Original Article

Assessment of Serum Levels of Vitamin D and Selected Biochemical and Hematological Parameters in Rats Post-Immunized with Inactivated COVID-19 Virus

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ABSTRACT

Serum levels of vitamin D (VD) are inversely correlated with the incidence or severity of COVID-19. This study aimed to investigate the effects of inactivated COVID-19 virus (ICoV-19) immunization on VD levels, as well as biochemical and hematological parameters in adult male Sprague-Dawley rats. Twenty rats were randomly divided into two groups of 10: the control group (group I) and the ICoV-19-receiving group (group II). Aluminum hydroxide and a single dose of ICoV-19 (10^7 TCID50 of the HB02 strain of the COVID-19 virus) were administered subcutaneously to rats in groups I and II, respectively. After two weeks (on day 14), booster doses were administered to both groups. Blood samples were collected from the rats in both groups primarily for the experiment and 21 days after the second administration (on day 35). Samples were then stored frozen until analysis. There were no significant differences in VD levels between the two groups on day 0, but on day 35, VD levels in group II had significantly decreased, compared to group I (P<0.05). Additionally, group II had higher concentrations of fibrinogen, blood urea nitrogen, creatinine, and lactate dehydrogenase activity than group I (P<0.05). Group II also showed a significant increase in neutrophil counts and neutrophil-to-lymphocyte ratio after 35 days (P<0.05), while lymphocyte counts decreased. These findings suggest that VD may play a role in preventing COVID-19 and can thus be a potential candidate for managing and controlling the disease.

Keywords: Biochemical parameters, Hematological parameters, Inactivated COVID-19 virus, Rats, Vitamin D

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1. Introduction

An adequate supply of some micronutrients, such as vitamins C and D, as well as the essential trace elements (zinc and selenium), plays a significant role in human health, particularly in the immune system (1). Vitamin D (VD) interacts with cells of both the innate and adaptive immune systems, which accounts for its potential as a preventive or therapeutic measure against SARS-CoV-2. Aside from its protective effects on the immune system, VD is also reported to protect ACE2 expression and the renin-angiotensin system (RAS) (2). Several successful clinical studies support the experimental findings, establishing VD as a highly effective adjuvant in COVID-19 treatment and prevention (3). As a stable form of VD, 25-hydroxycholecalciferol (25(OH)D) is often used as a biomarker for VD levels in the body. Most observational studies suggest a higher risk of infection and hospitalization associated with low 25(OH)D concentrations. VD deficiency is related to an increased risk of disease and mortality in European and Asian countries (4). Patients with low 25(OH)D are especially at risk of COVID-19 infection. VD supplementation was found to reduce the risk of upper respiratory tract infections before the COVID-19 pandemic (5). Taking 400-1000 IU of VD daily for one year reduced acute respiratory infections safely and effectively (6). In addition, the use of high doses of 5000 IU following COVID-19 accelerated recovery from cough and ageusia (7). In patients with COVID-19, pulse therapy with 60,000 IU VD suppressed several inflammatory markers with a rise in 25(OH)D levels (8). VD activates B lymphocytes that produce virus-specific IgG1 antibodies, alters adaptive immunity by reducing T helper 17 lymphocytes, and increases interleukin-10 production. Furthermore, it inhibits a cytokine storm by inhibiting CD4+ T cells' release of pro-inflammatory cytokines through nuclear factor B signaling. VD also increases the bioavailability and expression of soluble ACE2, causing virus entrapment and inactivation. Besides inhibiting renin expression, VD negatively regulates the RAS (9). A recent article published in the United Arab Emirates and Bahrain reported 72-78% vaccine efficacy against COVID-19 using BNT162b2 and mRNA-1273 vaccines, which are two approved COVID-19 mRNA vaccines with excellent safety and effectiveness (10). Based on data from the US, where VD food fortification has been mandatory for several years, the two mRNA vaccines have shown satisfactory safety and efficacy profiles (11). Therefore, low VD levels in the study population (in the Middle East) could have contributed to the low vaccine efficacy of inactivated SARS-CoV-2 vaccines. An essential part of diagnosing and monitoring disease conditions is a simple blood test since it provides information regarding inflammatory processes and disease severity, including leucocyte counts and neutrophil-to-lymphocyte ratios (N/L) as markers of inflammation. It may be possible for a physician to determine the etiology of pneumonia based on the results of blood tests, which provide information regarding the nature of pneumonia. A complete blood count, including platelet count, neutrophil count, lymphocyte count, and monocyte count, is a good indicator of an inflammatory process. In the immune system, neutrophils play a crucial role. It may be worthwhile to use circulating biomarkers representative of inflammation as prognostic indicators in COVID-19-positive patients. Since there is no data about serum VD levels after vaccination against COVID-19, it is crucial to identify the importance of taking VD supplements even after vaccination. This study aims to investigate the effects of inactivated COVID-19 virus (ICoV-19) immunization on VD levels, as well as biochemical and hematological parameters, in adult male Sprague-Dawley rats.

