ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2022; 21(5):687-703.
Doi:

Specific Clinical and Immunological Changes Following Mesenchymal Stem Cell Transplantation in COVID-19–induced Acute Respiratory Distress Syndrome Patients: A Phase-I Clinical Trial

Najmeh Kaffash Farkhad[1,2], Alireza sedaghat[3], Hamidreza Reihani[4], Amir Adhami Moghadam[5], Ahmad Bagheri Moghadam[6], Nayereh Khadem Ghaebi[7], Mohamad Ali Khodadoust[1], Amin Reza Nikpoor[8], and Jalil Tavakol-Afshari[1]

[1] Department of Immunology, Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Student Research Committee, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Lung Disease Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[4] Department of Emergency Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[5] Department of Internal Medicine and Critical Care, Islamic Azad University, Mashhad, Iran
[6] Department of Anesthesiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[7] Neonatal Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[8] Molecular Medicine Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

Received: 27 August 2022; Received in revised form: 17 September 2022; Accepted: 22 September 2022

ABSTRACT

Acute respiratory distress syndrome (ARDS) is a systemic inflammation resulting from immune system overactivity. ARDS is also a fatal complication of COVID-19. Mesenchymal stem cells (MSCs) have immune modulatory properties. This study evaluated the safety and efficacy of three times transplantation of umbilical cord-derived MSCs (UC-MSCs) in terms of specific immunological and clinical changes in mild-to-moderate COVID-19-induced ARDS patients.

In this single-center, open-label, phase 1 clinical trial, 20 patients diagnosed with COVID-19 and mild-to-moderate ARDS were included and were divided into two groups: a control group receiving standard care and an intervention group receiving UC-MSC in addition to standard care. Three consecutive intravenous transplants of UC-MSC (1×10^6 cells/kg body weight per each transplant) were performed in the intervention group on days 1, 3, and 5. The biological assay was investigated four times (days 0, 5, 10, and 17).

UC-MSCs improved the patients' clinical and paraclinical parameters, including leukocytosis, lymphopenia, thrombocytopenia, and liver enzyme abnormalities, compared to the control group. They also decreased pro-inflammatory lymphocytes (TH1 and TH17) and increased anti-inflammatory T lymphocytes. Cell therapy also reduced the mean fluorescence intensity (MFI) in overactivated $CD8^+$ T cells.

Corresponding Author: Jalil Tavakol Afshar, MSC, PhD; Department of Immunology, Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: (+98 915) 1134 220, Fax: (+98 51) 3711 2596, E-mail: Tavakolaj@mums.ac.ir, tavakolafsharijalil@gmail.com

These findings show that three UC-MSC injections could regulate a hyperactivated immune system in COVID-19-induced ARDS patients by decreasing the inflammatory T lymphocyte subset and can improve the patient's hematological condition and liver function. However, more studies are needed in this area.

Keywords: Acute respiratory distress syndrome; Immunological biomarkers; Inflammation; Lymphopenia; Mesenchymal stem cells

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or COVID-19) is the most significant medical challenge today. It was first identified in Wuhan, China, in late 2019 and quickly spread worldwide.[1] SARS-CoV-2 is an enveloped positive-sense single-stranded RNA virus that exploits the endocytosis process with the help of angiotensin-converting enzyme 2 (ACE2) receptors, expressed on pulmonary epithelial cells to enter pulmonary epithelial cells. The virus can affect all organ systems, including the immune system.[2,3] It has been proven that immune system dysfunction is closely related to the severity of COVID-19. Lymphopenia,[4] thrombocytopenia, changes in the number and function of specific immune cells, and cytokine storm are among common observations in acute COVID-19. Several of these symptoms have also been described in infections with viruses such as SARS-CoV and MERS-CoV.[5-8] Acute respiratory distress syndrome (ARDS) is another deadly complication of COVID-19 that causes generalized inflammatory destructive lung damage.[9] It has also been proven that ARDS is one of the leading causes of COVID-19 patient admissions to the intensive care unit (ICU).[10] Unfortunately, despite global efforts, there is still no specific treatment for the disease, and the number of patients continues to increase. In this regard, finding new treatment strategies with anti-inflammatory and immunomodulatory properties can pave the way for the patient's treatment.

Mesenchymal stem cells (MSCs) comprise a small population of cells in the bone marrow with self-renewal and differentiation capabilities.[11] Therapeutic properties of these cells and their derivatives (eg, exosomes [12] are demonstrated in many diseases, including amyotrophic lateral sclerosi·[1] rheumatoid arthritis,[14] graft-versus-host disease (GVHD),[15] diabetes[16] systemic lupus erythematosus,[17] ARDS.[18-20] Furthermore, several clinical trials have been registered aiming to use MSCs in COVID-19, many of which are underway, and only a few results have been published.[21,22]

MSCs have anti-inflammatory and immunomodulatory properties and are ACE2-negative[9,11] and their effect on many clinical and immunological parameters of COVID-19-induced ARDS has been demonstrated[9,20] According to the available information, this trial aims to examine the impacts of UC-MSCs on specific clinical and immunological parameters of COVID-19-induced ARDS patients.

MATERIALS AND METHODS

Study Design

This clinical trial was designed as a single-center, open-label, phase 1 clinical trial conducted at Imam Reza Hospital, Mashhad University of Medical Sciences, Mashhad, Iran, from July 2020 to May 2021. A total of 20 COVID-19 patients with mild-to-moderate ARDS (according to the WHO classification guideline for ARDS: SPO2/FIO2 between 120 and 315),[23] were admitted to the ICU. All participants signed an informed consent form before entering the study. They were also divided into two groups: the control group receiving standard care and the intervention group receiving standard care plus three intravenous injections of UC-MSCs on days 1, 3, and 5 (1×10^6cells/kg body weight per transplant). Standard care included the accepted treatment protocols of that time (ie, complementary treatments, dexamethasone, remdesivir, and antibiotics when necessary.

The exact time of the disease onset was unknown due to the delay in performing PCR. However, in all patients, the intervention was started at least one week after the onset of symptoms.

Having an age range between 18 to 75 years, signing the informed consent form, being positive for COVID-19, suffering from ARDS (non-severe), need for supplemental oxygen, not having severe underlying

diseases or other concurrent viral infections, and full patient participation from the beginning to the end of the study are among the most important criteria for patients to enter and exit the study.

For more detailed about the inclusion and exclusion criteria of patients, please refer to our previous article.[24]

UC-MSC Processing and Transplantation

Allogenic UC-MSCs were isolated and cultured from the umbilical cords of 10 healthy mothers. Characterization (using flowcytometry, special differentiation and staining methods for osteogenic and adipogenic differentiation), transplantation, and cell quality control before each infusion were performed according to our previous protocol.[24] Three times intravenous injections (1×10^6 cells/kg body weight per injection) through the peripheral veins were performed for patients at 3 different time points (days 1, 3, and 5). Also, patients received standard treatments based on their special conditions in all phases of the study. A grade B cleanroom with GMP requirements was used for all the above steps.

Clinical Evaluation and Follow-up

Paraclinical parameters, including complete blood cell count (CBC), liver enzymes (alanine aminotransferase and aspartate aminotransferase) and bilirubin levels, were examined on days 0, 5, 10, and 17. Blood (15 mL) was collected from each patient where 5 mL whole blood was used for CBC and 5 mL for flow cytometry. Blood was collected in EDTA tubes to determine the CBC and was analyzed by Sysmex KX-21N hematology analyzer. The remaining 5mL of the serum samples were used to check the other parameters (bilirubin and liver enzymes).

Flow Cytometry

For flow cytometric analysis, the peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation (Ficoll-Paque PREMIUM; GE Healthcare Bio-Sciences, Uppsala, Sweden). Mononuclear cells were diluted in an equal volume of phosphate-buffered saline (PBS), and were washed and centrifuged several times. The cell precipitate is then resuspended in 1 mL of RPMI culture medium (Gibco, USA) to which a cell activation cocktail with brefeldin A (2 μL of PMA and ionomycin solution [Sigma-Aldrich, USA]; final concentration of 50 ng/ml PMA and 1 μg/mL ionomycin) was added for cytokine stimulation. The homogeneous suspension was incubated for 4-6 hours in a 37°C incubator with standard conditions. Then 100 μL of cell suspension in flow cytometry tubes was used to determine the $CD4^+$ and $CD8^+$ T cell populations by flow cytometry (BD Biosciences FACSCalibur, USA) according to the manufacturer's protocols. Cell surface antibodies included anti-CD3 conjugated with peridinin-chlorophyll-protein (PerCP/cy5.5) and CD25. Fluorescein isothiocyanate (FITC) was conjugated with both CD4 and CD8a, and allophycocyanin (APC) was conjugated with CD127. Intracellular phycoerythrin antibodies were also used for interferon (IFN)-γ, interleukin (IL)-17, and FOXP3 (forkhead box P3) staining (all antibodies were from BioLigands, USA). Each counting cycle consisted of approximately 10 000 cells. FlowJo software version 7.6 (Treestar, Ashland, OR, USA) was used to analyze the flow cytometry results. Also, the mean fluorescence intensity (MFI) of $CD4^+$ and $CD8^+$ T cells were analyzed.

