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Exosomes from Adipose Tissue-derived Mesenchymal Stem Cells Induce Regulatory T Cells in COVID-19 Patients

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ABSTRACT

An imbalance between regulatory T (Treg) and T-helper (Th)-17 cells has been implicated in the pathogenesis of coronavirus disease 2019 (COVID-19). Mesenchymal stem cells (MSCs) exert immunomodulatory properties through secreting exosomes. This study aimed to assess the effect of MSC-derived exosomes (MSC-Exo) on the differentiation of peripheral blood mononuclear cells (PBMCs) into Tregs from patients with COVID-19.

Exosomes were isolated from adipose tissue–derived MSCs. PBMCs were separated from the whole blood of COVID-19 patients (n=20). Treg frequency was assessed before and 48 hours after treatment of PBMCs with MSC-Exo using flow cytometry. Expression of *FOXP3* and cytokine genes, and the concentration of cytokines associated with Tregs, were assessed before and after treatment with MSC-Exo.

The frequency of $CD4^{+}CD25^{+}CD127^{-}$ Tregs was significantly higher after treating PBMCs with MSC-Exo (6.695±2.528) compared to before treatment (4.981±2.068). The expressions of transforming growth factor (TGF)-β1, interleukin (IL)-10, and *FOXP3* were significantly upregulated in MSC-Exo–treated PBMCs. The concentration of IL-10 increased significantly after treatment (994.7±543.9 pg/mL) of PBMCs with MSC-Exo compared with before treatment (563.5±408.6 pg/mL). The concentration of TGF-β was significantly higher in the supernatant

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of PBMCs after treatment with MSC-Exo (477.0±391.1 pg/mL) than PBMCs before treatment (257.7±226.3 pg/mL).

MSC-Exo has the potential to raise anti-inflammatory responses by induction of Tregs, potentiating its therapeutic effects in COVID-19.

Keywords: COVID-19; Exosomes; Immunomodulation; Mesenchymal stem cells; Peripheral blood mononuclear cells; Regulatory T cells

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a significant global health issue caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2).[1] Over the last year, this epidemic has impacted human health and the economy. One of the most critical characteristics of this virus is the speed and ease of dissemination between individuals.[2] Despite the virus' ease of transmission and few cases with severe disease presentations, 80% of infected cases are almost asymptomatic or show mild flu-like symptoms. Meanwhile, around 15% of individuals infected with the virus experience a severe form of the disease, such as pneumonia, and 5% suffer from acute respiratory distress syndrome (ARDS).[3] Cytokine storms, severe lung damage owing to inflammation, and impaired immune system homeostasis are prominent causes of morbidity and mortality from COVID-19.[4]

At the cellular level, the virus interacts with angiotensin-converting enzyme II receptors and transmembrane receptor serine protease-2 through the spike glycoprotein, resulting in an overactivation of the inflammatory pathway by the transcription factor nuclear factor (NF)-κB.[5,6] This molecule is involved in promoting the transcription of several cytokines and chemokines and contributes to the pathophysiology of the disease by triggering the components of the cytokine storm. The cytokine storm causes circulating immune cells, such as T cells, neutrophils, and monocytes, to infiltrate into the lungs. The recruitment of these activated immune cells causes considerable lung tissue damage, which may lead to ARDS, the major cause of disability in COVID-19 patients.[7] Evidence suggests that T helper (Th) 17-mediated inflammation plays a critical role in the etiology and pathogenesis of COVID-19–mediated pneumonia.[8]

Regulatory T (Treg) cells are critical immune players in maintaining homeostasis in the immune system.[9] These cells are required for maintaining self-tolerance and avoiding autoimmune responses by exploiting diverse immunosuppressive mechanisms.[10]

The most important transcription factor involved in Treg development is forkhead box P3 (FoxP3). Tregs exert their function through direct contact with other immune cells and the induction of inhibitory signaling in target cells via inhibitory immune checkpoint receptors, such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), and secretion of anti-inflammatory mediators such as interleukin (IL)-10 and transforming growth factor (TGF)-β.[11] The frequency of Tregs in the peripheral blood of severe COVID-19 patients is lower than in those with a moderate presentation.[12] One explanation for the reduced quantity of Tregs in the circulation of COVID-19 patients is the migration of Tregs to the lungs to prevent lung tissue damage. As a result, lower Treg counts in the circulation might be the underlying cause of the immune system's overactivation and lung injury in subjects with severe COVID-19 disease.[13] Therefore, the induction of Treg differentiation might confer a therapeutic tool in the treatment of COVID-19.