2. Materials and Methods

2.1. Animals

Twenty healthy male Sprague-Dawley rats (weighing 200-250 g) were purchased from the Laboratory Animal Department of the Pasteur Institute of Iran and placed in rat cages, according to standard conditions (12 h of light, a temperature of 19-25°C, and a relative humidity of 45-55%). The rats had been acclimated for one week before the experiment, and they were fed pellets of food. They also had free access to water.

2.2. Generation of Inactivated COVID-19 Virus

The COVID-19 Vaccine (Vero Cell), Inactivated is made from the SARS-CoV-2, 19nCoV-CDC-Tan-HB02 strain, which is inoculated on Vero cells for culturing, harvesting, as well as performing propiolactone-inactivation, concentration, and purification, followed by adsorption with aluminum hydroxide adjuvant to form the liquid vaccine. The vaccination was devoid of antibiotics and preservatives. In order to protect against COVID-19, HB02 vaccines may be able to produce a significant increase in neutralizing antibody titers in mice, rats, guinea pigs, rabbits, and primates other than humans. The stock virus was replicated efficiently and reached a titer of over 7 log10 infectious doses at 50% within 72 h of infection. Beta-propiolactone was thoroughly mixed with viral solutions from cell cultures at a ratio of 1:4000 at 2-8°C to inactivate the harvested virus (26). Vaccine formulations contained 4 mg of total protein, which was quantified by the Bradford method, along with aluminum hydroxide adjuvant (0.45 mg/mL) per 0.5 mL (17). The vaccine was obtained from the Virology Research Center of Baqiyatallal Hospital, Tehran, Iran.

2.3. Inoculation of Inactivated Viral Particles

Rats were randomly assigned to two groups of 10: group I, which received 0.5 mL of saline containing aluminum hydroxide adjuvant, group I, which received 0.5 mL of saline containing aluminum hydroxide adjuvant, and group II, which received 0.5 mL of ICoV-19 (4 μg of total protein and aluminum hydroxide adjuvant) subcutaneously on days 0 and 14. Blood samples were collected from the rats in both groups primarily for the experiment and 21 days after the second administration (on day 35). and group II, which received 0.5 mL of ICoV-19 (4 μg of total protein and aluminum hydroxide adjuvant) subcutaneously on days 0

and 14. Before virus administration on day 0, two rats from each group were serologically tested for a basic antibody titer against COVID-19. ELISA kits were used to measure the antibody levels in three rats from each group three weeks after their second inoculation (on day 35), and the level of neutralization of sera against the COVID-19 virus was tested using the Anti-SARS-CoV-2 S1-RBD (IgG) ELISA kit (Diazist, Iran).