Statistical Analysis

Normality of distribution was checked by the Shapiro-Wilk test. To test the effect of time on the normally distributed data, generalized linear model and repeated measures analysis of variance (RM ANOVA) were applied. Paired t-test or Wilcoxon signed-rank test were used to compare the medians of two related groups. All statistical results were reported as mean±SEM. Statistical IBM SPSS package version 23.0 was used to analyze the data, and $p<0.05$ was considered significant.

RESULTS

Patient Characterization

This study consisted of 10 patients in the control group (4 women and 6 men; mean age, 61.3±5.34) and 10 patients in the intervention group (3 women and 7 men; mean age, 62.00±2). Diabetes, mild anemia, and hypertension were among the underlying diseases in both groups, but none of them were serious. Also, at the baseline, all patients were diagnosed with moderate-to-severe COVID-19 or mild-to-moderate ARDS based on a clinician's diagnosis.

One death was reported in the control group on the day 13 and 2 deaths in the intervention group on days 6 and 17. Aggravation of ARDS from moderate to severe was the main reason for patients' death in the intervention group, and pulmonary embolism was the

cause of death in the control group. Also, in this regard safety results are mentioned in our previous article. Briefly, no serious side effect was observed in any of the patients after cell transplantation.[24]

One out of the 10 patients in the intervention group left the study before receiving the complete course of injections, and was replaced by another patient. Therefore, the results are related to the patients who completed the protocol.

Specific Hematological Parameters of Patients

Table 1 summarizes the specific hematological changes of patients in the two groups during the study period.

Table 1. Changes in patients' specific hematological parameters in comparison with the control group during the study.

Parameters	Before Injection	*p*	5th day	*p*	10th day	*p*	17th day	*p*
WBC (cells/ µL)	C: 10675±566.8	0.14	10412±435.65	0.23	C:8750±176.27	0.08	C:8812±447.78	<0.001*
	IV:11860±634.5		IV:10460±552.06		IV:7580±393.50		IV:5462±248.95	
RBC (10^6 cells/µL)	C: 5.16 ± 0.13	0.17	C: 5.54 ± 0.09	0.34	C: 4.79 ± 0.14	0.38	C: 4.57 ± 0.14	0.004*
	IV: 5.22 ± 0.13		IV:5.52 ± 0.1		IV:4.81 ± 0.09		IV:5.32 ± 0.5	
Hb (g/dL)	C: 12.82 ± 0.53	0.18	C: 13.9 ± 0.44	0.15	C: 12.85 ± 0.37	0.31	C: 12.4 ± 0.39	0.32
	IV:13.54 ± 0.73		IV:14.6 ± 0.62		13.17 ± 0.49		IV:12.72 ± 0.41	
HCT (%)	C: 40.02 ± 1.26	0.44	C: 42.82 ± 1.83	0.38	C: 37.88 ± 1.47	0.22	C: 36.75 ± 1.45	0.46
	IV:38.96 ± 1.19		IV:43.08 ± 1.64		IV:38.93 ± 0.77		IV:36.55 ± 0.65	
Lymphocytes (%)	C: 5.2 ± 0.35	0.16	C: 5.55 ± 0.28	0.002*	C: 5.82 ± 0.19	0.004*	C: 6.04 ± 0.3	0.005*
	IV: 5.57 ± 0.28		IV:8.02 ± 0.23		IV:15.36 ± 0.54		IV:21.84 ± 1.02	
Neutrophils (%)	C: 92.81 ± 0.8	0.11	C: 93.55 ± 0.83	0.003*	C: 91.77 ± 0.46	0.004*	C: 90.66 ± 0.88	0.004*
	IV: 93.48 ± 0.81		IV:82.12 ± 1.72		IV:77.22 ± 1.34		IV:69.34 ± 1.41	
Platelets ($\times10^3$/µL)	C: 257.77±4023	0.04*	C: 208.666±3454	0.14	C: 165.000±2424	0.002*	C: 115.666±11439	0.003*
	IV:189.44±1219		IV:207.555±1403		IV:232.111±1449		IV:242.777±11698	

*Significant difference compared to the control group

C, control group; IV, intervention group; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; HCT, hemathocrit;

Leukocyte Counts

At the beginning of the study, WBC counts in both groups were higher than normal (Table 1), and in the course of treatment (day 17), a significant decrease was observed in both groups (p<0.001), which was also significantly different between groups(p<0.001). The trend of this decline was also more rapid after the first injection in the intervention group compared with the control group.

Erythrocyte, Hemoglobin, and Hematocrit Levels

RBC count in each group showed an increase with no statistical significance during the first 5 days of the study. However, the number decreased significantly in both groups during the rest of the study (until day 17); this decrease was greater in the control group compared with the intervention group. Similar patterns were observed for hemoglobin and hematocrit levels as well: an initial increase was followed by a consequent decrease with no statistical significance.

Lymphocyte, Neutrophil and Platelet Levels

The lymphocyte percentage increased significantly in both groups during the treatment period. Furthermore, a significant increase in the number of lymphocytes in the intervention group compared to the control group was observed on days 5, 10 and 17.

The pattern of change in the neutrophil counts was completely different compared to the pattern of change in lymphocytes: the percentage of neutrophils decreased in both groups during the study period, whose rate was significantly higher in the intervention group compared to the control group on days 5, 10 and 17.

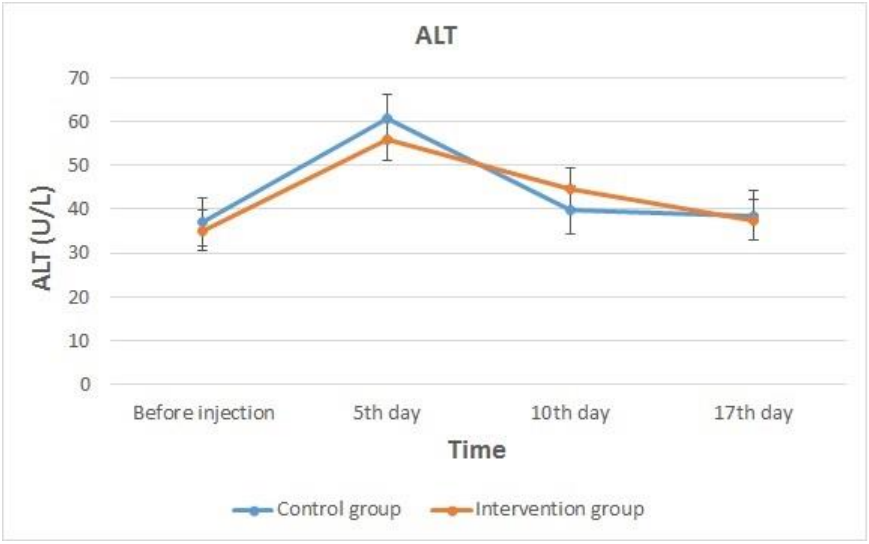
Platelet counts were significantly lower in the intervention group compared with the control group at the onset of the study. This number decreased significantly in the control group but increased in the intervention group. On the other hand, a significant difference was observed between the intervention and control groups on days 10 and 17.

Results of Liver Factors

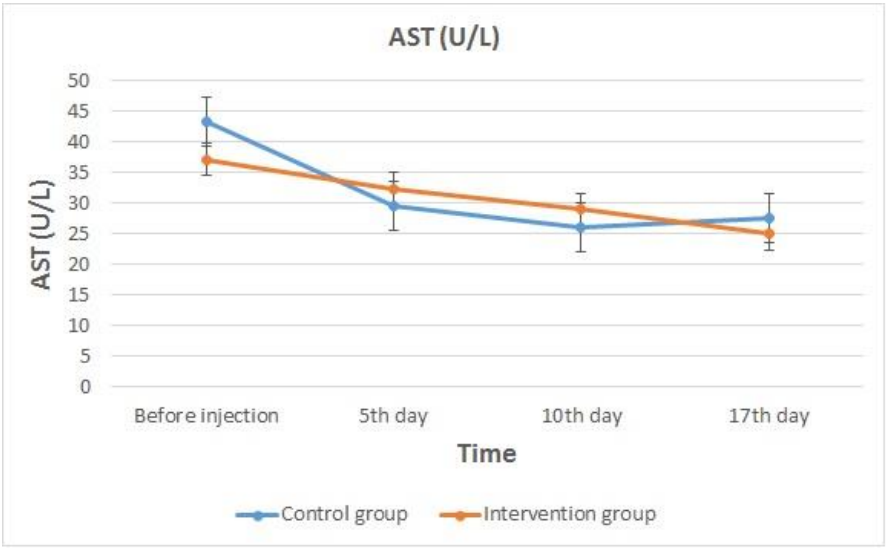
Alanine aminotransferase (ALT) levels increased during the first 5 days in both groups. Following that period, a significant decrease in the enzyme was observed in both groups (p<0.001). No significant difference was observed between groups during the study. ALT levels later returned to normal; ALT levels were not significantly different from the beginning of the study.