Mesenchymal stem cells (MSCs) possess immunomodulatory capabilities that suppress immunological responses by producing various factors, like exosomes.[14] Coculturing MSCs and Tregs from asthmatic patients promotes the suppressive capability of Tregs.[15] Studies also show that the hepatocyte growth factor produced by MSCs alters the Th17 to Treg ratio by converting fully differentiated Th17 cells into functional Tregs.[16] However, due to limitations in cell transplantation, the application of MSC-derived exosomes (MSC-Exo) has increased in recent years.[17,18]

Exosomes are extracellular vesicles (EVs) 30–150 nm in diameter. These vesicles contain the same contents as MSCs and may easily merge with target cells due to their membrane's resemblance to the cell membrane.[19] Adipose tissue-derived MSCs produce exosomes with a variety of therapeutic that can be utilized in research.[20] MSC-Exo increases the secretion

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of various anti-inflammatory mediators, such as TGF-β and IL-10, and suppress the production of pro-inflammatory mediators, such as interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and IL-1 in target cells.[21] In addition, MSC-Exo contain a variety of anti-inflammatory, regenerative, and immunomodulatory growth factors, chemokines, microRNAs, and mRNAs.[22] MSC-Exo can also increase the proliferative and suppressive capacity of Tregs in patients with asthma by modulating the expression of anti-inflammatory mediators like TGF-β and IL-10.[23] Furthermore, COVID-19 patients have recently been treated with MSC-Exo.[24] However, the precise mechanisms underlying the therapeutic properties of MSC-Exo are not clear.

The potential therapeutic effects of adipose tissue-derived MSC (AD-MSC)-Exo have already been addressed but have not been explored in the context of COVID-19 regarding its anti-inflammatory properties. Taking into account the importance of Tregs in the outcomes of COVID-19 patients, this research aimed to look into the anti-inflammatory and immunomodulatory effects of MSC-Exo on PBMCs in COVID-19 patients by assessing its effect on the induction of Treg development and cytokine release.

MATERIALS AND METHODS

Study Design

In this in vitro before-and-after trial, PBMCs isolated from patients with COVID-19 (n=20) were treated with 2 µg/mL of phytohemagglutinin (PHA; eBiosciences Inc., San Diego, CA), in the presence or absence of 100 mg/mL exosomes (total protein measured by bicinchoninic acid [BCA]), for 48 hours in 96-well culture plates, according to our previous optimization study.(25) Within 48 hours after treatment, gene expression was measured by real-time polymerase chain reaction (PCR), and flow cytometry was performed to determine Treg frequency. The supernatant was also collected to measure the concentration of selected cytokines (Figure 1).