2.4. Serum Biochemical Analysis: Lymphocyte Counts, Neutrophil Counts, and Neutrophil-to-Lymphocyte Ratio

Blood samples were collected from the rats of both groups primarily for the experiment and 14 days after the second administration (on day 35), and then samples were stored frozen until analysis. Five biochemical parameters from the rats' serum were analyzed using an automated chemical analyzer (Mindray 20) during immunization to evaluate liver and kidney function. In addition, immune system functions were assessed by calculating lymphocyte counts (L%), neutrophil counts (N%), the N/L ratio, and serum fibrinogen concentration. Samples that displayed hemolysis, lipemia, or icteric discoloration were excluded from the analysis. Several liver-related parameters were also measured, including aspartate transaminase (AST), total bilirubin (TBIL), and lactate dehydrogenase (LDH). Moreover, blood urea nitrogen (BUN) and creatinine (CR) were used to assess kidney function.

2.5. 25-Hydroxyvitamin D Measurement

VIDAS 25-hydroxyvitamin D was used to assess VD status. VIDAS 25-hydroxyvitamin D is an automated quantitative test for the determination of 25-hydroxyvitamin D in serum or plasma using the ELFA technique (enzyme-linked fluorescent assay; reference number: 30 463). The measurement range of the assay extends from 8.1 to 126 ng/Ml. Readings below 20 ng/mL, between 20-29 ng/mL, and between 30-100 ng/mL were classified as VD deficient, insufficient, and sufficient, respectively.

2.6. Statistical Analysis

The serum levels of VD, as well as biochemical and hematological parameters, were depicted as mean±SD. The independent sample t-test was used for data analysis using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistically significant for differences between uninfected and infected animals.

3. Results

The serum levels of VD were not significantly different between group I (59.05±7.67) and group II rats (58.93±7.35) on day 0, while there was a significant decrease in VD serum levels in blood samples of rats in group II on day 35 (46.82±5.84; $P<0.05$), compared to group I rats (60.19±7.33) on day 35 (Figure 1). Serum TBIL concentration was not significantly different in groups I and II on days 0 (0.013±0.003 and 0.014±0.003, respectively) and 35 (0.014±0.004 and 0.016±0.004, respectively) of the study ($P>0.05$); however, its concentration increased in group II on day 35 (Figure 2).

The serum activity of LDH and AST increased significantly ($P<0.05$) on day 35 in group II rats (771.40±76.86 and 251.80±126.54, respectively [Figure 3]), compared to group I rats (481.90±122.23 and 118.50±21.64, respectively [Figure 4]). The concentration of BUN and CR increased in group II (25.22±1.76 and 0.65±0.10, respectively), compared to group I (21.30±2.24 and 0.55±0.05, respectively) on the 35th day of the experiment. However, this increase was significant ($P<0.05$) only in the concentration of BUN (Figures 5 and 6). In immune-associated markers, fibrinogen level (Figure 7), N% (Figure 8), and the N/L ratio (Figure 9) significantly increased, while L% (Figure 10) decreased on day 35 in group II rats ($P<0.05$).

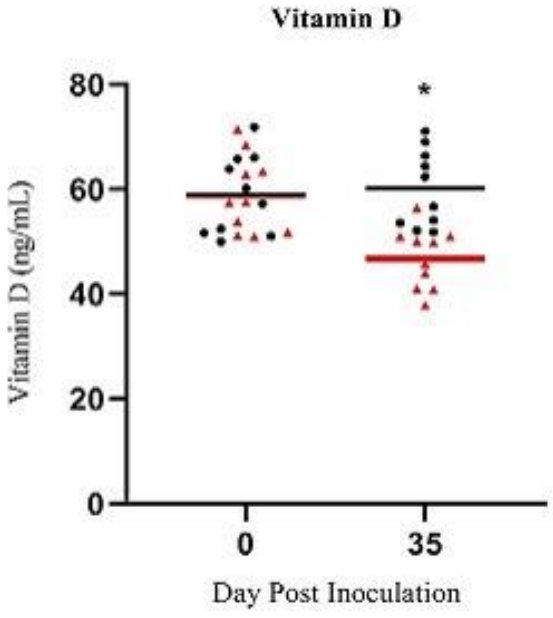

Figure 1. Comparison of changes in serum vitamin D levels in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of the serum vitamin D levels were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