Aspartate aminotransferase (AST) levels also showed a significant decrease from the beginning to the end of the study in both the intervention (p=0.004) and the control (p<0.001) groups. No significant difference was observed in bilirubin levels in either group (Figure 1A-D).

A



B



Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

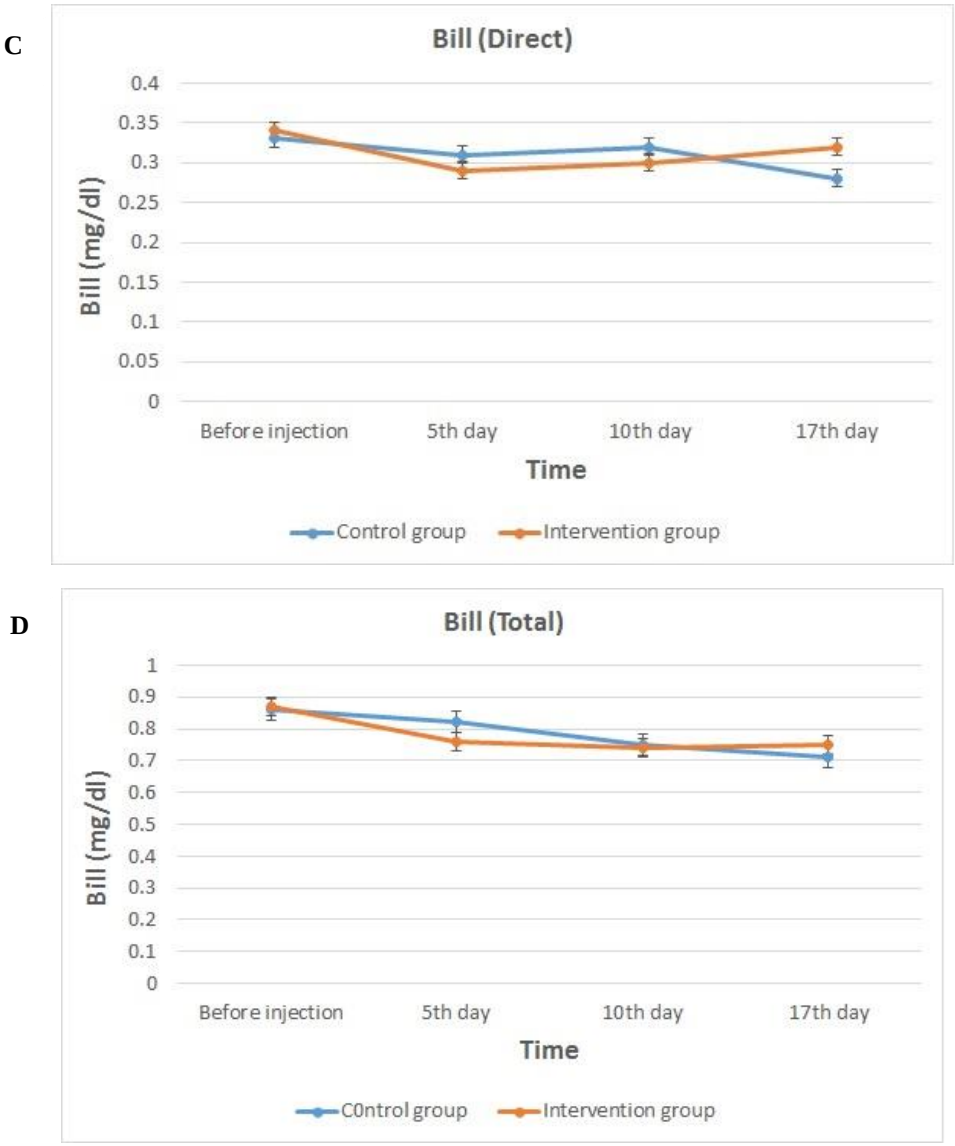


Figure 1. A-D Liver factors' change in COVID-19-induced ARDS patients during a 17-day follow-up period.
ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, Bill: Bilirubin, ARDS: Acute respiratory distress syndrome

T lymphocyte Analysis

$CD3^+$ T lymphocyte

Figure 2A displays the flow cytometry graph of patients in each group. Also, as shown in Figure 2B, at the onset of the study, there was no significant difference in $CD3^+$ T lymphocytes percentage between the control and intervention groups. At the same time, as the disease progressed to day 5, this percentage decreased significantly in the control group, and increased in the intervention group. Also, there was a significant difference between these two groups at that time.

On the 10th day, compared to the beginning of the study, non-significant increase in the control group but significant increase in the intervention group (p<0.0001) were observed. The difference between the two groups was also significant.

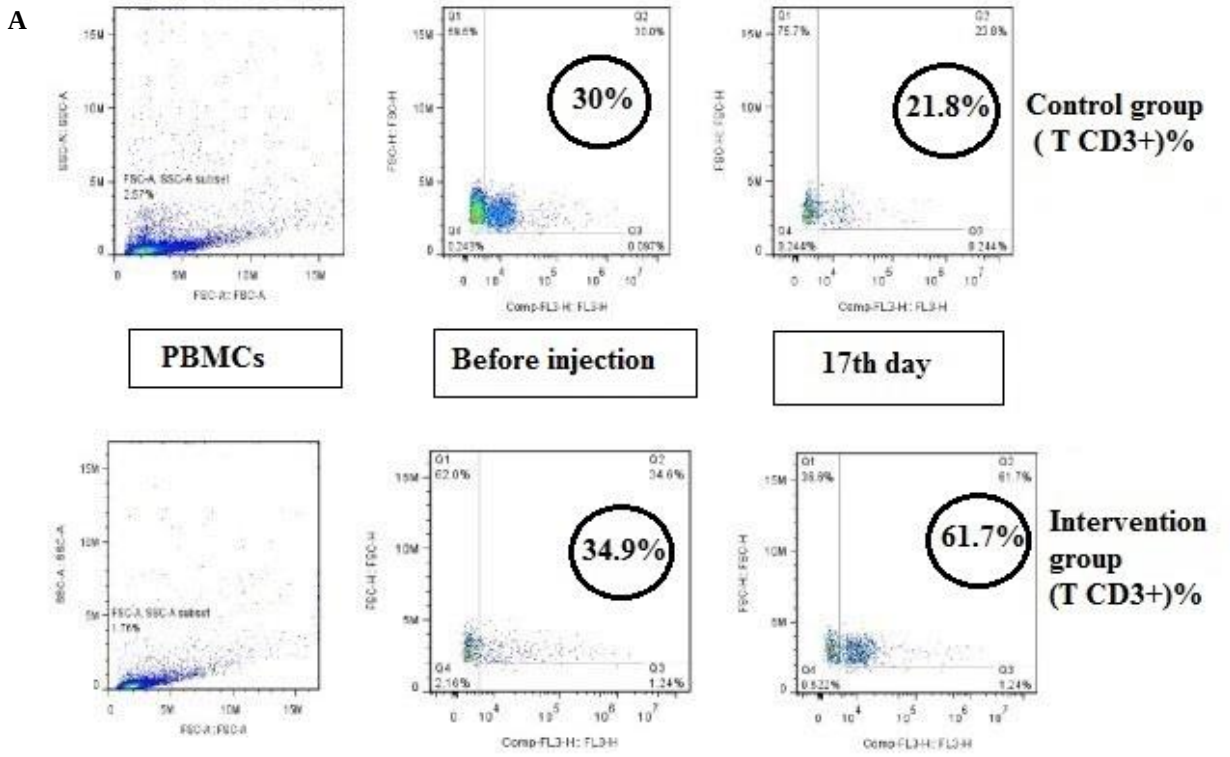
On the 17th day, a significant decrease in control group and an increase in intervention group was observed. Please refer to Table.2 for more details.

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

CD4⁺/IFN-γ⁺ T Lymphocytes (TH1)

Initially, there was no significant difference between the percentages of $CD4^+$ T cells between groups.

On the day 5, we saw a significant increase in in controls and a non-significant decreased in the intervention group. There was still no difference between the groups at this stage.



