Research Paper

Safety and Efficacy of Placental Mesenchymal Stem Cell-derived Extracellular Vesicle in Severe COVID-19 Patients: Phase I & II Clinical Trials

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ABSTRACT

Introduction: Most mortality in COVID-19 cases was due to the increased inflammatory cytokines and cytokine storm. As mesenchymal stem cells (MSCs) possess immunomodulatory properties, this study assessed the therapeutic effects of placental MSC-derived extracellular vesicles on the inflammation and pulmonary injury caused by COVID-19.

Methods: The study was carried out in phases I (safety study, 101 patients) and II (efficacy study, 80 patients) in a randomized, double-blind study at four hospital centers from April 2021 to August 2021. In addition to standard treatments, 15 mL of normal saline solution containing 15×10^9 vesicles was injected intravenously for five consecutive days.

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Results: No reaction or adverse events were observed in any patients. In the intervention group, after 5 days of treatment, patients' clinical status and oxygenation improved, and 75% of patients presented an increased SpO_2 after 5 days. Besides, inflammatory parameters assessment indicated a 21% decrease in neutrophil-lymphocyte ratio and a 54% reduction in C-reactive protein after day five of the intervention.

Conclusion: PMSC-derived extracellular vesicles were safe and well-tolerated, down-regulated cytokine storms, and restored oxygenation. Thus, they can be considered a promising therapeutic candidate for severe COVID-19.

1. Introduction

Since December 2019, coronavirus disease-2019 (COVID-19) caused by SARS coronavirus 2 (SARS-CoV-2) infections have spread rapidly across the globe and declared a global pandemic by the World Health Organization (WHO). While most patients experience mild symptoms like fever and dry cough [1], others go through more severe complications like acute respiratory distress syndrome, which may lead to intubation and death [2].

The reports indicate that chronic inflammation caused by COVID-19 is related to increased pro-inflammatory cytokines, acute-phase proteins, viral invasion of lymphocytes, activation of macrophages, and oxidative stress. Moreover, it can exacerbate and dysregulate immune responses. Hence, uncontrolled inflammation can lead to severe/irreversible damage to the respiratory tract, kidneys, liver, and heart [3].

Mesenchymal stem cells (MSCs) are multipotent progenitors capable of differentiating into various cell types [4]. Based on the studies, using MSCs is beneficial in decreasing inflammation due to their immunomodulatory characteristics [5]. The anti-inflammatory property of these cells is performed via stimulating the toll-like receptors by viral RNAs, leading to an inhibitory effect on the dendritic cells, lymphocytes, neutrophils, and monocytes [6]. Moreover, it can be isolated from various tissues and retain damaged tissue regeneration by migrating to the site of injury and differentiation [7]. Studies demonstrate that using MSCs for deregulated immune responses, cytokine storms, and lung injuries caused by COVID-19 can be a promising strategy [8-11]. The challenges of stem cell therapy, such as cellular survivability and scalability, limit the extensive use of MSCs, leading researchers to introduce an alternative for benefiting their properties without using the cellular compartment [12, 13].

MSCs-derived extracellular vesicles (EVs) are novel biological agents that can significantly reduce inflammation and cytokine storm caused by COVID-19. Indeed, extracellular vesicles possess immunomodulatory and anti-inflammatory functions due to their cargo of growth factors, micro-RNAs, mRNA, and chemokines [14]. Moreover, their scalability, stability, and safety have made these vesicles practical therapeutic options for COVID-19 disease [15]. Many studies on animal models in this field indicate that MSCs-derived vesicles are essential in decreasing acute lung injuries and inflammatory diseases [16].

The present study focused on the therapeutic effects of placental mesenchymal stem cell (PMSC)-derived vesicles on the inflammation and pulmonary injury caused by COVID-19 disease.

2. Materials and Methods

Placental mesenchymal stem cells-derived vesicle preparation

Passage-3 of placenta-derived mesenchymal stem cells was obtained from our previous study [17]. After cell culture in serum-free DMEM containing GlutamaX, NEAA, and penicillin/Streptomycin medium and incubated in 5% CO_2 at 37°C for 72 hours. The conditioned culture media was collected and centrifuged at 300 for 10 minutes. The obtained cell suspension was re-centrifuged at 20000 g for 10 minutes and then 10000 g for 30 minutes to remove dead cells, cell debris, and large particles. The supernatant was ultra-centrifuged using a W32Ti rotor (L-80XP; Beckman Coulter, USA) at 110000 g for 70 minutes to pellet the vesicles. The pellet was washed in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) and centrifuged again at 110000 g for 70 minutes to eliminate contaminating proteins. Finally, the vesicles were re-suspended in 100 µL sodium chloride 0.9% (Sigma-Aldrich, USA) for intravenous infusion.