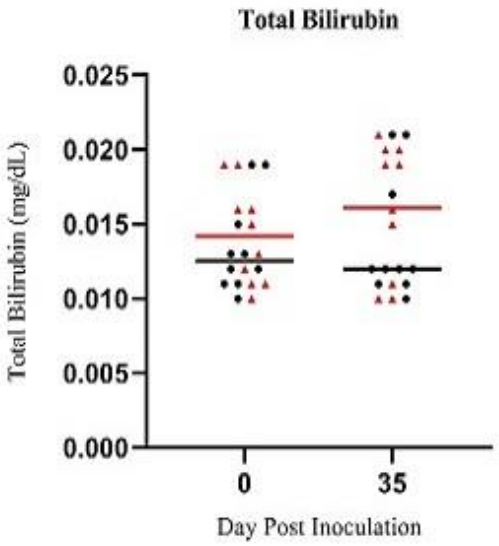

Figure 2. Comparison of changes in serum total bilirubin levels in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum total bilirubin levels were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

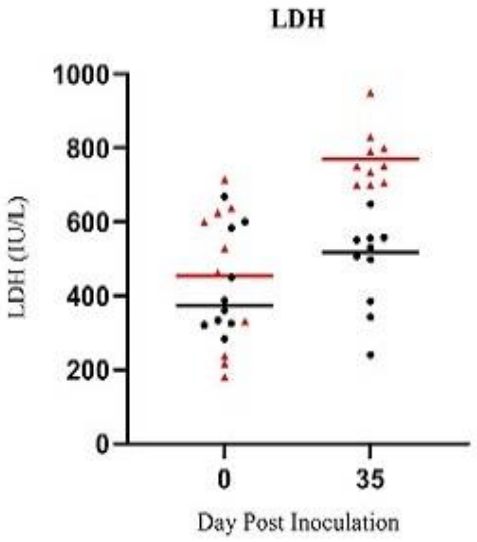

Figure 3. Comparison of changes in serum LDH activity in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum LDH activity were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

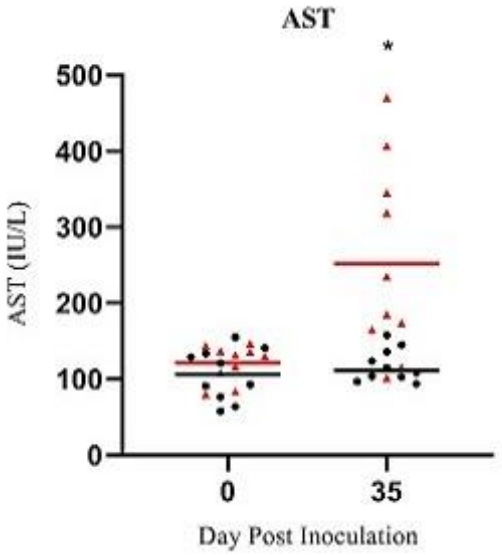

Figure 4. Comparison of changes in serum AST activity in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum AST activity were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

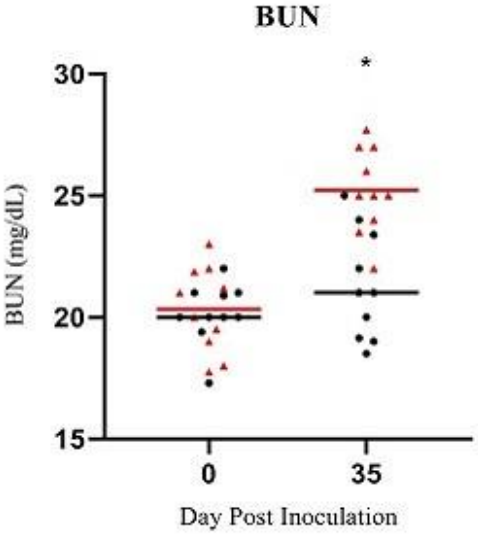

Figure 5. Comparison of changes in serum BUN levels in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum BUN levels were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