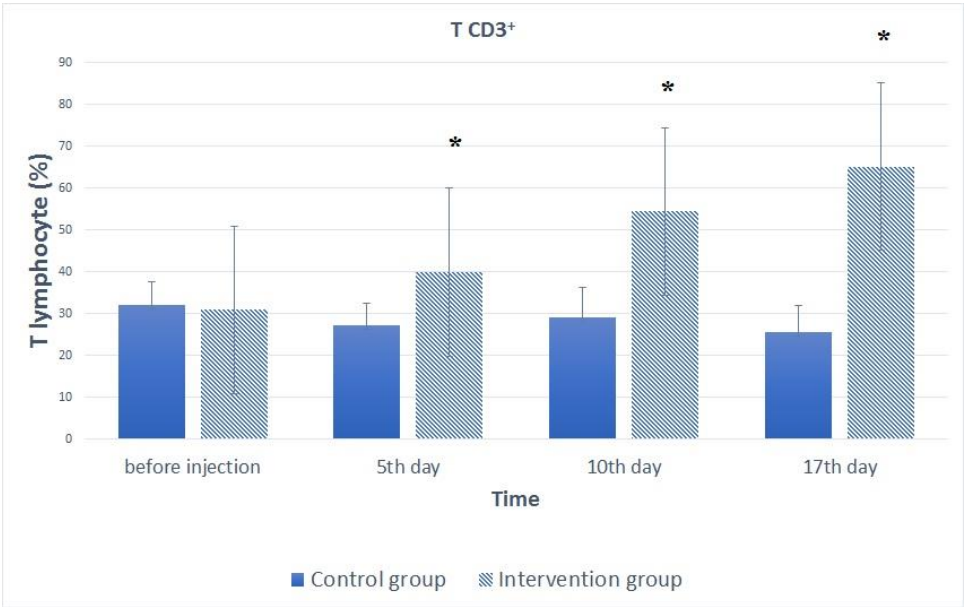


Figure 2. A) A flow cytometry graphs of $CD3^+$ T lymphocytes of a patient from each group. B) T lymphocyte percentage changes in the groups.

***Significant difference compared to the control group (p<0.05)**

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

On day 10 a decreasing trend was observed in both group which was not significant in control group but was significant in intervention group (p<0.0001) compared with the beginning of the study. The difference between groups was also significant at this stage.

On day 17, a significant decrease was observed in both the control (p=0.006) and intervention groups (p<0.0001) (Figure 3). Please refer to Table 2 for more details

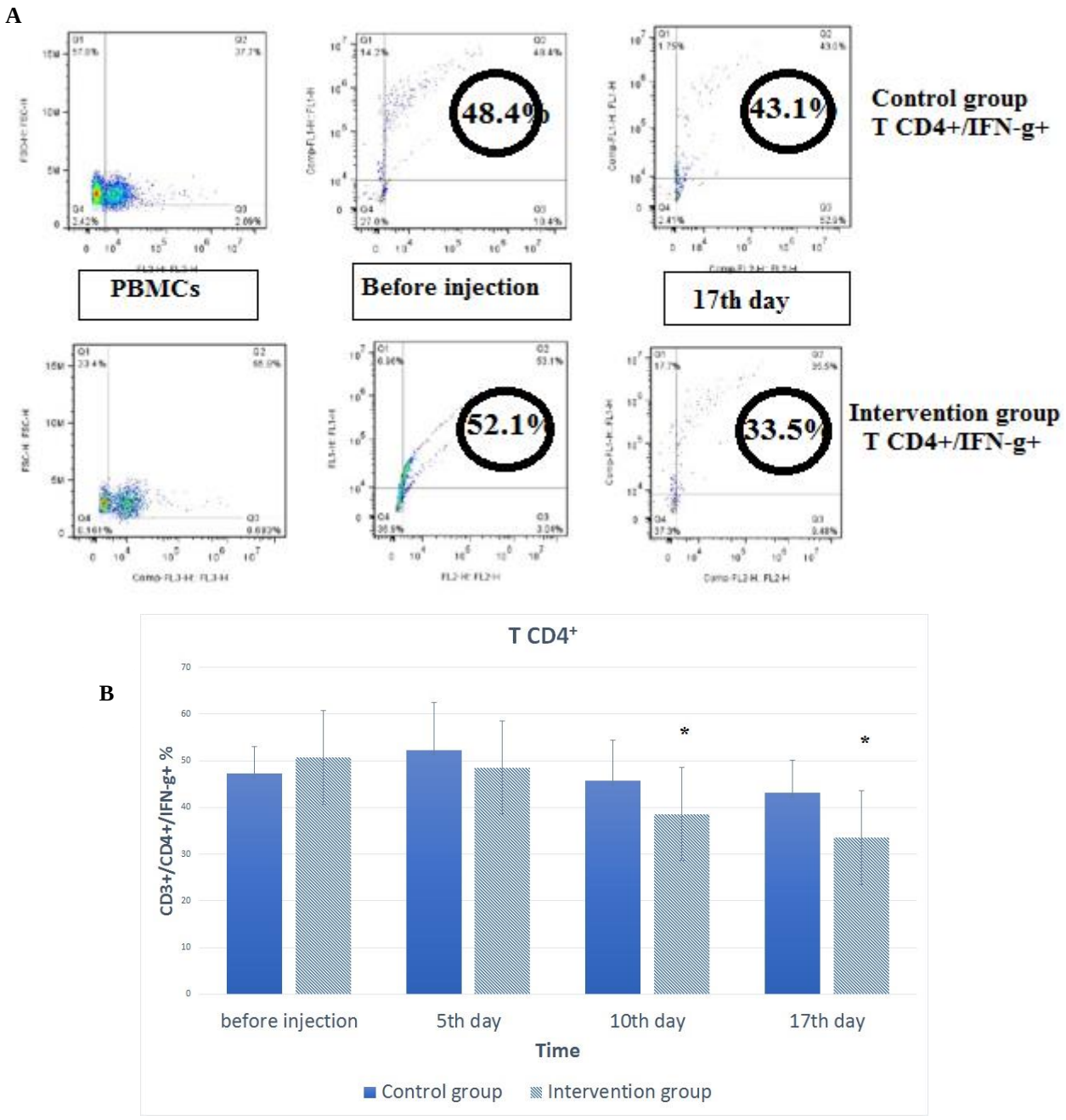


Figure 3. A) Flow cytometry of CD4$^+$IFN-γ$^+$ T lymphocytes of a patient from each group. B) Diagram of CD4$^+$IFN-γ$^+$ T lymphocyte percentage changes in the two groups.

***Significant difference compared to the control group (p<0.05)**

T CD8$^+$/IFN-γ$^+$ Lymphocytes (Cytotoxic T Lymphocytes)

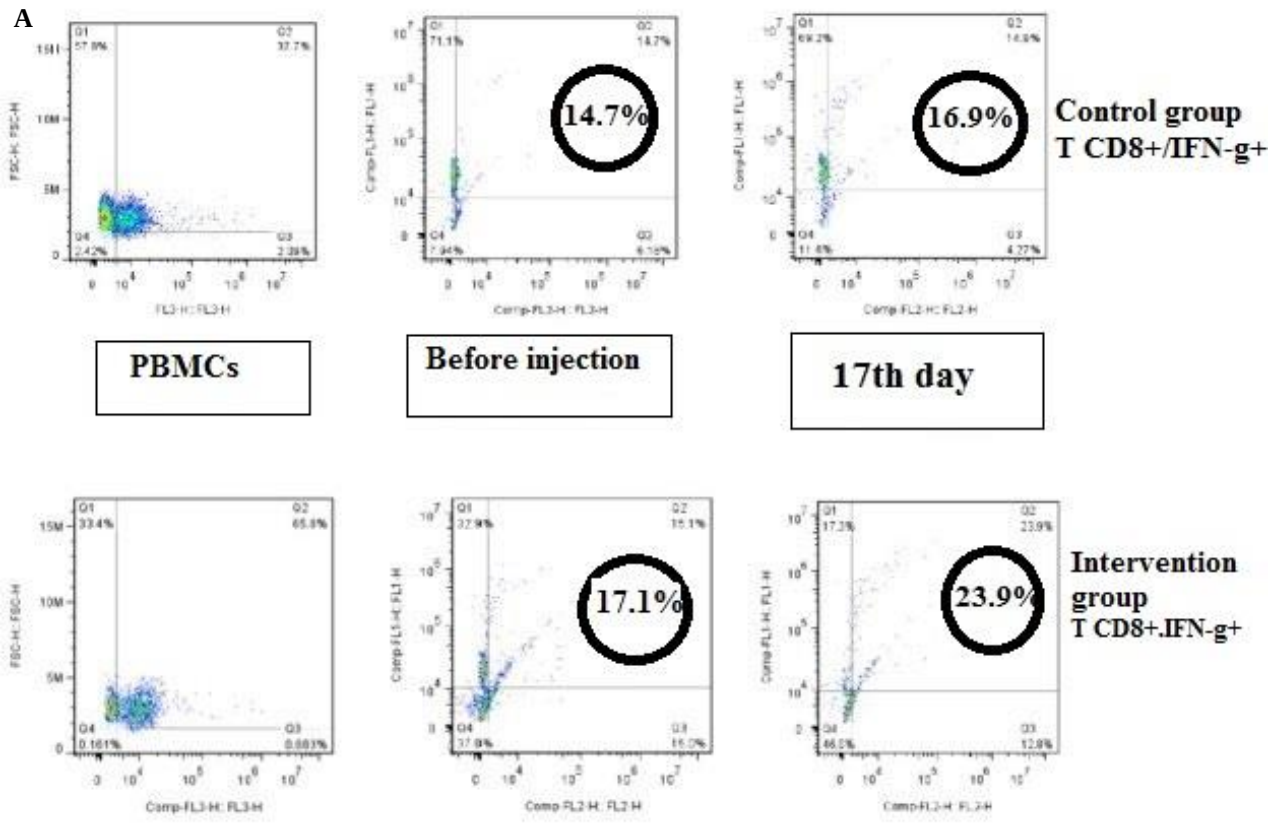
At the beginning of the study, the percentage of CD8$^+$ T cells were 15.56±2.4 in the control group and 18.6±4.9 in the intervention group, with no significant change on day 5. A significant difference was observed between the two groups at this time (p=0.002).

