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β-D-mannuronic Acid (M2000) and Inflammatory Cytokines in COVID-19; An in vitro Study

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ABSTRACT

coronavirus disease of 2019 (COVID-19) can be complicated by acute respiratory distress syndrome (ARDS) and may be associated with cytokine storm and multiorgan failure. Anti-inflammatory agents, such as systemic corticosteroids, monoclonal antibodies, and nonsteroidal anti-inflammatory drugs (NSAIDs) can be used for this purpose. In this study, we evaluated the immunomodulatory effect of mannuronic acid (M2000), which is a novel NSAID, on COVID-19-related cytokine storms.

This study was conducted in vitro on blood samples of 30 COVID-19 patients who presented with ARDS to a referral center. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples and incubated with phorbol myristate acetate for 24 hours. M2000 was administered with the dosages of 25 μg/well and 50 μg/well after 4 hours of incubation at 37°C. The quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess mRNA gene expression. Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the supernatant PBMC levels of interleukin (IL)-6, IL-17, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ.

Both mRNA expression and the supernatant PBMC levels of IL-17, TNF-α, IL-6, and IFN-γ were decreased in PBMCs of COVID-19 patients treated with M2000 compared with the control group.

For the first time, it was observed that M2000 could be effective in alleviating the inflammatory cascade of COVID-19 patients based on an in vitro model. After further studies in vitro and in animal models, M2000 could be considered a novel NSAID drug in COVID-19 patients.

Keywords: Coronavirus disease of 2019; Mannuronic acid; Severe acute respiratory syndrome coronavirus 2

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INTRODUCTION

COVID-19(coronavirus disease of 2019) infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a pandemic since December 2019.[1-6] Until 19 October 2022, SARS-CoV-2 infected more than 623 million individuals and caused almost 6.5 million deaths worldwide.[7] Several preventive measures have been applied in the last two years to control this disease, whose burden has decreased gradually, especially after vaccine development at the end of 2020.[8] SARS-CoV-2 binds to cells by the angiotensin-converting enzyme (ACE)-2 receptor. After infecting the cells, it uses the host's replication machine to produce new viruses. The release of cellular virions from host cells stimulates the cells of the immune system, which activates a cascade of inflammatory cytokines (i.e., cytokine storm) causing systemic inflammation.[9-11]

Some COVID-19 patients may experience a severe course of the disease, characterized by acute respiratory distress syndrome (ARDS), which is associated with high morbidity and mortality due to multiorgan failure.[12] Multiorgan failure is an adverse event of COVID-19 that is associated with acute inflammation resulting from the activation of the angiotensin II (Ang II)/angiotensin receptor type 1 axis.[13] Anti-inflammatory–based treatments such as systemic corticosteroids and monoclonal antibodies against inflammatory cytokines have been used vastly in this context.[14]

Nonsteroidal anti-inflammatory drug (NSAID) treatment in COVID-19 has been challenging. It has been suggested that these drugs have an adverse impact on the early immune response against COVID-19. However, NSAIDs can also alleviate the inflammatory response later during the disease.[15] Previous studies have shown that mannuronic acid (M2000) can be used as a novel NSAID to treat several inflammatory diseases. Also, in in vitro studies, it was found that M2000 has no toxic effects on cells and is completely safe in the doses used (25 µg/well and 50 µg/well).[16-22] In this study, we evaluated the immunomodulatory effect of M2000 on the COVID-19 cytokine storm in an in vitro setting.

MATERIALS AND METHODS

Study Design

This in vitro study was conducted on blood samples of COVID-19 patients who presented to Imam Reza Hospital, Tehran, Iran, in 2021. We included patients aged between 18 and 65 with positive SARS-CoV-2 infection detected by qRT–PCR, who were referred to our medical center with ARDS, diagnosed based on the Berlin criteria,[23] and accordingly, required supplemental oxygen, had $SPO_2/FiO2 \leq 300$ mmHg and had sequential organ failure assessment (SOFA) scores between 2 and 3. Patients with malignancies, other viral respiratory coinfections, underlying immune-compromised diseases, and those with a history of systemic corticosteroid or other immune suppressant drug administrations before the presentation were excluded from the study.