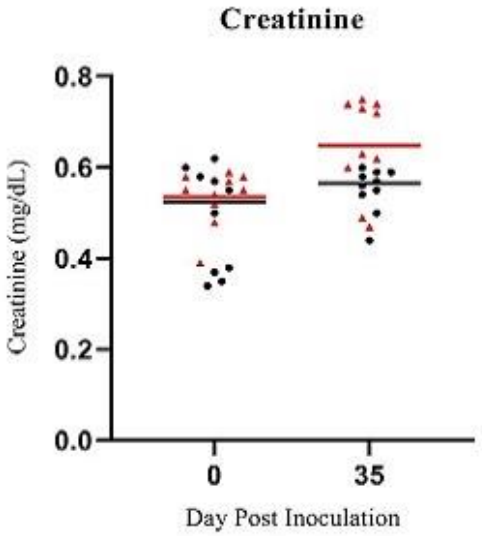

Figure 6. Comparison of changes in serum creatinine levels in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum creatinine levels were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

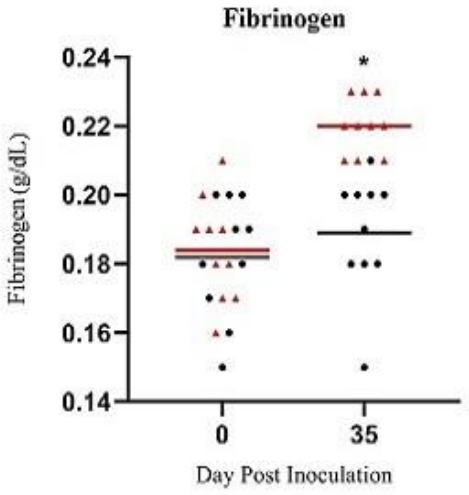

Figure 7. Comparison of changes in serum fibrinogen levels in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of serum fibrinogen levels were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

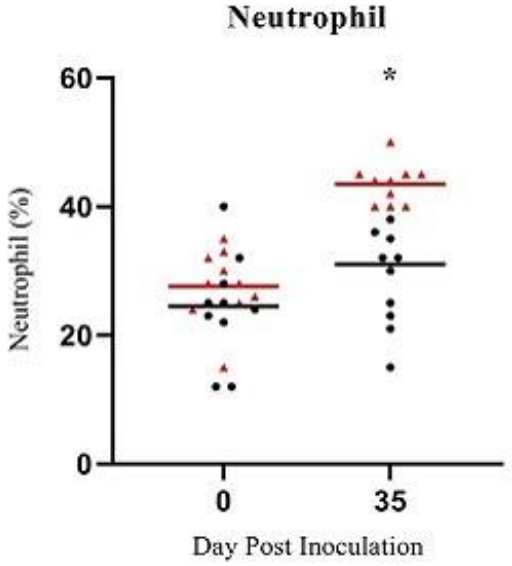

Figure 8. Comparison of changes in neutrophil% in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of neutrophil% were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

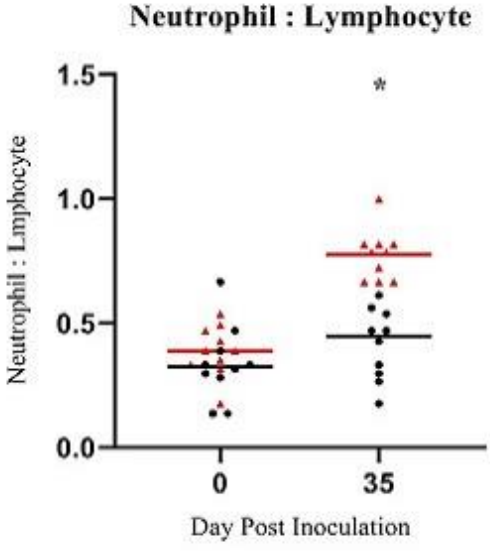

Figure 9. Comparison of changes in the neutrophil-to-lymphocyte ratio in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of neutrophil-to-lymphocyte ratio were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