We observed a significant increase in this percentage compared to baseline on days 10 (p=0.0001) and 17 (p<0.0001).

Also, in the control group on the tenth day, there was a non-significant increase, while on the 17th day, a significant increase compared to the onset of the study was observed. Besides, on the fifth, tenth and seventeenth days, a significant difference was observed in the intervention group compared to the control group, respectively. Figure 4A is a typical representative flowcytometric graph of T CD8$^+$/IFN-γ$^+$ lymphocytes from a patient of each group. Also, all these data are summarized in Figure 4B. Please refer to Table 2 for more details.

TH17 Lymphocytes

As shown in Figure 5, the primary percentage of the TH17 cells in the control group had not significant difference compared to the intervention group. On the fifth day this percentage decreased non-significantly in both groups with an insignificant difference between this two groups at this time .In the control group on the tenth and seventh days compared to the study'-onset non-significant difference was observed. Also, In the intervention group on the tenth (p=0.012) and seventh days (p=0.008) compared to the beginning of the study significant difference was observed. The differences between this two groups on the tenth and seventh days was also statistically significant. Figure 5A is a typical representative flowcytometric graph of TH17 lymphocytes from a patient of each group. Also, all these data are summarized in Figure 5B. Please refer to Table 2 for more details.



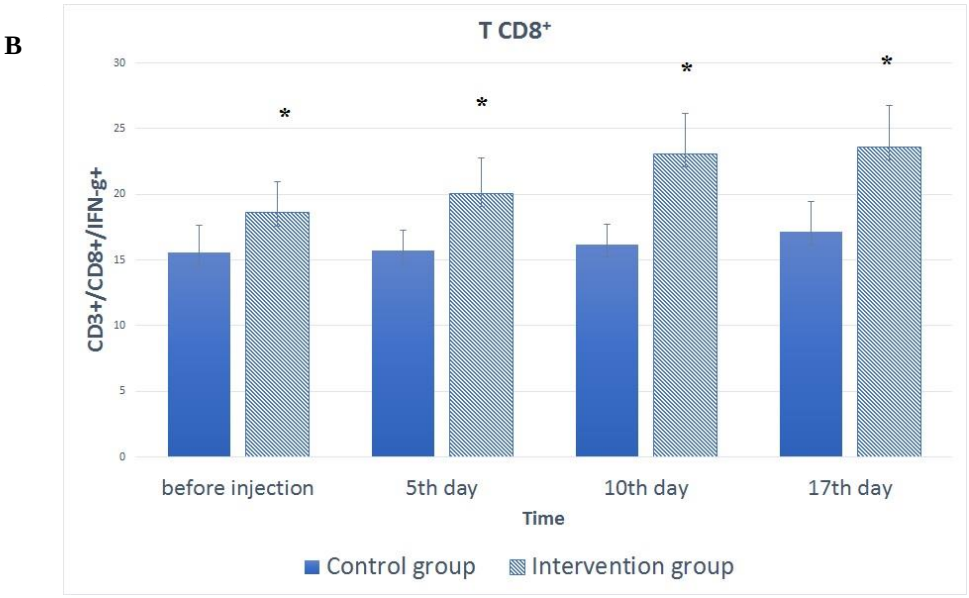
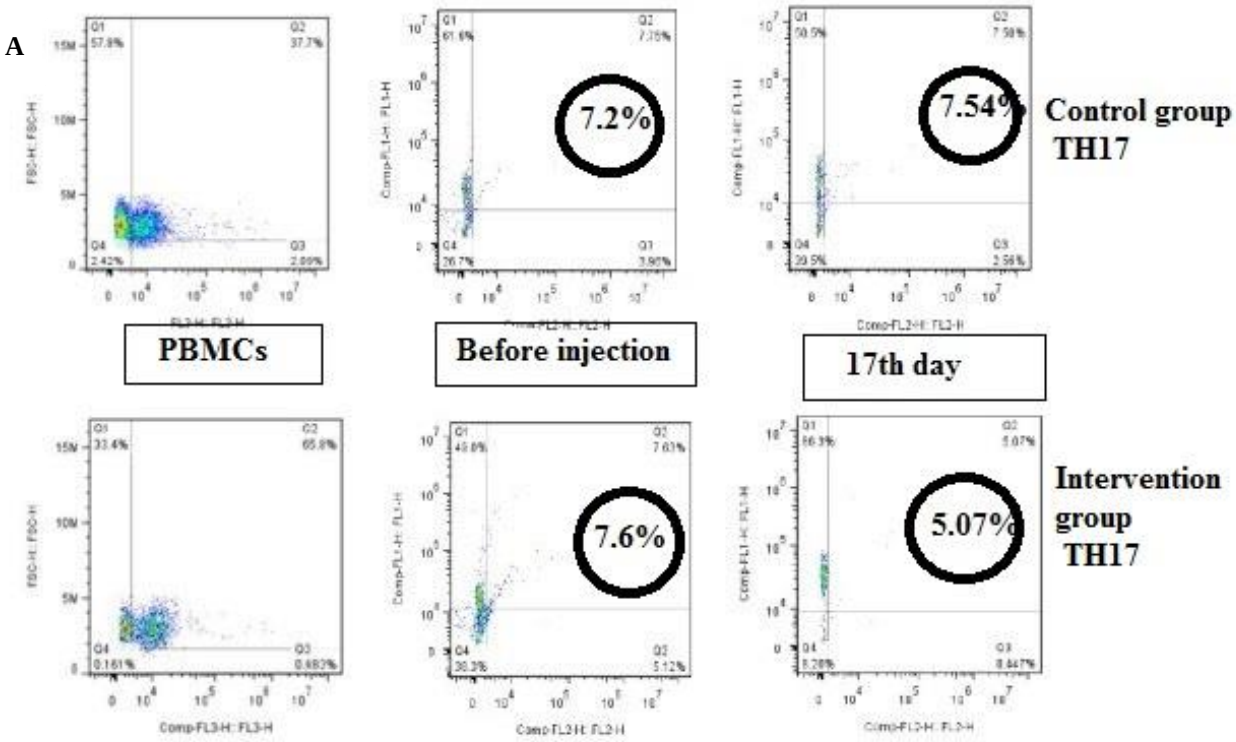


Figure 4. A) Flow cytometry graphs of T CD8$^+$/IFN-γ$^+$ lymphocytes of a representative patient from each group. B) Diagram of CD8$^+$/IFN-γ$^+$ T lymphocyte percentage changes in the two groups.
***Significant difference compared to the control group (p<0.05)**



B

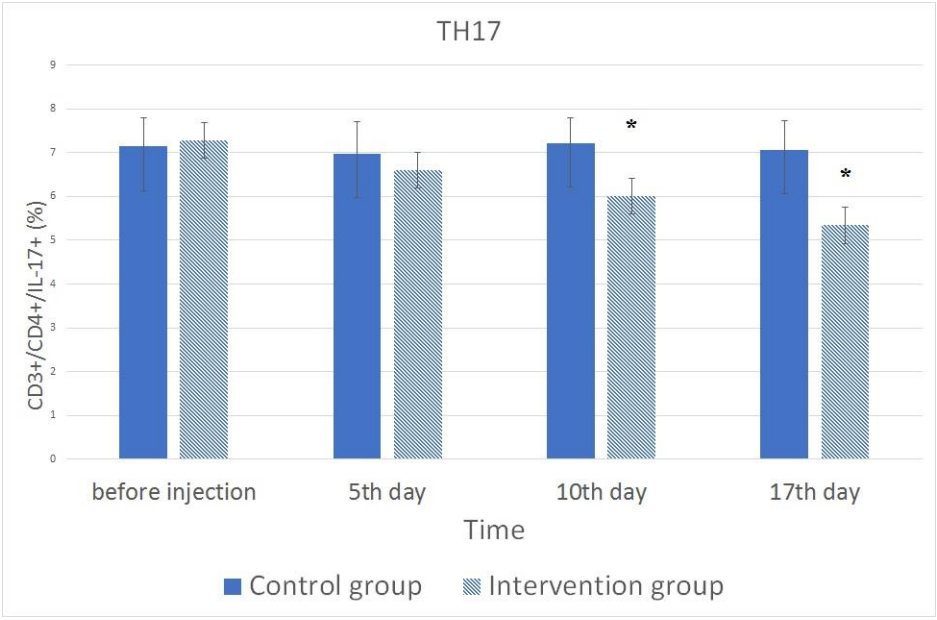


Figure 5. A) Flow cytometry graph of TH17 lymphocytes of a representative patient from each group. B) Diagram of TH17 lymphocyte percentage changes in the two study groups.
***Significant difference compared to the control group (p<0.05)**