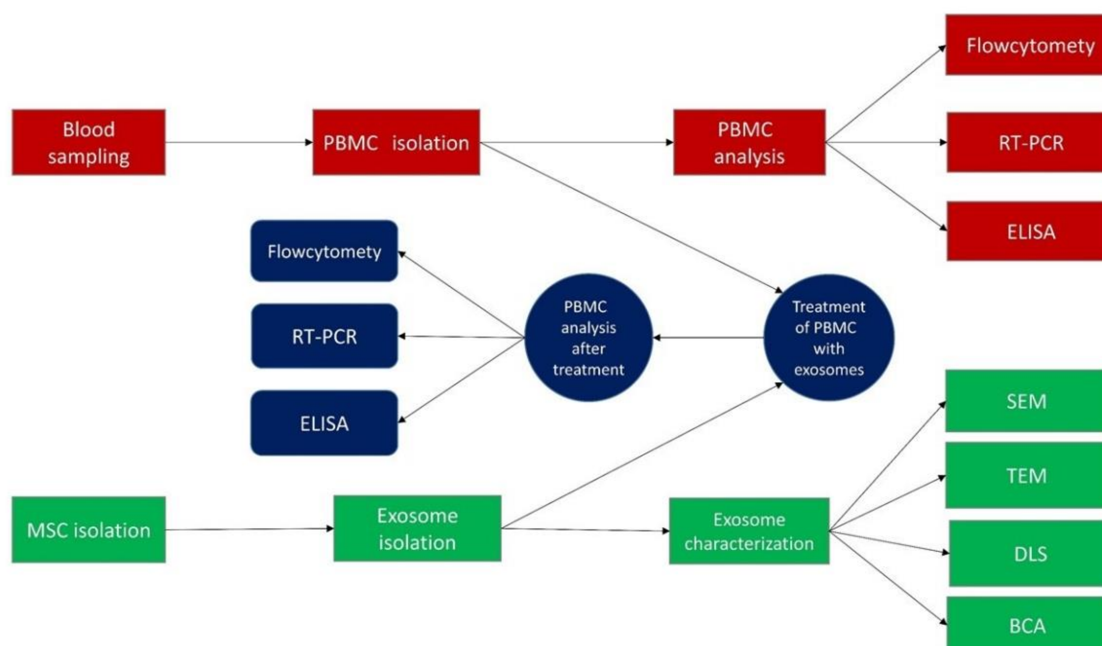

Figure 1. Flow diagram of study design and procedures. PBMCs were separated from the whole blood of 20 COVID-19 patients. Exosomes were also isolated from adipose tissue-derived MSCs and characterized for their specifications using SEM, TEM, and DLS. Before and 48 hours after the treatment of PBMCs with MSC-Exo, flow cytometry was used to assess the frequency of Tregs. mRNA expression of *FOXP3* and cytokines, as well as concentrations of cytokines related to Tregs, were assessed before and after the treatment. BSA, bicinchoninic acid; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; Exo, exosome; MSC, mesenchymal stem cell; PBMC, peripheral blood mononuclear cells; RT-PCR, real-time polymerase chain reaction; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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Patients

A total of 20 patients with COVID-19 (10 women and 10 men, mean age: 45.5 years old) were recruited from the outpatient clinic of Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran. Peripheral blood samples were taken from each patient following written informed consent. The diagnosis of COVID-19 was confirmed using laboratory tests, clinical symptoms, real-time PCR analysis of nasal or pharyngeal swab specimens, and a computerized tomography (CT) scan of the lungs. All subjects had mild COVID-19 and were not hospitalized in the ICU. All individuals were free of any current anti-inflammatory or immunosuppressive medications, had no other viral or bacterial infections, and had not used any vitamin supplements in the last month leading up to the sampling. Table 1 summarizes the subjects' demographic, clinical, and laboratory findings.

Table 1. Baseline, clinical, and laboratory findings of the study subjects (n=20).

Parameter	Result	Parameter	Result
		White blood cell count (× 10^9/L)	
Age in years; range (mean± standard deviation)	19-69 (53.3±8.4)	**<4**	4 (20%)
		4–10	10 (50%)
		>10	6 (30%)
Sex		**Lymphocyte count (× 10^9/L)**	
Men	10 (50%)	**<1.0**	12 (60%)
Women	10 (50%)	**≥1.0**	8 (30%)
		Platelet count (× 10^9/L)	
Current smoker	4 (20%)	**<100**	9 (45%)
		≥100	11 (55%)
		Creatinine (µmol/L)	
Diabetes mellitus	1 (5%)	**≤133**	18 (90%)
		>133	2 (10%)
		Lactate dehydrogenase (U/L)	
Hypertension	1 (5%)	**≤245**	14 (70%)
		>245	6 (30%)
Cardiovascular disease	2 (10%)	**Bilateral involvement of chest radiographs**	20 (100%)
Chronic kidney disease	1 (5%)	**Cough**	11 (55%)
Fever			
<37.3°C	2 (10%)	**Headache**	4 (20%)
37.3–38.0°C	8(40%)		
38.1–39.0°C	7 (35%)	**Dyspnea**	6 (30%)
>39.0°C	3 (15%)		

Blood Sampling and PBMC Isolation

PBMCs were isolated from 10 mL of blood samples. A density gradient method was employed using Ficoll-Hypaque density gradient solution (Biosera, Heathfield, East Sussex, UK) at 1.077 g/mL gradient density in a ratio of 2:1 in falcon conical tubes (SPL, Korea). The samples were then centrifuged at 3000 rpm for 20 minutes and washed twice using phosphate-buffered

saline (PBS; Sigma Aldrich, Germany). Then, 10^6 PBMCs were cultured in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% decomplemented fetal bovine serum (FBS), 1% L-glutamine, and 1% Penicillin-Streptomycin (all from Gibco, USA), and incubated at 37°C with 5% CO_2 for 24 hours.

AD-MSC Culture

AD-MSCs were purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% decomplemented FBS, 1% L-glutamine, and 1% Penicillin-Streptomycin (all from Gibco, USA) in T25 cell culture flasks (SPL, Korea). The cell culture media were changed twice a week. When they reached 80% confluency, they were collected using trypsin (Invitrogen, Basel, Switzerland), and the cells were washed twice with FBS-containing DMEM/F12 medium to inactivate trypsin. Then, cells were centrifuged at 1700 rpm and transferred to three T25 cell culture flasks.

MSC Exosome Isolation and Characterization

We cultured MSCs in T75 flasks (SPL, Korea) until they reached 80% confluency. Then, we decreased the FBS level from 10% to 0% over 3 days to starve the cells. The cells were then incubated with 0% FBS for 72 hours. Then, the cell supernatant was collected and centrifuged (2000*g*, 30 minutes) to remove debris and cells. The supernatant, which contained cell-free culture medium, was then transferred to another clean tube. For every 1 mL of supernatant, 500 μL of Total Exosome Isolution Kit (Cib Biotech Co., Tehran, Iran) was added to the tubes. After vortexing, the tubes were incubated at 2–8°C for one day. Following incubation, tubes were centrifuged at 10,000*g* and 4°C. The supernatant was removed, and the exosomal precipitate was dissolved in PBS. After separation, the exosome concentration was assessed using a BCA kit (DNAbiotech Co., Tehran, Iran). Then, a standard solution for serial dilution of exosomes was prepared in 9 tubes. This solution's concentration ranged from 1000 μg/mL in tube 1 to 6.25 μg/mL in tube 9. A working solution containing BCA (reagent A) and Capers (reagent B) that was previously prepared in a 50:1 ratio was added to all tubes.