Vesicle characteristics

The morphology assessment of the vesicles was performed by a scanning electron microscope using Philips XL30 SEM) (Germany). The vesicles were imaged at an

accelerating voltage of 20.0 kV, and the mean diameter was measured by Clemex Vision software with at least 100 vesicles. Dynamic light scattering (DLS) measurement was carried out to study the size distribution of vesicles using an SZ-100 Nanopartica Series Instrument (Horiba, Japan), with a dynamic range of 0.3 nm–8 µm and scattering angle of 90°.

The flow cytometric test was performed using anti-CD81and anti-CD63 monoclonal antibodies (Abcam Co, UK) and FACScan Becton Dickinson device (BD Biosciences, USA) and analyzed using flowing software-2.5.1 (BD Company, USA). The western blot assay was performed using anti-CD9 and anti-CD81 (Abcam, UK) and observed using an increased chemiluminescence kit (Bio-Rad, USA) using Chemidoc Touch (Bio-Rad, USA). CD9 and CD81 are the surface proteins expressed on the outer membrane of the EVs [18].

Clinical trial

From April 2021 to August 2021, the study was carried out in phases I and II to evaluate the safety and efficiency of PMSC-derived EVs. COVID-19 patients admitted to four hospital centers (Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran; Shariati Hospital, Tehran, Iran; Noor Afshar Hospital, Tehran, Iran; and West Nikan Hospital, Tehran, Iran) were enrolled in a blinded randomized, double-blind study. Inclusion criteria included age 20–75 years, positive SARS-CoV-2 polymerase chain reaction results, patient clinical deterioration, and down-trending in peripheral blood oxygen saturation (SpO_2). Exclusion criteria involved pregnancy, body weight index higher than 35, chronic heart failure, and severe pre-existing pulmonary, hepatic, or renal disease. Written informed consent was obtained from patients or their families. Initial screening involved reviewing medical histories, physical examinations and monitoring vital signs during hospitalization. The treatment was applied to patients with severe COVID-19 hospitalized in the intensive care unit; among them, 15 cases were intubated, 15 were on non-invasive ventilation (NIV) and 2 received oxygen with a reserve bag. The intervention was not initiated until the patient reached a stable condition.

Administration dose and route

As Hashemian et al. [17] confirmed that multiple infusions of high-dose allogeneic prenatal MSCs are safe and can rapidly improve respiratory distress and reduce inflammatory biomarkers in some severe COVID-19-induced ARDS cases, we determined the optimum dose based on that study, in which patient received intravenous infusions of a total dose of 600×10^6 allogeneic human MSCs in three doses every other day. In the present study, 1800×10^6 MSCs were cultured, and the released EVs were isolated. A total number of $1\times10^{11}\pm10\%$ vesicles were obtained, dissolved in 100 mL of sterile sodium chloride 0.9% (normal saline) solution. For five consecutive days, in addition to standard treatments, 15 mL (1×10^9 vesicles/1 mL) of the solution, which contained a total of 15×10^9 vesicles, was injected intravenously with an injection rate of 4-5 mL/minute.

Vital signs assessments and baseline tests

In phase I, the respiratory, hepatic, and cardiac system function, consciousness level, hemoglobin level, white blood cell count, pulse rate, respiratory rate, ferritin, creatinine, blood urea nitrogen (BUN), C-reactive protein (CRP) levels, partial Thromboplastin time, platelet-lymphocyte ratio, and neutrophil-lymphocyte ratio (NLR), were performed before infusion and also on minutes 10, 30, 60, on the hour 12 h, and on days 6, 7, 14, and 28 after the first infusion. Chest x-rays were performed on day zero (before intervention) and on days 1-5 (from the first day after the intervention to the fifth day of it).

In phase II (efficacy assessment), all the mentioned tests were performed on the days of treatment. The axial chest CT scan was scored following the involvement percentage of lung lobes (A<5%, B: 5–30%, C: 31–50%, D: 51–80%, and E>80%).