T-reg Lymphocytes

T-reg percentage at the beginning of the study was (2.79±0.11) in control and (2.48±0.08) in the intervention group, which shows a statistically significant difference (p=0.028). On the fifth day of the disease, this percentage in the control group significantly decreased (p=0.06) while in the intervention group significantly increased (p=0.0001). Also, on the tenth day compared to the first day of the study, it decreased significantly in the control group and increased significantly in the intervention group. On the seventh day, significant decrease in the control group (p=0.03) and significant increase in the intervention group (p=0.0001) was observed. Also, on the fifth, tenth and seventeenth days, a significant difference was observed between the two intervention and control groups and T-reg percentage in the intervention group compared to the control group showed a significant increase. Figure 6A is a typical representative flowcytometric graph of TH17 lymphocytes from a patient of each group. Also, all these data are summarized in Figure 6B. Please refer to Table 2 for more details.

$CD4^+$ and $CD8^+$ MFI Analysing

As shown in this Figure 7 , mean floerence intensity (MFI) of $CD8^+$ T cells at the end of the study significantly decreased in intervention group (Figure 7B) compared to the control group (Figure7A) , which indicates the decrease in their overa ctivity at the end of the study.

Also, there was no significant difference between the two groups based on their final MFI of total $CD4^+$T lymphocytes at this time (Figure 7C and D).

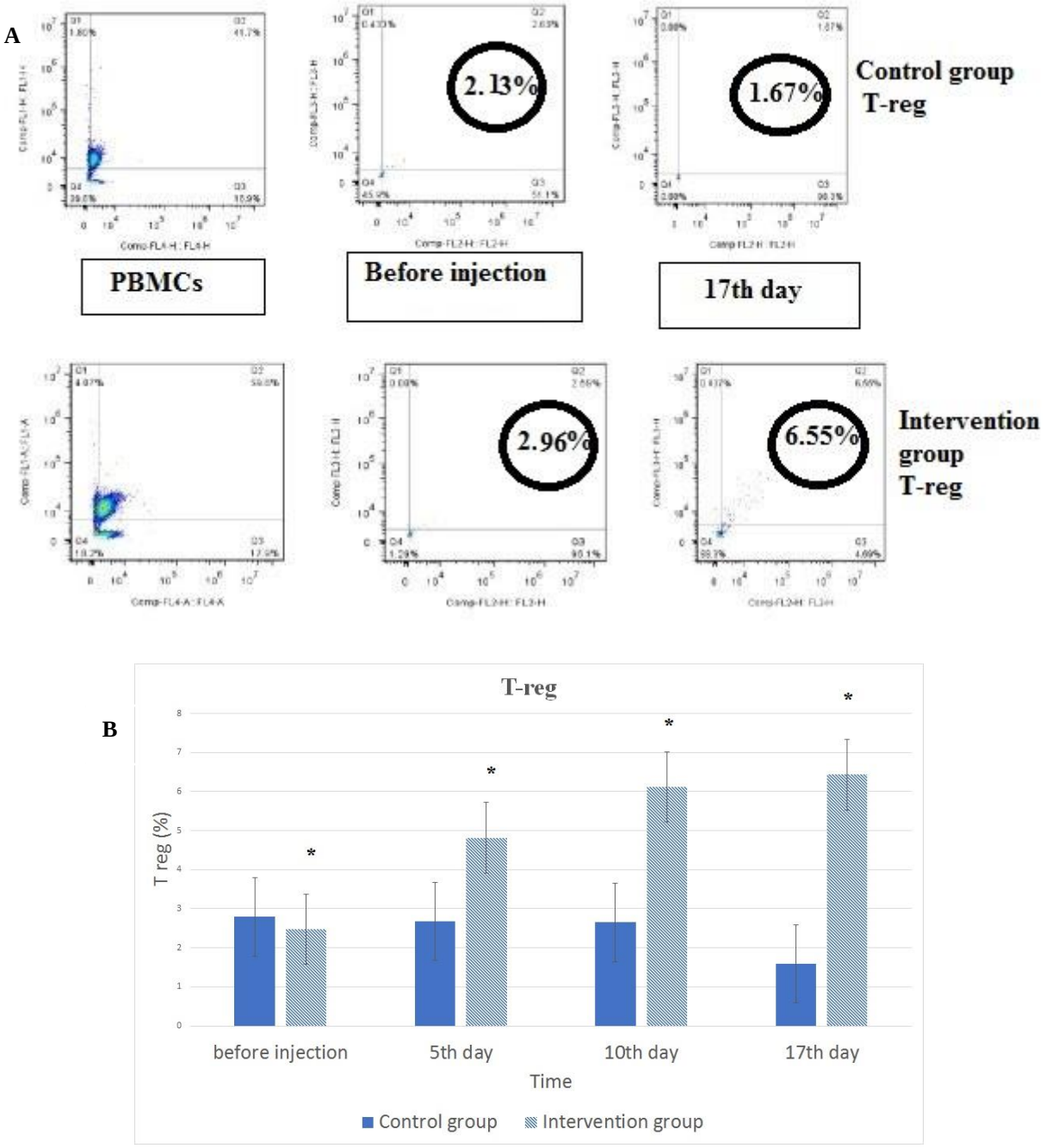


Figure.6. A) A typical representative flowcytometeric graph of T-reg lymphocytes from a patient of each group. B) Diagram of T-reg lymphocytes' percentage in two groups during the study.
***Significant difference compared to the control group (p<0.05)**

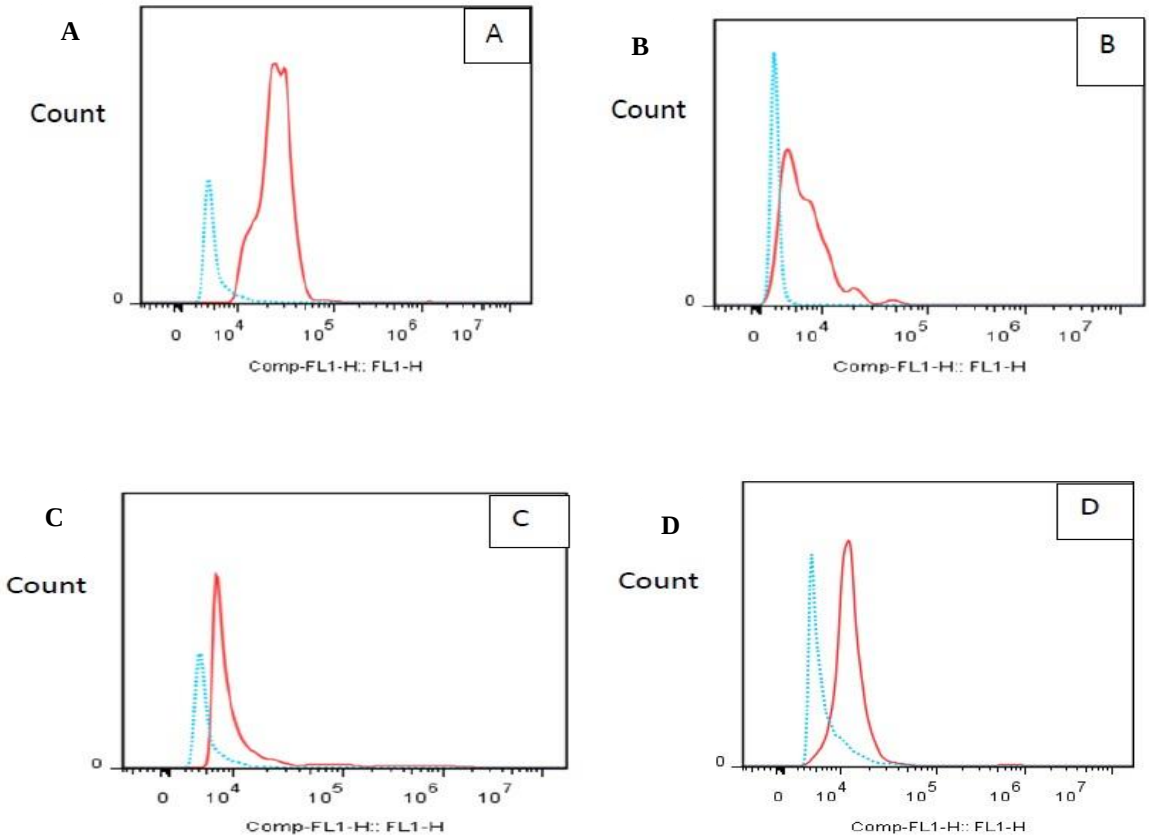


Figure.7. MFI analysing. A) Final $CD8^+$ MFI of control group. B) Final $CD8^+$ MFI of intervention group. C) Final total $CD4^+$ MFI of control group. D) Final total $CD4^+$ MFI of intervention group. Both CD4 and CD8 were stained by fluorescein isothiocyanate (FITC) , read in the FL-1 channel. (Dott line: unstained control, Solid line: a marker of interest).