After 60 minutes of incubation at 60°C in a water bath, each tube's optical density was read at 562 nm using spectrophotometric measurement (NanoDrop; Agilent Technologies, USA). The form and approximate size of the isolated exosomes were assessed using scanning electron microscopy (SEM). For this purpose, the samples were fixed on the slide with glutaraldehyde for 15 minutes, and after washing with PBS, ascending dilutions of ethanol were used to dehydrating the samples. After ethanol evaporation, samples were kept at room temperature for 24 hours to dry completely. After gold-palladium sputtering, they were examined by SEM. Also, exosome size distribution was determined using a well-established dynamic light scattering (DLS) approach (Malvern Instruments, Malvern, UK). Exosome morphologies were examined using transmission electron microscopy (TEM).

Flow Cytometry

Flow cytometry was employed to measure the number of Tregs in the culture of PBMCs before and after treatment with MSC-Exo. Cells were collected after 48 hours of treatment with MSC-Exo. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD25, and phycoerythrin (PE)-conjugated anti-CD127 antibodies, or isotype-matched immunoglobulin G controls (all from eBioscience, San Diego, CA, USA) for 20 minutes at room temperature. A FACSCalibur flow cytometer (Becton Dickinson, CA, USA) was used to measure the mean fluorescence intensity. FlowJo Software (version 10) was used to analyze the data.

Real-time PCR

Total RNA was isolated from PBMCs (before treatment) and a pool of cells 48 hours after treatment with MSC-Exo) using SinaClon RNX-PLUS solution (Tehran, Iran) and assessed for its quality and quantity using a nanodrop spectrophotometer (NanoDrop; Agilent Technologies, USA). The ratio of absorbance at 260 and 280 nm (A260/280) and A260/230 was also measured to determine the quality of extracted RNA. Thermo Fisher Reverse Transcriptase Kit (Waltham, MA, USA) was used for complementary DNA (cDNA) synthesis. Real-time PCR using SYBR Green master mix (Ampliqon, Denmark) and StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) were employed to determine the amounts of FoxP3, IL-10, and TGF-β mRNAs. Supplementary Table 1 lists primers used in the real-time measurement of target genes. The content of the reaction mixture in

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each tube included 10 μL SYBR Green master mix, 8 μL cDNA, 1 μL primer (0.5 μL forward and 0.5 μL reverse), and RNase-free H_2O, collectively equaling 30 μL. The thermocycling conditions comprised 10 minutes at 95°C, followed by 40 cycles of denaturation for 10 seconds at 95°C, annealing for 30 seconds at 58°C (for *FOXP3*) and 60°C (for *TGFB1* and *IL10*), and expansion for 20 seconds at 72°C. The transcript level of beta-actin (*ACTB*) was utilized as the housekeeping gene to normalize mRNA expression levels of target genes. The relative amounts of mRNA expression were quantified via comparative C_T method applying the $2^{-\Delta\Delta Ct}$ formula. For each sample, a duplicate PCR reaction was set up.

Measurements of Cytokine Levels

A commercial enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, USA) was used to measure the concentrations of TGF-β and IL-10 in the supernatant of PBMCs before and 48 hours after treatment. The predicted sensitivity for IL-10 and TGF-β was 4.69 pg/ml and 18.75 pg/ml, respectively, based on the concentration gradients of positive controls or standard solutions of the kit. The optical density was measured at 450 nm using a Medgenix ELISA reader (BP-800, Biohit, USA). Using proper standard calibration lines, concentrations in each sample were measured by Microplate Reader SoftMax software. The experiments were conducted in duplicate.

Statistical Analysis

GraphPad Prism for Windows version 8.3 (GraphPad Software Inc., San Diego, CA) was used to analyze data and design graphs. Using the Shapiro-Wilk test, the normal distribution of quantitative variables was assessed (the data had a normal distribution). To compare means between before and after treatment groups, the paired *t*-test was used. The statistical significance was set at $p<0.05$. Data is presented as the mean ± standard deviation (SD).

RESULTS

MSC-Exo Characterization

Exosomes isolated from AD-MSCs were characterized for size using DLS (Figure 2A) and shape using SEM (Figure 2B1) and TEM (Figure 2B2). Exosomes ranged in size from 50 to 200 nm. The mean size of the isolated exosomes was 157 nm. Additionally, SEM and TEM images revealed a uniform spherical shape for exosomes with no significant deformities.