Statistical analysis

Statistical analysis was performed using the t-test and one-way analysis of variance (one-way ANOVA, Tukey-test) and two-way ANOVA by GraphPad Prism software, version 6. Data were calculated using three measurements and were expressed as Mean±SD.

3. Results

Vesicle characteristics

The vesicles' morphology, diameter, and size distribution were evaluated using SEM images and the DLS method. As shown in Figure 1A, the vesicles showed a spherical shape with a mean diameter of 34.56±10.74 nm (min: 13.79 nm, max: 84.57 nm), confirming the structure of extracellular vesicles. The DLS results also demonstrated that the Z-average of vesicles was 35.59 nm with a PDI (polydispersity index) of 0.251 (Figure 1B). Flow cytometric analysis indicated sufficient CD63

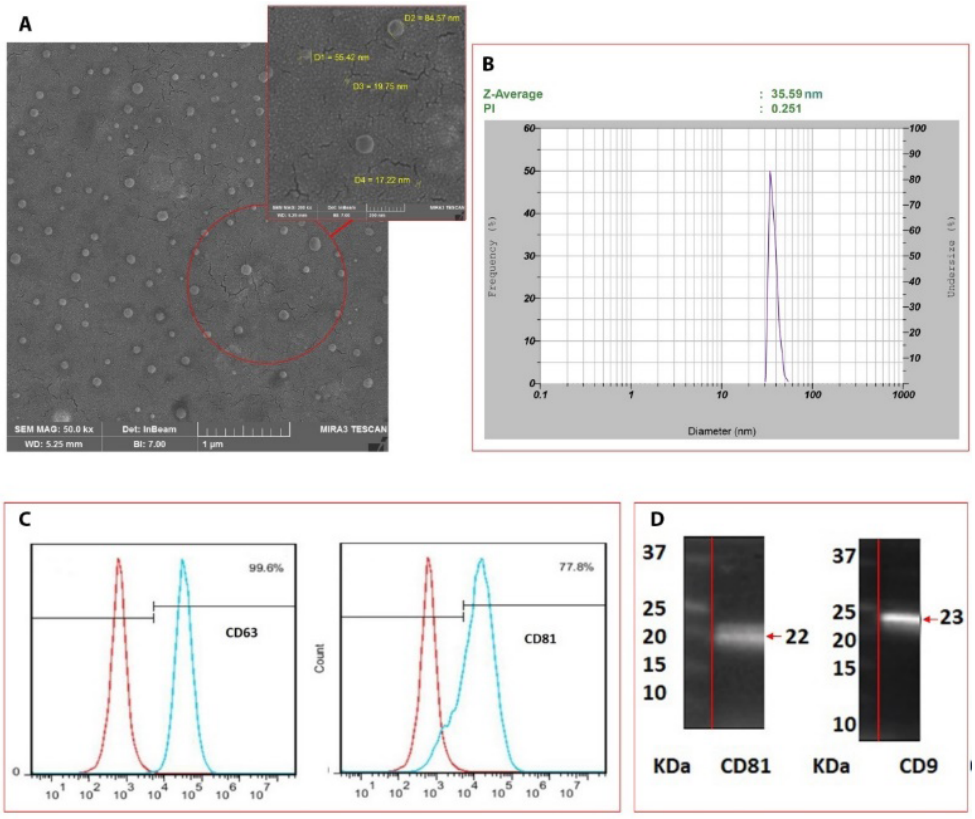

Figure 1. Vesicle characteristics

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A) SEM images: The EVs showed a spherical shape with a mean diameter of 34.56±10.74 nm and intact membrane, B) DLS analysis: The Z-average of vesicles was 35.59 nm, C) Flow cytometry analysis: The EVs express a sufficient amount of CD63 and CD81 which are the EVs surface markers, D) Western blot analysis: The EVs express the sufficient amount of EVs surface proteins, CD9 and CD81

and CD81 signals (Figure 1C). CD63 and CD81 were expressed at higher levels than 98% and 75%, respectively. The Western blot results also confirmed the presence of extracellular vesicles (CD9 and CD81) (Figure 1D).

Clinical characteristics of patients

Among 101 patients enrolled in phase I of the study, 63% and 37% were male and female, respectively. The age range was 36–75 years, averaging 54. The mortality rate and hospitalization period were 23% and 11 days, respectively. A total number of 80 cases were enrolled in phase II, divided into intervention and control groups (40 in each group). Besides, among 40 patients enrolled in the intervention group of phase II, 60% and 40% were female, with an age range of 38–75 years (average age: 54). In this group, the mortality rate and hospitalization period were 25% (8 subjects) and 14 days, respectively. The control group of phase II included 40 cases, 61% male and 39% female, with an age range of 36–72 years (with an average of 52 years). The mortality rate and hospitalization period were 33% (4 subjects) and 12 days, respectively.