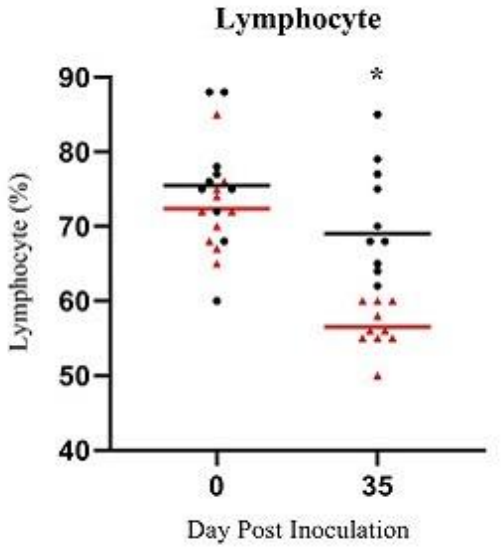

Figure 10. Comparison of changes in lymphocyte% in the control rats and those receiving the inactivated COVID-19 virus on days 0 and 35. The means of lymphocyte% were noted, as indicated by a horizontal bar. Sprague-Dawley rats are inoculated with ICOV-19 (red triangle) and control rats (black circle). *, $P<0.05$.

4. Discussion

This case-control study was conducted on rats to examine serum VD, as well as biochemical and hematological parameters, 35 days after inoculation with two doses of ICoV-19. Several studies have recently investigated the clinical characteristics of COVID-19 patients. These studies differed in their sample sizes and the clinical characteristics they examined, in particular the biochemical indices. Recent studies have also focused on laboratory abnormalities, including increased lymphocytes, prolonged prothrombin times, increased CRP, and a rise in LDH, in addition to epidemiology and clinical symptoms (12). Multiple organ dysfunction has been linked to abnormalities in laboratory indicators, especially serum biochemical parameters. This is the first study that examines the N/L ratio, fibrinogen as an inflammatory indicator, and biochemical parameters such as VD, TBIL, LDH, AST, BUN, and CR in rats 35 days after ICoV-19 inoculation. It has been shown that VD supplements can help protect against respiratory infections, and VD levels are related to the severity of the disease during the COVID-19 pandemic (13). The COVID-19 virus attaches to cells through angiotensin II, which contributes to the complex manifestations of this viral infection. VD can induce angiotensin II and regulate the immune system through multiple mechanisms (14). Based on the available information, VD may play a role in preventing COVID-19. Despite VD being readily available and affordable and VD poisoning being extremely rare, VD deficiency is a worldwide health problem. Therefore, VD supplements can be considered a global strategy to increase serum levels, especially in COVID-19 patients (14).

In a previous retrospective study, a significant relationship was observed between VD deficiency and poor clinical outcomes in COVID-19 (15). It was also reported that the highest level of VD deficiency was present in the most severe cases of COVID-19, and with increasing serum levels of VD, there was a significant increase in the improvement of the disease (15).