Table 2. Changes in patients' T-lymphocytes percentage in comparison with the control group during the study.

Parameters	Before Injection	*p*	5th day	*p*	10th day	*p*	17th day	*p*
% $TCD3^+$	C: 32.15±5.6 IV: 30.92±7.6	0.16	27.28±5.12 IV: 39.94±6.5	0.002*	C: 29.07±7.3 IV: 54.4±10.1	0.002*	C: 25.66±6.4 IV: 65.2±15.6	<0.0001*
% TH1	C: 47.36±5.63 IV: 50.74±11.2	0.18	C: 52.25±10.3 IV: 48.55±8	0.59	C: 45.75±8.7 IV: 38.6±8.5	0.01*	C: 43.19±6.9 IV: 33.51±5.3	0.03*
% CTL	C: 15.56±2.4 IV: 18.6±4.9	0.002*	C: 15.7 ± 3.1 IV: 20.05±5.1	0.002*	C: 16.2±2.7 IV: 23.06±4.7	0.004*	C: 17.14±3.5 IV: 23.59±5.1	0.003*
% TH17	C: 7.14±0.89 IV: 7.28±0.65	0.4	C: 6.98±0.78 IV: 6.6±0.73	0.08	C: 7.21±0.9 IV: 6.01±0.09	0.004*	C: 7.07±0.15 IV: 5.34 ±0.27	0.004*
% T-reg	C: 2.79 ± 0.11 IV: 2.48 ± 0.08	0.028*	C: 2.68 ± 0.07 IV: 4.82 ± 0.16	0.14	C: 2.65±0.05 IV: 6.11 ± 0.06	0.002*	C: 1.59±0.03 IV: 6.43 ± 0.1	<0.0001*

* Significant difference compared to the control group

DISCUSSION

This study examined the clinical and immunological changes following 3 UC-MSC injections in ARDS patients infected with the SARS-COV-2.Since the therapeutic effects of MSCs won't last for an entire lifetime[25] in this study, multiple injections (3 times, every other day) were used to increase the therapeutic efficacy.No serious side effects were observed during the study and injections were well tolerated in all patients.

Positive results were observed in several parameters, includingdecreased total WBC and neutrophil counts (Table 1) in the intervention group after the first injection and in both groups after 10 days. This decrease was greater in the intervention group compared with the control group and these results were similar to the results from the studies by Hashemian et al,[26] and Liang et al.[27]

Furthermore, ASTlevels were decreased on the 5th day with no significant decrease in ALT and bilirubin levels 17 days. New coronavirus strains has been shown to impair liver function and cause elevated liver enzymes[28] Systemic inflammation and the direct attack of thevirus are two hypotheses for liver malfunction in COVID-19 patients. The results of several studies show that MSC-transplantation can improve the liver function in COVID-19 patients.[29,30] In accordance with our results, in a study by Meng et al. on 18 patients with COVID-19 following 3 doses of UC-MSC injections, a significant decrease in liver enzymes was observed.[29] Anti-inflammatory and immunomodulatory features of MSCs lead to the improvement of liver damages.[31]

Another advantageous change in both groups following the treatment process progression was a significant increase in the number of lymphocytes with a greater increase in the intervention group (21.84±1.02) compared with the control group (6.04±0.3) on day 17. This increase indicates an improvement in the immune function after cell transplantation. Significant increase in peripheral T lymphocyte percentages in the intervention group following cell injection and a significant decrease in the control group following disease progression (Figure 3) indicate the stimulatory role of MSCs in improving the immune function.

These results were consistent with previous cell therapy studies in this field.[27,32,33] For instance, in a study of Liang et al., an increase in T lymphocytes percentages ($CD3^+$, $CD4^+$, $CD8^+$) was observed along with the improvement in the patient's clinical condition.[27] Besides, the percentage of IFN-γ secreted by T_H1 lymphocytes significantly decreased in both groups at the end of the study (Figure 4).

MSC therapy can reduce the release of proinflammatory cytokines (eg, IFN-γ, IL-6, IL-8, IL-17) from activated immune cells due to their immunomodulatory properties.[34] In particular, it has been proven that MSC can reduce the secretion of IFN-γ by T_H1 cells.[35] Additionally, in the present study this decreasing trend was observed following the progress of treatment for T_H17 lymphocytes in both groups (Figure 6). However, at the end of the study, this decrease was significant in the intervention group and statistically insignificant in the control group. On the other hand, increasing the percentage of regulatory T cells (T-reg) (Figure 6) and cytotoxic (CD8) T lymphocytes (Figure 4) and their over-activity compared to $CD4^+$ T lymphocytes (Figure 7) based on their MFI, which is common in viral infection[36] was another outcome of cell therapy, which is consistent with other studies.[37]

Nowadays, COVID-19 disease is not only known as an infectious lung disease, but is also defined as an inflammatory disorder capable of causing ARDS and multiorgan failure through the abundance of ACE2 on various cells like hepatocytes, cardiomyocytes, alveolar cells and renal cells.[38] Also, like other respiratory viruses, SARS-CoV-2 dramatically triggers the host immunity, causing dysfunction in the host immune system. Indeed, many alterations such as lymphopenia,[4] thrombocytopenia, changes in the number and function of specific cells of the defense system, as well as the cytokine storm are common observations in severe COVID-19, some of which have already been observed in infections with viruses like SARS-CoV and MERS-CoV.[5-7]

MSC therapy also has crucial role in regulating the impaired immune system by various mechanisms. By secreting soluble factors like nitric oxide (NO), IL-10, and prostaglandin E2 (PGE2).[39] MSCs can interact with the injured tissue leading to an increase in Treg population and inhibition of T_H1 and T_H17 proliferation.[40] Protection of endothelial cells against oxidative stress and inflammation is another regulatory mechanism of MSC therapy.[41] Furthermore, by shifting the inflammatory T cell subsets to the regulatory subsets, MSC can increase the level of IL-10, which plays an eminent immune-regulatory role in COVID-19 patients.[42] Promising results of MSC therapy for COVID-19 patients in several recent clinical trials,[22,43] corroborate their immunoregulatory role.

The limitations of the present study include the short follow-up period and small sample size, which was due to the lack of enough beds in the ICU at the onset of the disease peak. However, shorter follow-ups have been reported in some studies.[21,32] Also having a control group in the study design, using fresh cells instead of frozen, and the use of UC-MSCs instead of bone marrow or adipose stem cells, which is a less invasive method are the advantageous of the present study.

To sum up, our results reveal that UC-MSC treatment can improve specific clinical and immunological parameters of COVID-19–induced ARDS patients. In particular, MSC therapy could improve liver function, leukocytosis, lymphopenia, neutrophilia and thrombocytopenia in COVID-19 patients. Furthermore, it could regulate impaired immune system through decreasing the inflammatory T cell subsets (T_H1, T_H17), decreasing the MFI of overactivated $CD8^+$ T cells, and increasing the CTL and Treg population. Nevertheless, larger studies are definitely needed to confirm these results.

STATEMENT OF ETHICS

This study was performed following the Declaration of Helsinki Ethical Principles and approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.REC.1399.150). Also, this clinical trial was registered with the Iranian Registry of Clinical Trials (ID: IRCT20160809029275N1). All patients or their representatives signed an informed consent form to participate in this clinical trial.