Safety assessment

No intervention-related reaction or adverse events were observed in any patient (n=101) during the infusions. The improvement in respiratory rate was observed in two subjects. The paraclinical assessments (hemoglobin, coagulation factors, inflammatory parameters, renal, respiratory, and cardiovascular functions based on pulse rate, SpO_2, and chest x-ray) showed no side effects, except for three patients who showed a significant rise in creatinine levels, although two of them showed increased creatinine levels even before injection. No drug-drug interaction or reduced drug effect was observed.

Efficacy study

Inflammatory factors: Results indicated a significant difference in NLR between the control and intervention groups on each day (Figure 2A). While the intervention

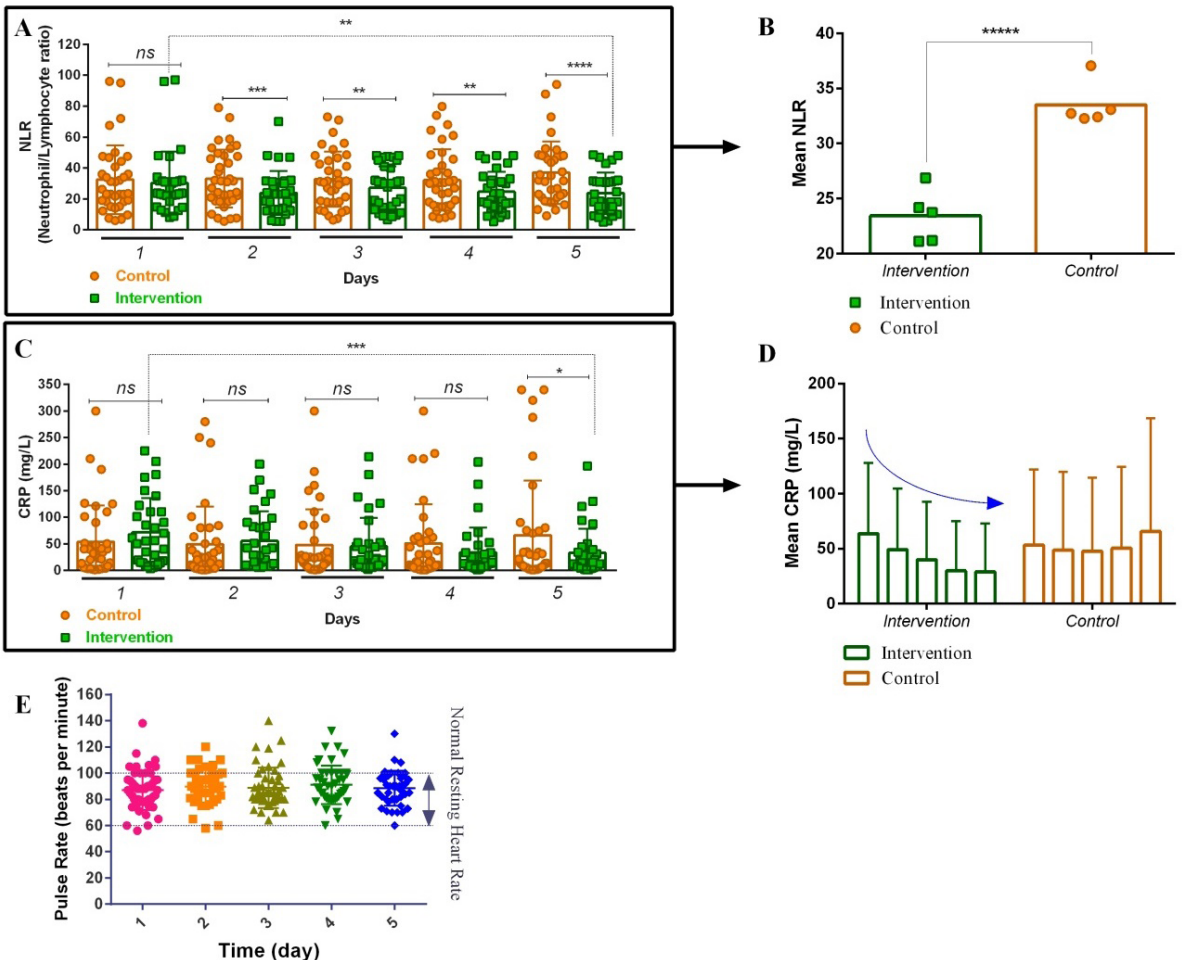

Figure 2. Inflammatory parameters in the intervention and control groups
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A) NLR on each day, B) The mean NLR on days 1-5, C) CRP (mg/L) on each day, D) The mean CRP (mg/L) on days 1-5, E) Heart rate (beats/minute)