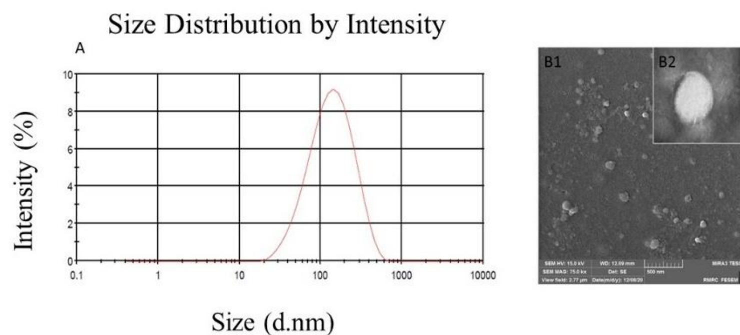

Figure 2. Characterization of mesenchymal stem cell exosomes (MSC-Exo). Measuring the mean size of exosomes by dynamic light scattering (A). Scanning electron microscopy (SEM) of exosomes to illustrate their shape (B1). Transmission electron microscopy (TEM) of MSC-Exo to confirm their regular morphology (B2). The mean size of isolated exosomes was 157 nm. Additionally, SEM and TEM images indicated that exosomes had a uniform spherical shape with no significant deformities.

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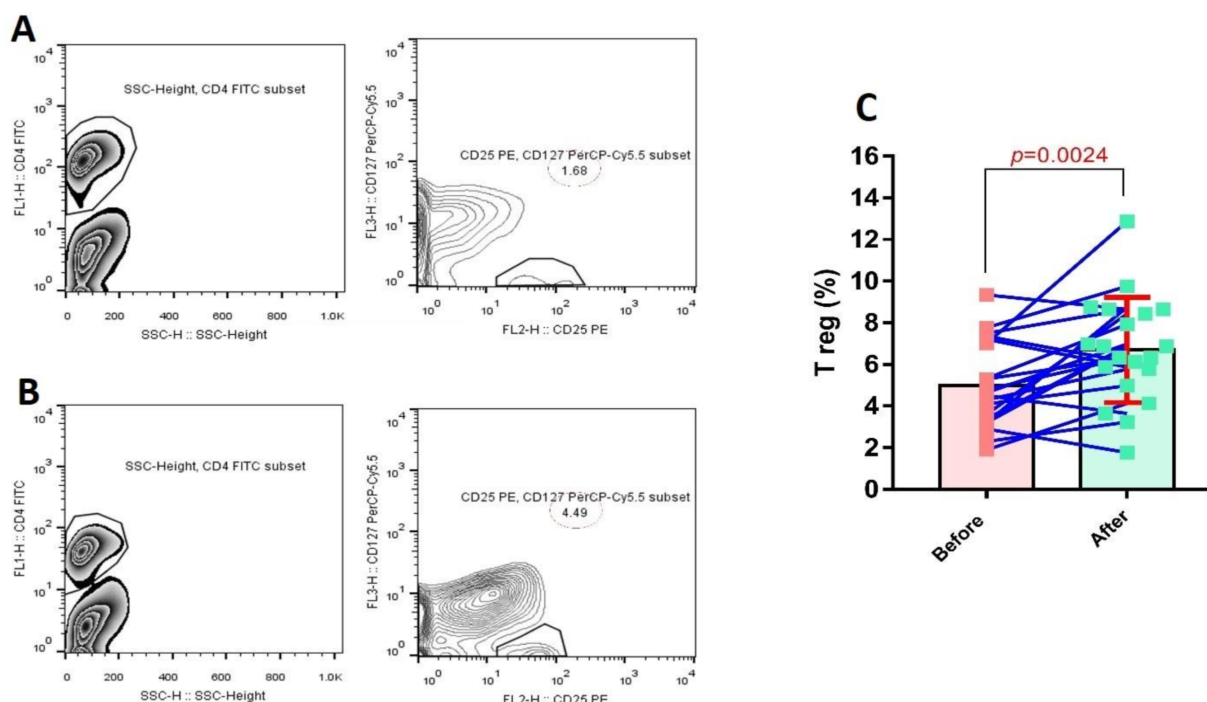

Figure 3. Frequency of Tregs. Treg population (CD4⁺CD25⁺CD127⁻) was enumerated according to the representative dot plots, which indicate the percentage of Tregs before (A) and after (B) the treatment of PBMCs with MSC-Exo. Flow cytometric findings indicated a significant increase in Treg percentage among the PBMC pool after treatment with MSC-Exo (C). Each group was composed of 10 cases, experiments were conducted in a singular order, and paired t-test was used to compare means between groups. MSC-Exo, mesenchymal stem cell exosomes; PBMC, peripheral mononuclear cell; Treg, regulatory T cell; FITC: fluorescein isothiocyanate; PE: phycoerythrin; APC: allophycocyanin

MSC-Exo Induce Treg Development

Figures 3A and 3B show micrographs from the flow cytometric assessment of Tregs. Figure 3C demonstrates that the frequency of Tregs ($CD4^+CD25^+CD127^-$ cells) was significantly higher after treating PBMCs with MSC-Exo (6.695±2.528) compared to before treatment (4.981±2.068), showing a statistically significant difference (*p*=0.0024).