Peripheral Blood Collection and M2000 Administration

Intravenous blood of COVID-19 patients was collected on sodium heparin. After centrifugation of blood samples at 2000 revolutions per minute (RPM) for 30 min, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats based on the density gradient of Ficoll-Hypaque (Lymphodex Innotrain, Germany).[24] The PBMCs were washed 3 times with phosphate-buffered saline (PBS). About 2×10^6 PBMCs were isolated and incubated in 2 mL of RPMI-1640 medium, containing 10% heat-inactivated bovine fetal serum, 1% penicillin/streptomycin, and 2 mM L-glutamine, for 24 hours, in 37°C and under 5% pressure of CO_2. Also, 10 ng/ml of phorbol myristate acetate (PMA) (Sigma, Germany) was added to the culture medium to stimulate cytokine secretion.[25] A 3-well cell culture plate was used where each well contained 2×10^6 PBMCs. We administered M2000 with 2 doses of 25 µg/well and 50 µg/well after 4 hours of incubation at 37°C. One well was treated as control. We conducted the sample collection 24 hours after the M2000 administration.

RNA Extraction

Total RNA was extracted from blood samples using the total RNA Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions by RNase-free (DNase 1) reaction buffer eluted in 40 µL of nuclease-

free water. RNA purity was assessed by electrophoresis (agarose gel), and its absorption was determined on A260/280 nm and A260/230 nm by NanoDrop one C spectrophotometer (Thermo Fisher, USA).

cDNA Synthesis and RT–qPCR

Total RNA was stored at –20°C until cDNA synthesis. TAKARA (Primer Script™ RT reagent Kit, Japan) cDNA Synthesis Kit was used to convert RNA to cDNA. The main cDNA synthesis reaction components were 10-µL Template RNA Primer Mixture (Random 6 mers, dNTP, and Template RNA), 5X PrimeScript Buffer (4 µL), RNase Inhibitor (0.5 µL), PrimeScript RTase (1.0 µL) and RNase-Free dH_2O. Gene expression of IL-17, TNFα, IL-6, and IFNγ were assessed with ABI StepOne Plus Real-Time PCR System (ABI, USA) based on SYBR Green Premix Ex Taq (Tii RNaseH Plus, Takara). The Beacon designer, OligoAnalyzer (IDT), and NCBI Primer-BLAST tools were used to design primers for RT-qPCR reactions (supplementary). GAPDH was used as the housekeeping gene. 20 µL of real-time PCR reactions (10 µL of 2X standard SYBR Green, 5 µL of cDNA, and forward and reverse primers, 0.8 µL each) were amplified using a single cycle at 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 30 s, and 70°C for 20 s. Melting curve analysis of each sample involved heating the PCR product from 60 to 95°C. The $2^{-\Delta\Delta Ct}$ method was used to estimate the gene expression of the target genes.[26]

ELISA

Enzyme-linked immunosorbent assay (ELISA) kits were used to quantify the supernatant PBMC levels of IL-17 (Elabscience E-EL-H0105, USA), tumor necrosis factor (TNF)-α (RayBiotech ELH-TNFa-1, USA), IL-6 (RayBiotech ELH-IL6-1, USA), and interferon-gamma (IFN-γ) (Invitrogen 88-7316-22, USA). The ELISA kits contained two uncoated microtiter plates, prematched antibody pairs, and reagents for performing quantitative ELISA tests. The assays were performed according to the manufacturer's instructions. The detection ranges for IL-17, TNF-α, IL-6, and IFN-γ were 31.25–2000 pg/mL, 30–6000 pg/mL, 3-1000 pg/mL, and 4–500 ng/mL, respectively.

Statistical Analysis

We used the statistical package for social science (SPSS Inc. Version 19) for the statistical analysis. We reported quantitative and categorical variables as mean±SD and number (percentage) values, respectively. We used the Kruskal–Wallis test to compare differences in immunologic factors between M2000-treated samples. The graphs were drawn by GraphPad Prism Version 9.00 (GraphPad Software, La Jolla, CA, USA). *p* values less than 0.05 were considered statistically significant.

Ethical Consideration

This study was conducted after obtaining permission from the Medical Ethics Committee (Reg. No. IR.AJAUMS.REC.1400.303). Samples were collected from the patients after filling out the informed consent form.