*P< 0.1, **P< 0.05, ***P<0.01, ****P<0.0005, *****P<0.0001. Ns: No significant.

led to a 21% decrease in NLR on day five compared to day one, it increased by 14% in the control group. The comparison of the mean NLR of both groups during five days demonstrated a 21.36% decrease in the intervention group compared to the control group (Figure 2B).

CRP results illustrated no significant difference between the control and intervention groups on days 1-4 (Figure 2C). In contrast, after day five, CRP decreased by 54.23 % in the intervention group and increased by 23% in the control group (Figure 2D).

Table 1. Oxygen supplementation

Respiratory System	Groups									
	Control					Intervention				
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Nasal canula	0	0	0	0	0	0	0	0	1	2
Reserve bag	0	0	0	0	0	2	7	8	10	11
NIV*	36	36	36	33	29	15	11	10	10	9
INTUBE	0	0	0	3	7	15	14	14	11	10

*Non-invasive ventilation.
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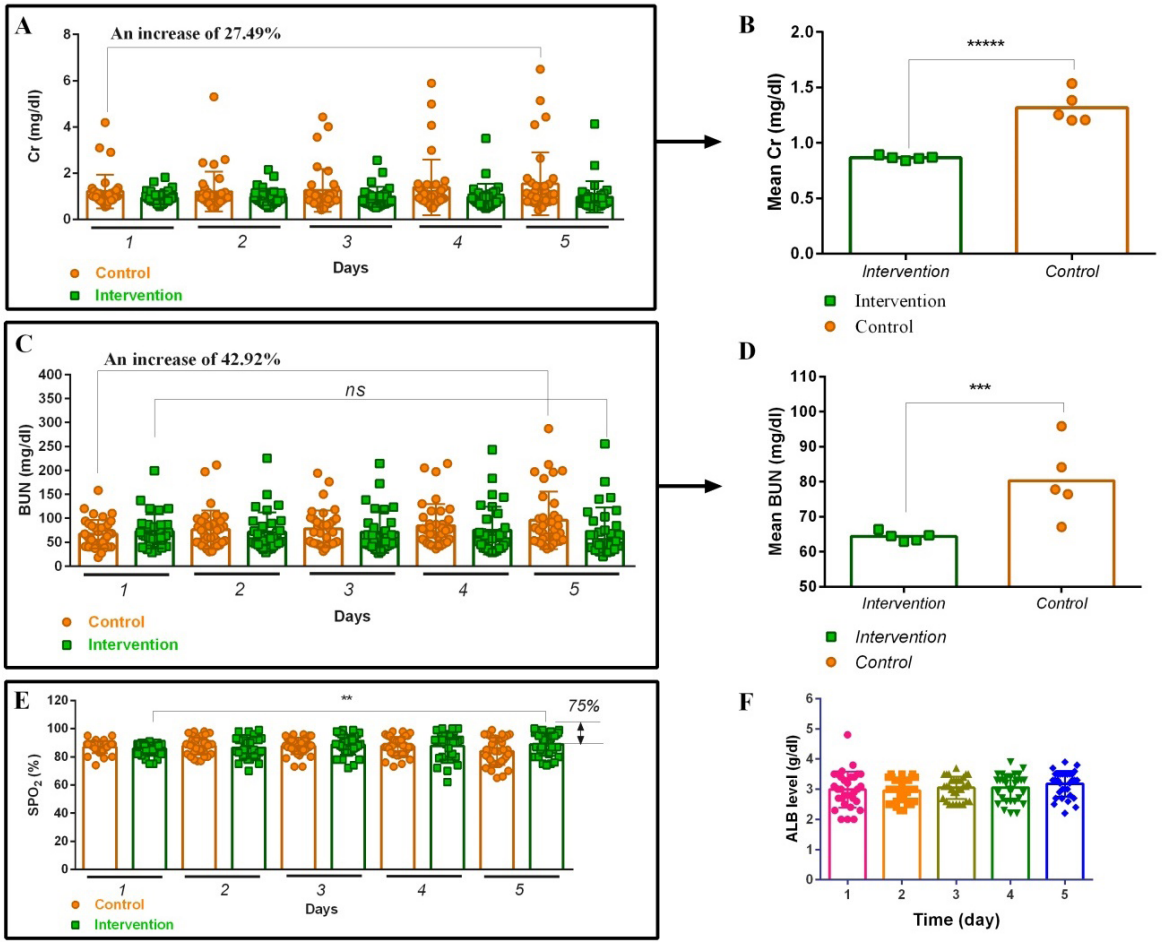