MSC-Exo Stimulates TGF-β, *FOXP3*, and IL-10 Expression

Results of the real-time PCR demonstrated that the mRNA expression levels of *FOXP3* were higher in PBMCs after treatment with MSC-Exo compared to before-treatment PBMCs (1.018±0.1052 vs. 1.405±0.5761, *p*=0.025, Figure 4A). As such, IL-10 mRNA expression increased significantly in MSC-Exo–treated PBMCs compared to before treatment (1.035±0.1418 vs. 1.672±0.957, *p*=0.015, Figure 4B). The relative mRNA expression of TGF-β (Figure 4C) in the supernatant of PBMCs treated with MSC-Exo (1.497±0.7258) was significantly (*p*=0.0083) higher than before treatment (0.9984±0.07388). Table 2 summarizes the results of the real-time PCR and ELISA.

MSC-Exo Stimulates TGF-β and IL-10 Secretion

IL-10 levels in the supernatant of PBMCs isolated from COVD-19 patients were 563.5±408.6 pg/mL, which increased to 994.7±543.9 pg/mL after treatment with MSC-Exo (*p*=0.033, Figure 5A). Furthermore, the concentration of TGF-β was higher significantly (*p*=0.028, Figure 5B) in the supernatant of PBMCs after 48 hours of treatment with MSC-Exo (477.0±391.1 pg/mL) in comparison to the PBMCs prior to treatment (257.7±226.3 pg/mL).

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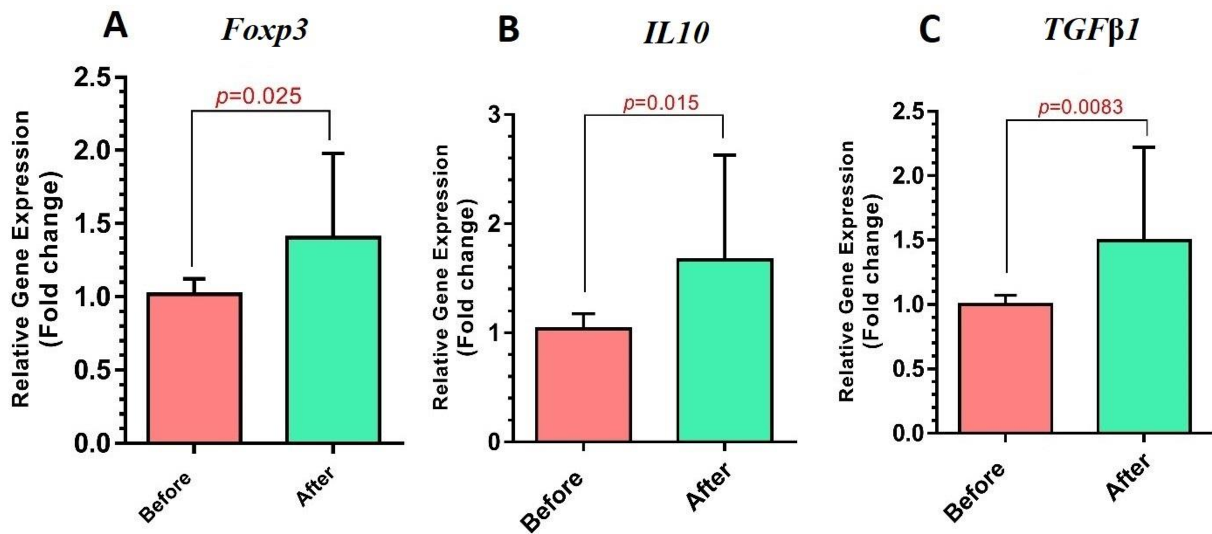

Figure 4. mRNA expression. Before and 48 hours after treatment of PBMCs with MSC-Exo, mRNA expression of *FOXP3* and cytokines were assessed by real-time PCR. Treg-specific transcription factor *FOXP3* (A) and IL-10 (B) and TGF-β (C) increased in the COVID-19 PBMCs treated with MSC-Exo. Each group comprised 10 patients; experiments were conducted in duplicate; and paired t-test was used to compare means between groups.