RESULTS

Thirty patients with ARDS due to COVID-19 (18 men and 12 women with a mean age of 63.34 ± 14.21; range 18–65 years) were included in this study. The baseline laboratory data of patients are presented in Table 1.

mRNA Expression of Inflammatory Cytokines

The mRNA expression levels of IL-17, TNF-α, IL-6, and IFN-γ decreased in the PBMCs of COVID-19 patients treated with M2000 groups (25 µg/well and 50 µg/well) compared to the control ($p<0.001$ for IL-17, TNF-α, IL-6, and IFNγ; Figure 1).

Quantified Levels of Inflammatory Cytokine

Supernatant PBMC levels of IL-17, TNF-α, IL-6, and IFN-γ were assessed and compared with PBMCs of COVID-19 patients treated with M2000 (25 µg/well and 50 µg/well) groups. Our results showed that the levels of all four inflammatory cytokines were decreased in PBMCs of this group compared with the control ($p<0.001$ for IL-17, TNF-α, IL-6, and IFNγ; Figure 2).

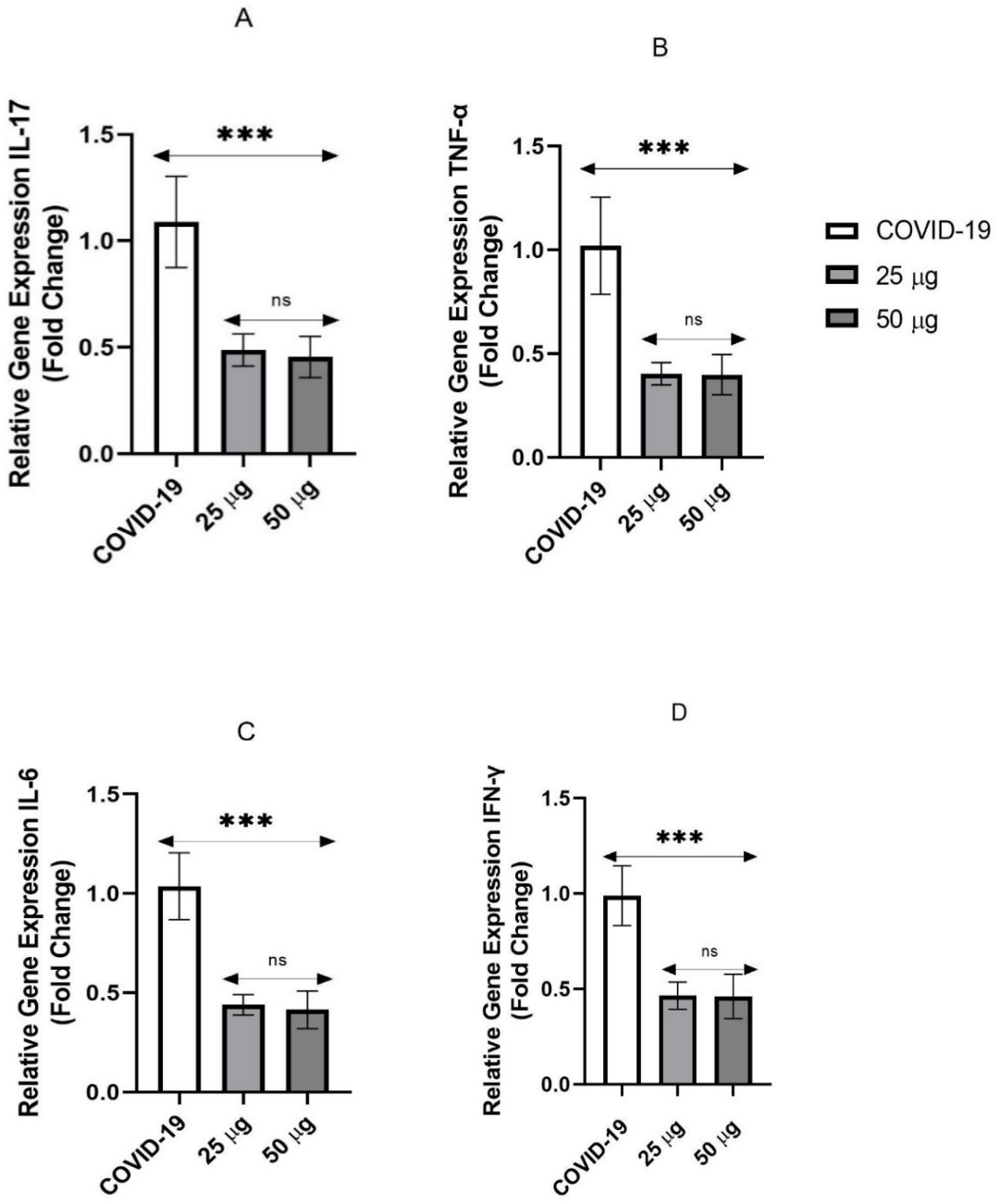

Figure 1. The mRNA expression levels of IL-17, tumor necrosis factor (TNF)-α, IL-6, and interferon-gamma (IFN)-γ in peripheral blood mononuclear cells (PBMCs) of COVID-19 patients treated with mannuronic acid (M2000). The expression levels of all inflammatory cytokines decreased after treatment with M2000 (25 µg/well and 50 µg/well) compared with the pretreatment state (p<0.001). There were no statistically significant differences in the expression level of any of the inflammatory cytokines between the PBMCs of COVID-19 patients treated with M2000 (25 µg/well and 50 µg/well). Evaluation of the mRNA expression level was performed using real-time PCR. The analysis was based on the Kruskal–Wallis test. ***p<0.001