Figure 3. Kidney function in intervention and control groups on days 1-5

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A) The creatinine level (mg/dL), B) The creatinine mean (mg/dL), C) The BUN level (mg/dL), D) The BUN mean (mg/dL), E) The SpO_2 level (%), F) The albumin level (g/dL)

**P<0.05, ***P<0.01, *****P<0.0001.

Cardiovascular function: Pulse rate results showed a normal resting heart rate (60-100 beats/minute) after five days in 87.5% of patients (Figure 2E).

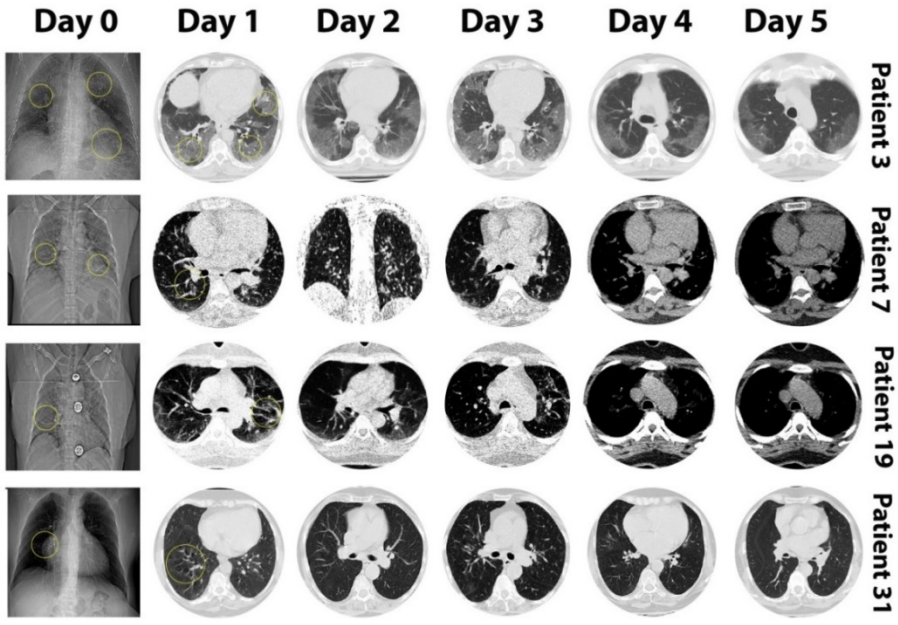
Kidney and liver function: Creatinine and BUN results, showing a 1% reduction in the intervention group, indicated that the intervention had no side effects on the kidney. In contrast, in the control group, after five days, an increase of ~27.5% and ~43% was observed in creatinine and BUN values, respectively (Figure 3A-D). Albumin results illustrated that its level was in the range of 3.3-5 g/dl in all patients before and after five days (Figure 3F), indicating that the intervention did not deteriorate kidney and liver function.

Oxygen supplementation: The SpO_2 results demonstrated that after 5 days, from 32 patients in the intervention group, the SpO_2 level of 24 patients (75%) increased compared to day one (Figure 3E). Moreover, the number of intubated patients decreased from 15 to 10 subjects (Table 1).

Lung function: Day zero chest x-ray demonstrated a significant lung involvement in all patients, varied from mixed ground-glass opacity, crazy paving patterns, vascular dilation, pleural effusion, honeycombing, and traction bronchiectasis. After five days of intervention, a significant improvement was observed in the chest x-ray images (Figure 4). Five out of 15 (~34%) intubated patients and six of 14(40%) non-invasive ventilation-supported patients recovered. A 4.5-fold increase was observed in the number of patients supported by reserve bags.