Soltani-Zangbar, Mahmoodpoor (16) found a possible association between VD concentration, immune system performance, and infection risk in COVID-19. VD can be considered a candidate for managing and controlling COVID-19 because of its antiviral and cytokine effects. Jenei, Jenei (17) demonstrated that VD deficiency is associated with mortality in COVID-19 patients. As a result of the COVID-19 infection, people over 60 years of age with other risk factors, such as cancer or diabetes, have lower VD levels. In Asia, VD deficiency is associated with mortality in COVID-19 patients, as demonstrated by Sooriyaarachchi, Jeyakumar (4). Based on the results of the present study, rats inoculated with ICoV-19 had an 11.6% reduction in VD, compared to rats in the control group ($P<0.05$). The hematological, biochemical, and immunological markers of COVID-19 patients were evaluated by Henry, De Oliveira (18). In this comprehensive meta-analysis, a clear pattern of abnormalities in inflammatory, hematologic, biochemical, and immune biomarkers was observed between patients with and without a severe disease. Rats with COVID-19 showed increased levels of AST, TBIL, BUN, and CR. Cui, Zhao (19) reviewed the clinical and laboratory findings of children with COVID-19 and revealed that the rate of lymphocytes was reduced less in children than in adults, which could be due to differences in the immune response. The pathogenesis, symptoms, and diagnostic methods of COVID-19 have been studied by Mohammadian, Chiti (20). They observed that in the severe form of COVID-19, the lymphocyte counts, as well as AST and LDH levels, decreased and increased, respectively. COVID-19 patients exhibited elevated levels of various biochemical parameters, including alanine transaminase (ALT), AST, LDH, BUN, TBIL, CR, amylase, lipase, procalcitonin, CRP, D-dimer, albumin,

and total protein. Over 10% of patients had CR, glucose, ALT, AST, potassium, and LDH levels above the reference range after recovering from the disease (21). A significant observation in COVID-19 patients was abnormal liver function (21). The AST/ALT ratio was elevated in infected hamsters, characterized by liver structural abnormalities and large vacuoles (22). However, rats inoculated with ICoV-19 demonstrated significantly higher levels of AST than the control group after day 35, while the level of TBIL did not change significantly. In hamsters, amylase, lipase, and ALT levels were comparable to those of patients with COVID-19. There were, however, differences between humans and hamsters in AST, BUN, TBIL, urea, chromium, and LDH (22). Similar to the findings of a previous study on Syrian-infected COVID-19 hamsters, in this study, the BUN levels of rats immunized for 35 days with ICoV-19 increased significantly (22). Additionally, serum CR levels increased slightly during the 35 days of the ICoV-19 infection but not considerably, compared to the control group. A host's susceptibility and permissiveness to SARS-CoV-2 may explain the differences between rats and hamsters. Rats inoculated with ICoV-19 for 35 days had higher N%, fibrinogen levels, and N/L ratios. Rats with COVID-19 also had a lower L%. These results are consistent with those found in earlier studies on COVID-19 patients (23). A significant increase in fibrinogen levels has also been reported by Zou, Guo (24) in 5.7% of mild COVID-19 and 19.1% of severe COVID-19 patients. A prothrombotic diathesis associated with significantly higher fibrinogen levels has been reported in critically ill COVID-19 patients. Immunogenetic parameters should be taken into account for disease control models in view of the crucial role played by immunogenic genes against resistance to diseases and their influence on production characteristics (25). To the best of our knowledge, the present study is one of the first to analyze VD and some inflammatory and biochemical changes in ICoV-19-inoculated rats. The findings showed that VD levels and L% decreased while LDH, AST, BUN, fibrinogen levels, N%, and the N/L ratio increased in rats inoculated with ICoV-19. Moreover, the results demonstrated that SARS-CoV-2 infection had long-term effects on rats. Therefore, this model may provide insights into long-term COVID-19 syndrome causes and effects.

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Authors' Contribution
Study concept and design: M. A. H.
Acquisition of data: M. M.
Analysis and interpretation of data: K. K.
Drafting of the manuscript: H. S.
Critical revision of the manuscript for important intellectual content: M. A. H.
Statistical analysis: M. A. H.
Administrative, technical, and material support: M. H. Y.

Ethics
The animal experimental protocol was approved by the Animal Research Ethical Committee for Scientific Research of Semnan University, Semnan, Iran (IR.SU.REC.1400.6).

Conflict of Interest
The authors declare that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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