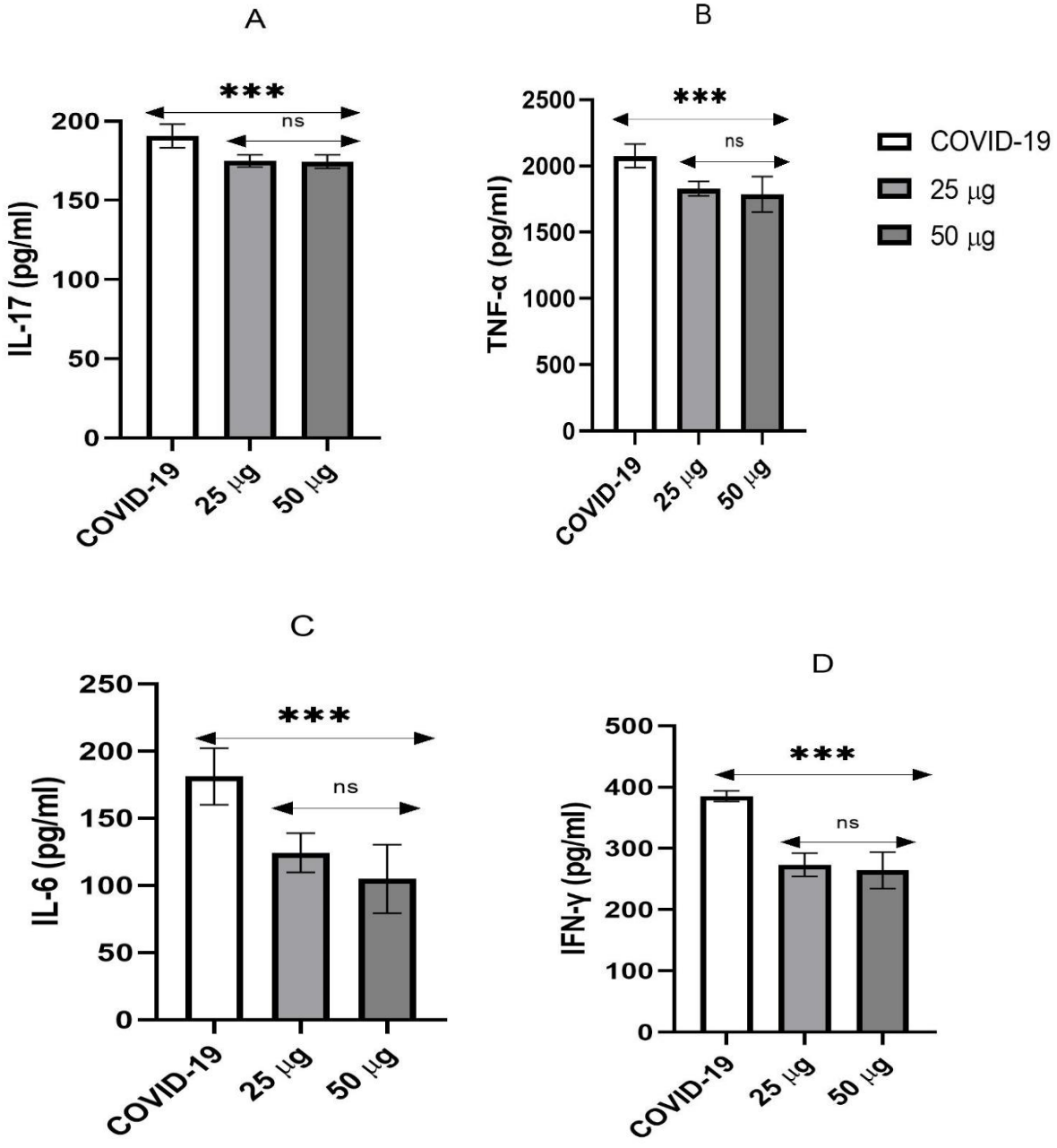

Figure 2. Quantifying inflammatory cytokine levels of IL-17, TNF-α, IL-6, and IFN-γ in the supernatant PBMCs of COVID-19 patients treated with M2000. The level of all inflammatory cytokines decreased after treatment with M2000 (25 µg/well and 50 µg/well) compared with the pretreatment state (p<0.0001). There were no statistically significant differences in the levels of any of the inflammatory cytokines between the PBMCs of COVID-19 patients treated with M2000 (25 µg/well and 50 µg/well). Evaluation of the mRNA expression level was performed using real-time PCR. The analysis was based on the Kruskal–Wallis test. ***p<0.001

Table 1. Laboratory parameters of patients

Laboratory parameters	Value	Range
White Blood Cells (WBC) ($\times 10^9$/L)	8.96 ± 5.24	3.75–23.76
Granulocytes ($\times 10^9$/L)	9.36 ± 4.48	4.06–22.69
Lymphocytes ($\times 10^9$/L)	1.02 ± 0.47	0.36–3.06
Monocytes ($\times 10^9$/L)	0.72 ± 0.53	0.32–2.94
Aspartate aminotransferase(AST) (IU/L)	42.12 ± 25.09	12–128
Lactate dehydrogenase (LDH) (U/L)	680.94 ± 152.43	370–984
C-Reactive Protein (CRP) (mg/L)	73.85 ± 54.37	29–356
Procalcitonin (PCT) (ng/mL)	0.23 ± 0.34	0.04–2.12
D-dimer (ng/mL)	2892 ± 465	2134–4205

IU: International Unit; U: Unit; L: Liter; ng:nanogram

DISCUSSION

This study assessed the in vitro effect of M2000 on the expression of inflammatory markers in PBMCs after COVID-19 infection. It showed that M2000 could effectively reduce the level of these markers. To the best of our knowledge, this is the first study to evaluate the effect of M2000 on PBMCs from COVID-19 patients.