4. Discussion

Cytokine storm is a lethal systemic inflammatory syndrome defined by hyperactivated immune cells and increased circulating inflammatory cytokines and chem-



Figuure 4. Axial chest CT scan images of four patients on day 0 and over 5 days of intervention
Note: The extensive mixed ground-glass opacities, crazy paving appearance, honeycombing, traction bronchiectasis, bilateral pleural effusion, and vascular dilatation.

ical mediators [19]. It is responsible for the disease severity and mortality in COVID-19 patients [20]. Various studies revealed that severe COVID-19 patients showed higher levels of pro-inflammatory and inflammatory cytokines compared to patients with mild and moderate infections. Hence, early control of the cytokine storm is critical in improving their survival rate [21]. Among the cytokine storm-attenuating therapies, MSC therapy exhibited a promising potential for rapid and significant improvement in clinical symptoms [22].

Due to the immunomodulatory properties of MSCs [23], they attract lots of attention during the pandemic [24-26]. Various studies have evaluated the safety and efficacy of MSCs in severe COVID-19 management (111 clinical trials until October 2022). Also, there are some clinical trials registered on ClinicalTrials data base, applying MSC-derived EVs (NCT05216562, NCT05191381, NCT04798716, NCT04602442, NCT04491240, NCT04493242) or placenta-derived MSCs (NCT04461925, NCT04614025, NCT04389450) in COVID-19. There is just one study (NCT05387278) that evaluated the PMSC-derived EVs in severe COVID-19 conditions, and no data published yet. In agreement with the mentioned clinical trials utilizing MSCs or MSC-derived EVs [27], we detected no intervention-related adverse effects during or after the infusions.

Liu et al. compared severe COVID-19 patients with mild patients and reported a significant and persistent decline in T helper and T cytotoxic lymphocyte counts, contrary to the increase in neutrophils. Therefore, the NLR is a predictive biomarker for COVID-19 outcomes [28]. In agreement with previous studies, NLR results demonstrated a decrease in the intervention group and an increase in the control group. The NLR downtrend through the intervention indicates the treatment's effectiveness.

CRP is an acute-phase protein that increases in infection or inflammation conditions and serves as a predictive biomarker in COVID-19 patients [29]. Ahnach et al. claimed that CRP level was significantly related to COVID-19 severity [29]. Besides, as CRP indicated the presence of systemic inflammation, Smilowitz et al. suggested that a high level of serum CRP is intensely related to morbidity and mortality in COVID-19 patients [30]. In the present study, CRP decreased in the intervention group and increased in the control group. Hence, the intervention is supposed to be effective in severe COVID-19 patients.

Increased serum creatinine and BUN are independent predictors of severe COVID-19 [31]. Although the increased creatinine level even before the intervention suggested that it was probably due to the COVID-19 infection, the decreased glomerular filtration of EVs and their possible aggregation can also lead to closure and increased creatinine levels.

Fathi-Kazerooni et al. applied MSC-derived Evs from allogeneic menstrual blood in severe COVID-19. Similar to our study, they detected no adverse events following three days of intervention. Also, they reported a significant decrease in CRP after the intervention. Also, consistent with our study, they reported a significant improvement in lymphopenia. Consistently with our findings, they demonstrated that five consecutive doses of MSC-derived secretome improved hypoxia, lessened pulmonary lesions, restored the immune system function, and eased the cytokine storm in severe COVID-19 [32].

Sengupta et al. conducted a cohort study that assessed the safety and efficacy of a single dose of intravenous EVs derived from allogeneic bone marrow MSCs in severe COVID-19. They concluded that EVs from bone marrow MSCs could restore oxygenation, down-regulating cytokine storm, and re-constituting immunity. In accordance with this cohort study, our findings illustrated that applying the MSC-derived EVs can increase SpO_2, improve lung injuries, and alleviate cytokine storms [33].

5. Conclusions

Generally, this study demonstrated that intravenous infusion of PMSC-derived EVs decreased cytokine storms, improved pulmonary function, and decreased invasive oxygen/mechanical ventilation support in hospitalized patients with severe COVID-19, with no associated side effects.

Ethical Considerations

Compliance with ethical guidelines

The clinical trial was approved by the Ethics Committee of the Tehran University of Medical Sciences (Code: IR.TUMS.MEDICINE.REC.1399.454).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflicts of interest.

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