FUNDING

This study was financially supported by Mashhad University of Medical Sciences, Mashhad, Iran. (Grant.NO:981880).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Mashhad University of Medical Sciences, Mashhad, Iran, has financially supported this study, for which we are grateful. Also, we are really appreciative to the Iranian Council for the Development of Stem Cell Sciences and Technologies for donation grant and financial support (Contract No. 11/55696)

REFERENCES

1. Metcalfe SM. Mesenchymal stem cells and management of COVID-19 pneumonia. Medicine in drug discovery. 2020;5:100019.
2. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. Intensive care medicine. 2020;46(4):586-90.
3. Hu B, Guo H, Zhou P, Shi Z-L. Characteristics of SARS-CoV-2 and COVID-19. Nature Reviews Microbiology. 2020:1-14.
4. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. The Journal of clinical investigation. 2020;130(5):2620-9.
5. Yin Y, Wunderink RG. MERS, SARS and other coronaviruses as causes of pneumonia. Respirology. 2018;23(2):130-7.
6. Jesenak M, Brndiarova M, Urbancikova I, Rennerova Z, Vojtkova J, Bobcakova A, et al. Immune parameters and COVID-19 infection–associations with clinical severity and disease prognosis. Frontiers in cellular and infection microbiology. 2020;10:364.
7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. Jama. 2020;323(11):1061-9.
8. Farkhad NK, Reihani H, Moghadam AA, Moghadam AB, Tavakol-Afshari J. Are Mesenchymal Stem Cells able to manage Cytokine Storm in COVID-19 patients? A review of recent studies. Regenerative therapy. 2021.
9. Leng Z, Zhu R, Hou W, Feng Y, Yang Y, Han Q, et al. Transplantation of ACE2-mesenchymal stem cells improves the outcome of patients with COVID-19 pneumonia. Aging and disease. 2020;11(2):216.
10. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. The lancet. 2020;395(10229):1054-62.
11. Farkhad NK, Mahmoudi A, Mahdipour E. How similar are human mesenchymal stem cells derived from different origins? a review of comparative studies. Current stem cell research & therapy. 2021;16(8):980-93.

12. Farkhad NK, Mahmoudi A, Mahdipour E. Regenerative therapy by using mesenchymal stem cells-derived exosomes in COVID-19 treatment. The potential role and underlying mechanisms. Regenerative Therapy. 2022.
13. Tavakol-Afshari J, Boroumand AR, Farkhad NK, Moghadam AA, Sahab-Negah S, Gorji A. Safety and efficacy of bone marrow derived-mesenchymal stem cells transplantation in patients with amyotrophic lateral sclerosis. Regenerative Therapy. 2021;18:268-74.
14. Ghoryani M, Shariati-Sarabi Z, Tavakkol-Afshari J, Mohammadi M. The sufficient immunoregulatory effect of autologous bone marrow-derived mesenchymal stem cell transplantation on regulatory T cells in patients with refractory rheumatoid arthritis. Journal of immunology research. 2020;2020.
15. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. The Lancet. 2008;371(9624):1579-86.
16. Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. Diabetes. 2008;57(7):1759-67.
17. Manchikanti L, Centeno CJ, Atluri S, Albers SL, Shapiro S, Malanga GA, et al. Bone marrow concentrate (BMC) therapy in musculoskeletal disorders: evidence-based policy position statement of American Society of Interventional Pain Physicians (ASIPP). Pain Physician. 2020:E85-E131.
18. Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, et al. Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. The Lancet Respiratory Medicine. 2015;3(1):24-32.
19. Liu KD, Wilson JG, Zhuo H, Caballero L, McMillan ML, Fang X, et al. Design and implementation of the START (STem cells for ARDS Treatment) trial, a phase 1/2 trial of human mesenchymal stem/stromal cells for the treatment of moderate-severe acute respiratory distress syndrome. Annals of Intensive Care. 2014;4(1):1-9.
20. Matthay MA, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, et al. Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): a randomised phase 2a safety trial. The Lancet Respiratory Medicine. 2019;7(2):154-62.
21. Chen X, Shan Y, Wen Y, Sun J, Du H. Mesenchymal stem cell therapy in severe COVID-19: A retrospective study of short-term treatment efficacy and side effects. The Journal of Infection. 2020;81(4):647.
22. Shu L, Niu C, Li R, Huang T, Wang Y, Huang M, et al. Treatment of severe COVID-19 with human umbilical cord mesenchymal stem cells. Stem cell research & therapy. 2020;11(1):1-11.
23. Organization WH. Clinical management of severe acute respiratory infection (SARI) when COVID-19 disease is suspected: interim guidance, 13 March 2020. WHO 2020.
24. Kaffash Farkhad N, Sedaghat A, Reihani H, Adhami Moghadam A, Bagheri Moghadam A, Khadem Ghaebi N, et al. Mesenchymal stromal cell therapy for COVID-19-induced ARDS patients: a successful phase 1, control-placebo group, clinical trial. Stem Cell Research & Therapy. 2022;13(1):1-13.
25. Özdemir RBÖ, Özdemir AT, Sarıboyacı AE, Uysal O, Tuğlu Mİ, Kırmaz C. The investigation of immunomodulatory effects of adipose tissue mesenchymal stem cell educated macrophages on the CD4 T cells. Immunobiology. 2019;224(4):585-94.
26. Hashemian S-MR, Aliannejad R, Zarrabi M, Soleimani M, Vosough M, Hosseini S-E, et al. Mesenchymal stem cells derived from perinatal tissues for treatment of critically ill COVID-19-induced ARDS patients: a case series. Stem cell research & therapy. 2021;12(1):1-12.
27. Liang B, Chen J, Li T, Wu H, Yang W, Li Y, et al. Clinical remission of a critically ill COVID-19 patient treated by human umbilical cord mesenchymal stem cells: A case report. Medicine. 2020;99(31).
28. Guan W-j, Ni Z-y, Hu Y, Liang W-h, Ou C-q, He J-x, et al. Clinical characteristics of coronavirus disease 2019 in China. New England Journal of Medicine. 2020;382(18):1708-20.
29. Meng F, Xu R, Wang S, Xu Z, Zhang C, Li Y, et al. Human umbilical cord-derived mesenchymal stem cell therapy in patients with COVID-19: a phase 1 clinical trial. Signal Transduction and Targeted Therapy. 2020;5(1):1-7.
30. Zhang Y, Ding J, Ren S, Wang W, Yang Y, Li S, et al. Intravenous infusion of human umbilical cord Wharton's jelly-derived mesenchymal stem cells as a potential treatment for patients with COVID-19 pneumonia. Stem Cell Research & Therapy. 2020;11:1-6.
31. Cho KA, Woo SY, Seoh JY, Han HS, Ryu KH. Mesenchymal stem cells restore CCl4-induced liver injury by an antioxidative process. Cell biology international. 2012;36(12):1267-74.
32. Tao J, Nie Y, Wu H, Cheng L, Qiu Y, Fu J, et al. Umbilical cord blood-derived mesenchymal stem cells in treating a critically ill COVID-19 patient. J Infection in Developing Countries. 2020;14(10).

33. Tang L, Jiang Y, Zhu M, Chen L, Zhou X, Zhou C, et al. Clinical study using mesenchymal stem cells for the treatment of patients with severe COVID-19. Frontiers of medicine. 2020;14(5):664-73.
34. Weiss ARR, Dahlke MH. Immunomodulation by mesenchymal stem cells (MSCs): mechanisms of action of living, apoptotic, and dead MSCs. Frontiers in immunology. 2019;10:1191.
35. Le Blanc K, Pittenger M. Mesenchymal stem cells: progress toward promise. Cytotherapy. 2005;7(1):36-45.
36. Jason J, Inge K. Modulation of CD8 and CD3 by HIV or HIV antigens. Scandinavian Journal of Immunology. 2001;53(3):259-67.
37. Ganji A, Farahani I, Khansarinejad B, Ghazavi A, Mosayebi G. Increased expression of CD8 marker on T-cells in COVID-19 patients. Blood Cells, Molecules, and Diseases. 2020;83:102437.
38. Pearce L, Davidson SM, Yellon DM. The cytokine storm of COVID-19: a spotlight on prevention and protection. Expert Opinion on Therapeutic Targets. 2020;24(8):723-30.
39. Le Blanc K, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. Nature Reviews Immunology. 2012;12(5):383-96.
40. Jiang X-X, Zhang Y, Liu B, Zhang S-X, Wu Y, Yu X-D, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005;105(10):4120-6.
41. Meng SS, Guo FM, Zhang XW, Chang W, Peng F, Qiu HB, et al. mTOR/STAT-3 pathway mediates mesenchymal stem cell–secreted hepatocyte growth factor protective effects against lipopolysaccharide-induced vascular endothelial barrier dysfunction and apoptosis. J Cell Bio. 2019;120(3):3637-50.
42. Li Z, Niu S, Guo B, Gao T, Wang L, Wang Y, et al. Stem cell therapy for COVID-19, ARDS and pulmonary fibrosis. Cell proliferation. 2020;53(12):e12939.
43. Shi L, Huang H, Lu X, Yan X, Jiang X, Xu R, et al. Effect of human umbilical cord-derived mesenchymal stem cells on lung damage in severe COVID-19 patients: a randomized, double-blind, placebo-controlled phase 2 trial. Signal transduction and targeted therapy. 2021;6(1):1-9.