SARS-CoV-2 can induce a cytokine storm by stimulating the production of proinflammatory cytokines.[27] Two main factors facilitate SARS-CoV-2 cell entry: 1) the interaction of SARS-CoV-2 surface (S) protein with ACE-2 receptor, and 2) the activation of S protein by transmembrane serine protease 2 (TMPRSS2), a host membrane serine protease.[28]

IFN-γ is one of the most important cytokines involved in the COVID-19 cytokine storm, which starts a chain reaction of inflammation throughout the body by attracting immune cells.[29,30] Membrane-bound immune receptors and subsequent signaling pathways mediate the inflammatory responses of $CD14^+CD16^+$ monocytes and T_H1 cells.[31] SARS-CoV-2, in particular, can quickly activate T_H1 cells causing the release of proinflammatory cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-6.[32] When T_H1 cells migrate to the area of inflammation in the lungs, IFN-γ secretion provokes the migration of other inflammatory cells.[30] Consequently, $CD14^+CD16^+$ inflammatory monocytes, under the influence of GM-CSF, produce large amounts of TNF-α, IL-6, and other cytokines, inducing positive feedback on inflammation.[33] Membrane-bound receptors, including Fc and Toll-like receptors, may be responsible for an unbalanced inflammatory response. Additionally, inadequate IFN-γ induction may serve as a significant cytokine production amplifier.[34]