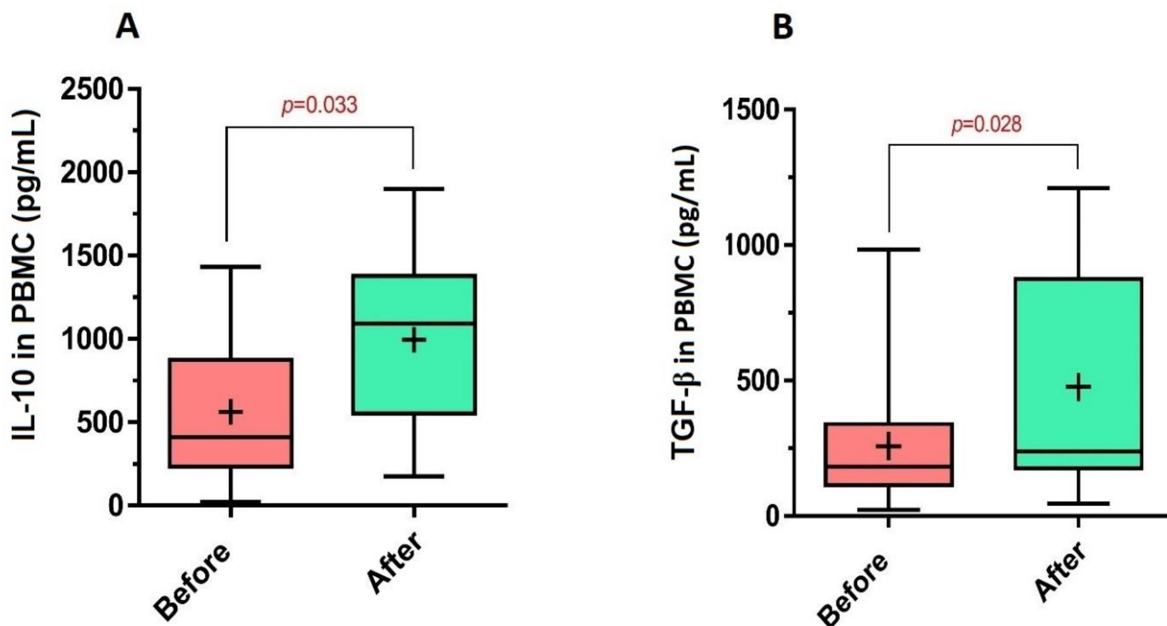

Figure 5. Concentration of cytokines. Before and 48 hours after treatment of PBMCs with MSC-Exo, concentration of cytokines in the supernatant media was assessed using ELISA. MSC-Exo treatment of COVID-19 PBMCs led to increased release of IL-10 (A) and TGF-β (B) in comparison to before treatment. Each group was composed of 10 cases, experiments were conducted in duplicate, and paired t-test was used to compare means between groups.

Table 2. Treg frequency, mRNA expression levels, and concentration of Treg-related factors in peripheral blood mononuclear cells of patients with COVID-19 and MSC-Exo treated groups.

Groups / Type	Before Treatment		After Treatment		Statistics
	Mean	SD	Mean	SD	*p*
Treg frequency	4.981	2.068	6.695	2.528	0.0024
FOXP3 expression (FC)	1.018	0.1052	1.405	0.5761	0.025
IL-10 expression (FC)	1.035	0.1418	1.672	0.957	0.015
TGF-β expression (FC)	0.9984	0.07388	1.497	0.7258	0.0083
IL-10 secretion (pg/mL)	563.5	408.6	994.7	543.9	0.033
TGF-β secretion (pg/mL)	257.7	226.3	477.0	391.1	0.028

FC: Fold change; SD: standard deviation; pg/Ml: picograms per mililiter; Treg: regulatory T cell

DISCUSSION

Cytokine storms are believed to be the major cause of lethal consequences in COVID-19. It is mediated by a variety of cytokines and chemokines released from effector immune cells.[26] A bulk of these cytokines are linked to Th17 cells. In addition to producing inflammatory cytokines, these cells induce the activation of Th2 responses, inhibit Th1 differentiation, and suppress Tregs. Many Tregs also appear to migrate to infected tissues such as the lungs and kidneys to control inflammation. This leads to a decreased number of Tregs in the circulation.[27,28] The Treg to Th17 cell ratio favors the Th17 cells in patients with COVID-19.[29]

Furthermore, recent studies have revealed that the Treg-Th17 balance is linked to the severity of uncontrolled systemic inflammation in ARDS and acute lung damage.[30,31] Therefore, an imbalance in the ratio of Treg to Th17 is one of the indicators of COVID-19 severity.[32] Thus, modulating the balance between these cells, such as through MSC therapy, can be a potential treatment for COVID-19. Here, we investigated the potential of MSC-Exo in promoting Treg differentiation from PBMCs from COVID-19 patients.

