the tissue in the desired shape. The paraffin blocks were then removed from the molds.

Tissues were sectioned using a microtome. The tissue blocks were cooled on ice for 10 minutes before sectioning. Sections of 10 µm thickness were initially cut, followed by sections of 5 µm thickness. Four 5 µm sections were prepared for histochemistry (mast cells) and immunohistochemistry (COX-2) studies.

2.3. Toluidine Blue Staining Method

Toluidine blue staining was used for histochemical studies to assess mast cell populations in tissue sections [9]. Using a 20x magnification objective, cell counts were performed in areas of 100 µm². The average cell count for these areas was calculated.

A toluidine blue solution (pH 2.0–2.5) was prepared by mixing 5 ml of toluidine blue stock solution (Ariateb, Iran) with 45 ml of 1% sodium chloride, adjusting the pH to ~2.3. Tissue sections were hydrated and incubated in the staining solution for 2–3 minutes. The samples were rinsed three times with distilled water, dehydrated with 95% ethanol, and then immersed twice in 100% ethanol. Tissue sections were cleared in xylene in two steps, each lasting 3 minutes. The slides were mounted using Entellan adhe-

sive and cover slips. Mast cells stained with toluidine blue appeared dark blue under a light microscope.

2.4. COX-2 Immunohistochemistry Staining

This method stained the cytoplasmic COX-2 protein brown [10]. Sections of 5 µm thickness were hydrated and underwent antigen retrieval using sodium citrate buffer. Fixation in formalin resulted in cross-linking, potentially masking antigens and reducing antibody binding. Antigen retrieval enhanced epitope exposure and improved antibody access.

Antigen Retrieval Process Slides were immersed in sodium citrate buffer and heated to 95–100°C for 20 minutes. They were then cooled for another 20 minutes before proceeding to staining. Non-specific binding sites were blocked using 3% hydrogen peroxide in methanol, followed by washing with PBS. The primary antibody (anti-COX2/cyclooxygenase; Abcam 15191) was applied at a 1:600 dilution, and slides were incubated overnight in a humid chamber. After PBS washing, an HRP-conjugated secondary antibody (Rabbit human IgG; Abcam 6759) was added and incubated for 1 hour.

Tissue sections were incubated in 3% diaminobenzidine in hydrogen peroxide in the dark for 30 minutes, and signals were checked microscopically. Cell nuclei were counterstained with hematoxylin for 5 minutes. Slides were dehydrated in graded ethanol, mounted with Entellan adhesive, and analyzed under a microscope. COX-2-positive areas appeared brown.

2.5. Statistical Analysis

Data were analyzed using SPSS software version 27. Descriptive statistics such as means, standard deviations, frequencies, and charts summarized the data. Outcome variables between the two groups (COVID-19-positive and COVID-19-negative) were compared using appropriate statistical tests. For continuous variables, normality was assessed using the Kolmogorov-Smirnov test. Parametric tests (independent sample t-tests) were used for normally distributed data, while non-parametric Mann-Whitney tests were applied for non-normal data. A significance level of 0.05 was considered for all tests.

3. Results

3.1. Demographic Information

A total of 41 azoospermic individuals participated in this study. The overall mean age was 37.2 ± 4.42 years. These individuals were divided into two groups for the evaluation of hormonal and histological outcomes:

Group 1 (Non-COVID-19): Included 20 participants with a mean age of 36.95 ± 4.50 years.

Group 2 (COVID-19 positive): Included 21 participants with a mean age of 37.09 ± 4.49 years.

3.2. Descriptive Statistics

Table 1 presents the descriptive statistics for COX-2 gene expression and the total number of mast cells across all participants.

3.3. Normality and Descriptive Indicators by Group

Table 2 provides descriptive and normality indices for the two variables (COX-2 gene expression and mast cell count) by group. Both factors were higher in the COVID-19 group compared to the non-COVID-19 group. Normality was confirmed for all variables across groups, with skewness, kurtosis, and the Kolmogorov-Smirnov test indicating no significant violations of normality.

3.4. Inferential Statistics

To compare the two groups (COVID-19 positive vs. non-COVID-19) for the two factors (COX-2 gene expression and mast cell count), independent t-tests and the Mann-Whitney U test were employed. The results are summarized in Table 3.

The COVID-19 group exhibited a significantly higher mean COX-2 gene expression compared to the non-CO-

Table 1. Descriptive Statistics for COX-2 Gene Expression and Mast Cell Count.

Variable	Minimum	Maximum	Mean	Standard Deviation
COX-2 Gene Expression	62	133	99.51	18.84
Mast Cell Count	53	123	88.53	17.57

Table 2. Descriptive and Normality Indices for COX-2 Gene Expression and Mast Cell Count.