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are also activated by SARS-CoV-2.[35] NF-κB, through its binding to ACE-2, decreases ACE-2 expression and stimulates the Ang II/angiotensin receptor type 1 axis. Besides NF-κB activation, this pathway can induce TNF-α and soluble IL-6 (sIL-6Ra) through disintegrin and metalloprotease 17 (ADAM17).[36,37] Signal transducer and activator of transcription 3 (STAT3) is activated in nonimmune cells by the binding of IL-6 to sIL-6R.[37,38]

Proinflammatory cytokines and chemokines, including monocyte chemoattractant protein-1, vascular endothelial growth factor, IL-8, and IL-6, can be produced after IL-6 amplifier activation by NF-κB and STAT3.[39] As a result, when IL-6 and IL-17 are produced during the systemic inflammatory response, they damage the lung tissue in ARDS by invoking other inflammatory agents of the immune system, such as neutrophils, macrophages, and specific T cells.

Anti-inflammatory agents, such as systemic corticosteroids and monoclonal antibodies against inflammatory markers, can be administered to treat COVID-19.[40] At the beginning of the pandemic, it was suggested that NSAIDs could upregulate ACE2 receptors.[41] Conversely, according to a systematic review and meta-analysis, NSAIDs are not associated with increased mortality, disease severity, intensive care unit stay, or the risk of mechanical ventilation.[42] Furthermore, NSAID administration in animal models was associated with a decrease in proinflammatory cytokine response to SARS-CoV-2 infection.[43] Various studies have focused on reducing inflammation in COVID-19 patients through medications such as pirfenidone,[44] nanocurcumin,[25]omega-3 fatty acids [45], etoricoxi,[46] andrographolide,[47] saikosaponins,[48] palmitoylethanolamide,[49] indomethacin, and resveratrol.[50]

M2000 is a novel NSAID with the lowest molecular weight (MW: 194.139 Da) and fewer complications than other NSAIDs, with promising anti-inflammatory and immunomodulatory effects.[20,51] The impact of M2000 has been evaluated in rheumatoid arthritis (RA) and multiple sclerosis and has been associated with a significant anti-inflammatory response and high safety [52]. However, it has not been evaluated in any infectious disease so far.

The anti-inflammatory effect of M2000 has been observed in previous human studies. In a randomized controlled trial in 2017, 1 gram of M2000 administered daily to 21 patients with RA was associated with a significant decrease in anti-cyclic citrullinated peptide antibodies and C-reactive protein (CRP).[53]Ahmadi et al. studied M2000 in 30 patients with RA, demonstrating a significant reduction in CRP and erythrocyte sedimentation rate (ESR).[54] Rezaieyazdi et al. reported similar results on ESR and CRP levels in RA patients treated with M2000.[55] Other clinical trials on autoimmune diseases revealed the significant effect of M2000 on other inflammatory markers such as IL-17,[56] matrix metalloproteinases,[57] STATs,[58] and NF-κB.[59]

M2000 is not associated with the systemic complications of other anti-inflammatory agents, such as corticosteroids, and has a lower cost of production than monoclonal antibodies. We evaluated the anti-inflammatory effect of M2000 on four inflammatory cytokines: IL-6, IL-17, TNF-α, and IFN-γ, which are the primary inflammatory cytokines in COVID-19.[60] However, as mentioned above, M2000 can effectively modulate other parts of cytokine storms. This issue should be considered in future studies.

This study was associated with several limitations, including 1) the failure to assess other parts of the cytokine storm due to high costs; 2) the inclusion of blood samples from only 30 patients; larger sample sizes may yield different results; and 3) performing our analysis only 24 hours after M2000 administration, which can confound the results.

Our study showed that M2000 could play a critical role in regulating the inflammatory cytokine storm in COVID-19 patients. However, more extensive in vitro and in vivo studies are needed to confirm that M2000 works to reduce inflammation in COVID-19.

STATEMENT OF ETHICS

Research was approved by the Aja University of Medical Sciences, and the foundation received was under Ethics Committee (Reg. No. IR.AJAUMS.REC.1400.303)

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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