MSCs are used in the treatment of various diseases due to their favorable properties, such as modulating the immune system, producing large amounts of exosomes, self-renewability, differentiation into cells of various types, and the potential to home in on the site of injury.[33] MSCs suppress the immune response primarily through the production of anti-inflammatory mediators, such as prostaglandin E2 (PGE2) and TGF-β, as well as immunomodulatory exosomes.[34,35] In addition, MSCs can inhibit the proliferation of antigen-activated T cells and reduce the function of cytotoxic T cells.[36] MSCs perform many of their functions by producing EVs like exosomes.[37] Therefore, MSC therapy may have the potential to reduce the inflammation in COVID-19.[38,39]

Several trials have evaluated the potential of MSC-based cell therapy in COVID-19.[40] Given the challenges of using cell therapy to treat inflammatory diseases, many studies suggest the use of exosomes as an alternative to cell therapy.

It appears that MSC-Exo implement their function mostly through microRNAs. Several preclinical studies have reported that the presence of different microRNAs, especially miR-145 in MSC-Exo,[41] increases lung tissue regeneration. A limited number of trials have evaluated the potential of MSC-Exo in the treatment of COVID-19.[40] In a phase I trial, Sengupta et al. showed that the administration of a single dose of MSC-Exo to COVID-19 patients through inhalation led to the reversal of hypoxia in these patients. In addition, exosome therapy improved patient outcomes by reducing cytokine storms and rebuilding the normal balance of the immune system in patients. Above all, no side effects were observed in patients receiving these exosomes during this experiment.[42] However, this study did not examine the

mechanisms underlying immune modulation by MSC-Exo. The results of other trials are yet to be published.

Therefore, we decided to investigate the mechanism behind the effect of MSC-Exo on the regulation of immune cells in COVID-19 patients. Previous investigations have partially revealed the immunomodulatory effects of MSC-Exo on immune cells in different diseases. Evidence shows that MSC-Exo are able to induce Tregs in patients with atopic dermatitis[43] and autoimmune type 1 diabetes mellitus.[44] Furthermore, the anti-inflammatory mediators IL-4, IL-10, and TGF-β, were increased in the serum of treated patients. Interestingly, the levels of inflammatory mediators associated with Th17 (i.e., IL-17) and Th1 cells (i.e., IFN-γ) in these patients receiving MSC-Exo were reduced. In addition to T cells, these exosomes affected macrophage function and increased the anti-inflammatory M2 phenotype.[45] In infectious disorders, exosomes reduce the migration and survival of eosinophils and mast cells while increasing the function and number of neutrophils.[46]

Our study showed that MSC-Exo was able to induce Treg development in PBMCs from COVID-19 patients after 48 hours of treatment. This accompanied the upregulation of *FOXP3* expression in the MSC-Exo–treated PBMCs, indicating the potential pathway through which MSC-Exo might stimulate Treg development. These Tregs were also functionally active as evidenced by increased mRNA expression and the release of anti-inflammatory cytokines TGF-β and IL-10. As a result, MSC-Exo possesses the potential to modulate the immune response and inflammation in COVID-19 patients. It should be noted that the balance between inflammatory and anti-inflammatory responses is precisely orchestrated to create a well-suited immune response for clearing viral infections. As an inflammatory response is not always a detrimental response, we believe that anti-inflammatory responses induced by MSC-Exo might act as a double-edged sword, preventing the development of proper humoral responses by B cells and the protective/memory arm of B cell-related immunity. Therefore, evaluating the potential of MSC-Exo in promoting or inhibiting inflammatory Th1- or Th17-based responses would be of interest in future studies.

The limitations and caveats of the study should be addressed. We did not evaluate the frequency of Th17 and Th1 cells in the MSC-Exo–treated PBMCs. Second, this was an in vitro study, which needs to be tested in appropriate trials. Third, we did not profile a comprehensive panel of cytokines, which could have yielded a more vivid picture of the immunomodulatory effects of MSC-Exo on the PBMCs from COVID-19 patients.

All in all, this research demonstrated the potential of AD-MSC-Exo in establishing an anti-inflammatory role in the immune system of COVID-19 patients by promoting the induction of Tregs. It should be noted that suppressing the immune system is not always the best way to help COVID-19 patients. Therefore, based on the clinical stage of the subjects (especially in those with intensive hyperactivation of inflammatory immune responses), immune suppression, potentially through the development of Tregs by MSC-Exo, might be effective in the therapy of COVID-19 patients. Further trials are needed to investigate the clinical efficacy of MSC-Exo in improving COVID-19 outcomes.

STATEMENT OF ETHICS

The Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1400.047) granted approval for this study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable.

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