Variable	Group	Minimum	Maximum	Mean	Std. Deviation	Skewness	Kurtosis	PP (Normality)
COX-2 Gene Expression	Non-COVID-19	62	121	90.60	17.37	0.02	-1.40	0.10
	COVID-19	75	133	108.00	16.38	-0.09	-0.74	0.20
Mast Cell Count	Non-COVID-19	53	110	81.75	15.81	-0.05	-1.12	0.20
	COVID-19	65	123	95.00	15.81	0.11	-0.96	0.19

Higher mean expression in the COVID-19 group (108.00±16.38) compared to the non-COVID-19 group (90.60±17.37). Also, higher mean mast cell count in the COVID-19 group (95.00±15.81) compared to the non-COVID-19 group (81.75±15.81). No significant deviations from normality were observed in the variables based on skewness, kurtosis, or the Kolmogorov-Smirnov test (P > 0.05).

Table 3. Independent t-test Results for COX-2 Gene Expression and Mast Cell Count.

Variable	Levene's Statistic	tt-value	dfdf	PP-value	Mean Difference	Std. Error	95% CI (Lower)	95% CI (Upper)	Cohen's dd
COX-2 Gene Expression	0.80	3.29	39	0.002	17.40	5.27	6.73	28.06	1.03
Mast Cell Count	0.03	2.57	39	0.01	13.25	5.13	2.87	23.62	0.80

The error bar charts for the two variables are presented below:

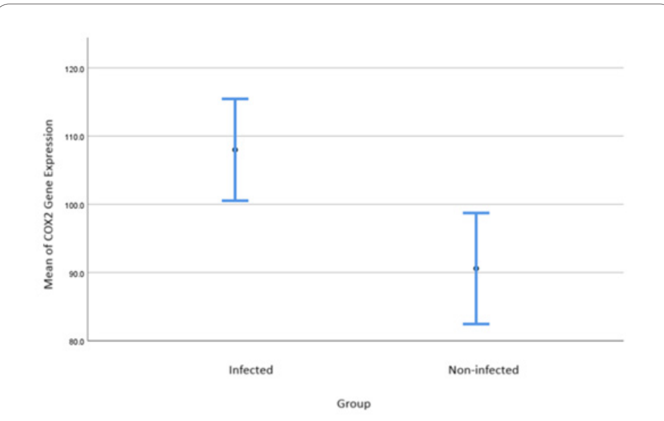

Fig. 1. Error bar chart for COX-2 gene expression, demonstrating higher values in the COVID-19 group compared to the non-COVID-19 group.

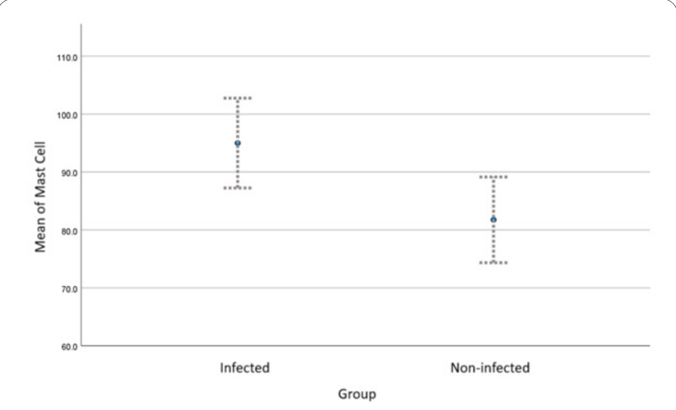

Fig. 2. Error bar chart for mast cell count, showing a significant increase in the COVID-19 infected group.

VID-19 group (P=0.002). The mean difference was 17.40 units. Cohen's d=1.03, indicating a large effect size, reflecting substantial differences between the two groups.

The COVID-19 group had a significantly higher mean mast cell count than the non-COVID-19 group (P=0.01). The mean difference was 13.25 units. Cohen's d=0.80, also indicating a large effect size.

4. Discussion

This study investigated the impact of COVID-19 infection on COX-2 gene expression and mast cell count in the testicular tissue of azoospermic individuals. Our findings demonstrate that both COX-2 gene expression and mast cell count were significantly higher in the testicular tissue of COVID-19-infected individuals compared to the non-infected group. These results underscore the role of inflammation in mediating testicular damage and suggest that COVID-19 may contribute to male infertility by triggering an inflammatory cascade within the testes.

The observed increase in COX-2 expression in the infected group is particularly significant, given the enzyme's pivotal role in the production of pro-inflammatory prostaglandins, which are known to disrupt normal cellular function and tissue homeostasis. Elevated COX-2 levels have been implicated in various inflammatory conditions, and their association with testicular damage is well-documented. For instance, Costa et al. (2023) reported that increased COX-2 expression in testicular tissue following COVID-19 infection was associated with decreased sperm quality and motility, aligning with the findings of our study [12]. Similarly, the heightened mast cell count observed in our study reflects an immune response to the viral invasion, as mast cells are key players in orchestrating inflammatory responses by releasing cytokines, histamines, and other inflammatory mediators [1].

Previous studies, such as that by Tian et al. (2021), have highlighted the destructive effects of inflammation on testicular function, particularly in animal models [13]. Tian et al. demonstrated that SARS-CoV-2 infection led to significant damage to the seminiferous tubules, the primary site of spermatogenesis, and a subsequent reduction in sperm count. These effects were attributed to the upregulation of inflammatory markers, including COX-2, further supporting the hypothesis that inflammation is a key mechanism by which COVID-19 impairs male fertility. Our findings are consistent with this hypothesis, as the significant increase in both COX-2 expression and mast cell infiltration suggests that similar inflammatory processes are at play in human testicular tissue.

One notable aspect of our study is the large effect size associated with the observed differences between infected and non-infected groups. The Cohen's d values of 1.03 for COX-2 gene expression and 0.80 for mast cell count indicate a strong impact of COVID-19 infection on these inflammatory markers. This underscores the potential severity of testicular inflammation in infected individuals and raises concerns about the long-term implications for male reproductive health. Chronic inflammation in the testes can lead to fibrosis, disruption of the blood-testis barrier, and hormonal imbalances, all of which are detrimental to spermatogenesis and overall fertility.

The findings of this study have important clinical implications. First, they suggest that men recovering from COVID-19, particularly those with severe infections, should be closely monitored for potential reproductive issues. Clinicians should consider evaluating the fertility status of male COVID-19 patients, especially those with pre-existing conditions such as azoospermia, which may make them more vulnerable to the effects of testicular inflammation [14]. Additionally, the identification of COX-2 and mast cells as key markers of testicular inflammation opens up potential therapeutic avenues [15]. Anti-inflammatory treatments targeting the COX-2 pathway or mast cell stabilization could be explored as strategies to mitigate testicular damage and preserve fertility in men affected by COVID-19 [3].

Moreover, the study emphasizes the need for multidisciplinary approaches in managing the long-term consequences of COVID-19. Collaboration between infectious disease specialists, reproductive endocrinologists, and urologists will be essential to develop comprehensive care plans for men at risk of infertility due to COVID-19. Early identification and intervention could help prevent irreversible damage to the reproductive system and improve fertility outcomes in affected individuals.

While this study provides valuable insights into the inflammatory mechanisms underlying COVID-19-induced testicular damage, it also has several limitations. The relatively small sample size may limit the generalizability of the findings to the broader population. Larger studies with diverse patient cohorts are needed to confirm and extend our results. Additionally, our study focused primarily on the molecular markers of inflammation without assessing the functional consequences on fertility, such as sperm

count, motility, and hormonal profiles. Future studies should incorporate these clinical parameters to provide a more comprehensive understanding of the impact of COVID-19 on male reproductive health.

Another important area for future research is the longitudinal assessment of fertility outcomes in men recovering from COVID-19. It remains unclear whether the observed inflammatory changes in testicular tissue are transient or if they persist long after recovery from the infection. Long-term follow-up studies are needed to determine the duration of testicular inflammation and its impact on fertility over time. Additionally, investigating the effects of different COVID-19 variants on testicular inflammation and fertility could provide important insights, as emerging variants may have varying degrees of virulence and tissue tropism.

Finally, exploring potential therapeutic interventions to mitigate testicular inflammation is an important next step. Anti-inflammatory agents, antioxidants, and lifestyle modifications could be evaluated for their efficacy in reducing inflammation and preserving testicular function in COVID-19 patients. Such interventions could have significant implications for the management of post-COVID-19 fertility issues and improve the quality of life for affected individuals.

5. Conclusion

The findings of this study provide compelling evidence of the impact of COVID-19 infection on male reproductive health, particularly in azoospermic men. The significant increase in COX-2 gene expression and mast cell count in testicular tissue among individuals infected with COVID-19 suggests a heightened inflammatory response that may adversely affect fertility. This aligns with existing literature indicating that inflammation plays a critical role in testicular damage and reduced sperm quality. Given the presence of angiotensin-converting enzyme 2 (ACE2) receptors in male reproductive tissues, the results underscore the vulnerability of this system to viral infections. The observed alterations in inflammatory markers highlight the need for further research to explore the long-term implications of COVID-19 on male fertility. In conclusion, this study emphasizes the importance of monitoring reproductive health in men who have contracted COVID-19. Future research should aim to investigate the clinical and functional aspects of fertility in this population, which could lead to improved strategies for preserving male reproductive health in the context of viral infections.

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