

Nebulization Therapy for COVID-19 Pneumonia with Embryonic Mesenchymal Stem Cells-derived Exosomes

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Research

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Abstract

Background: Scientists have been facing numerous challenges in the development of an effective therapeutic strategy for the treatment of COVID-19 pneumonia. Several studies have suggested that improving patient immunity and reducing lung injury induced by COVID-19 could be effective in treating the patients with COVID-19.

Methods: A pilot trial of nebulization therapy for COVID-19 pneumonia with exosomes of MSCs was performed on seven patients with COVID-19 infected pneumonia. Exosomes were collected and purified from secretion of MSCs using multiple ultrafiltration. All patients were treated with nebulization of MSC-derived exosomes. The primary safety and efficacy outcomes were observed.

Results: Our clinical study demonstrated that nebulization of mesenchymal stem cell (MSC)-derived exosomes is a novel method that could be utilized in the treatment of COVID-19 pneumonia. Nebulization of MSC-derived exosomes did not induce acute allergic and secondary allergic reactions. It could promote the absorption of pulmonary lesions, and reduce the time of hospitalization for minor cases of COVID-19 pneumonia.

Conclusions: Nebulization of MSC-derived exosomes is a safe, effective, and simple method. Nebulization of MSC-derived exosomes from the beginning of the treatment could be more beneficial to the patients.

Trial registration: Chinese Clinical Trial Registry, ChiCTR2000030261. Registered on 26 February 2020.

Background

COVID-19 pneumonia has been declared as a global pandemic by the World Health Organization (WHO) and has become a matter of public health emergency (1). Although the diagnostic efficiency and accuracy of the treatment modalities have improved, the overall therapeutic effect remains poor (2). The major causes of death were severe pneumonia, pulmonary edema, ARDS or multiple organ failure (3).

The two major characteristics of COVID-19 pneumonia are: 1. People with low immunity are more likely to be infected with COVID-19 (4); 2. The major target organ of attack is the lung (5). Respiratory failure has been reported as one of the major causes of death due to COVID-19 (6). Autopsy revealed the occurrence of pulmonary injury, significant exudative reaction, and pulmonary embolism in many patients (7). Therefore, COVID-19 induced lung injury could be reduced by improving the immunity of patients.

Mesenchymal stem cells (MSCs) have been shown to possess a comprehensive, powerful immunomodulatory function (8, 9). The exosomes of MSCs can not only regulate human immunity through immune cells, but also inhibit inflammatory response through cytokines (10, 11). Numerous studies have demonstrated that the exosomes of MSCs could be employed in the treatment of immune deficiency, inflammation, acute respiratory distress syndrome (ARDS), and other lung diseases (10, 12, 13). Therefore, exosomes of MSCs could also be employed in the treatment of COVID-19 pneumonia.

For stem cell therapy, the routine methods of administration are intravenous injection (14, 15). Exosomes are one of the major active components secreted by stem cells (16), with their size ranging from 30–200 nm (17). They can reach the bronchioles and alveoli directly after nebulization, which is conducive to the extent of drug absorption (13). Therefore, we hypothesized that nebulization of MSC-derived exosomes could be an effective treatment for COVID-19 pneumonia.

Methods

Study design

A pilot trial of nebulization therapy for COVID-19 pneumonia with exosomes of MSCs was performed on seven patients with COVID-19 infected pneumonia. The study was conducted in Wuxi No.5 people's Hospital, China. The safety and scientific validity of this study "have been issued in Chinese Clinical Trial Registry (ChiCTR2000030261).

Inclusion criteria:

We initially enrolled patients with COVID-19 (age 18–65 years) according to the guidance of National Health and Health Commission of China (18). We comprehensively considered the patient's epidemiological history, clinical symptoms, nucleic acid test. Informed consents were obtained from all the patients to participate in this clinical study.

Exclusion criteria:

(1) Age < 18 or > 65; (2) patients with severe heart, brain, lung, kidney dysfunction, endocrine disease, hematopoiesis system disease or other serious diseases and psychosis; (3) pregnant and lactating women; (4) patients who were participating in other clinical trials or who have participated in other clinical trials in the last 3 months; (5) patients who were unwilling or unable to sign the informed consent due to illness.

Patients

The patients were enrolled from Feb 26, 2020 to April 30, 2020. All the enrolled patients were confirmed with COVID-19 via the real-time reverse transcription polymerase chain reaction (RT-PCR) to detect SARS-CoV-2 RNA following the protocol outlined in a previous study. (3, 19).

All patients were treated with Ritonavir oral, Abidol oral, Interferon nebulization, or Clerking phosphate oral (Fig. 1). The clinical, laboratory, and radiological outcomes of all patients were recorded and certified by a trained group of doctors. The detailed record included primary safety data (allergic reactions, secondary infection and life-threatening adverse events) and the primary efficacy data (the level of CRP in plasma and the oxygen saturation). The secondary efficacy outcomes mainly included the total white blood cell count, total lymphocyte count (tested by Hitachi 7600-020 automatic biochemical analyzer), SARS-CoV-2 nucleic acid detection (tested by RT-PCR protocol, DAAN GENE Co., Ltd, China), chest CT (tested by 320-slice spiral CT scanner, Aquilion One, Toshiba Medical System, Japan), respiratory rate, patient symptoms (especially the fever and shortness of breath), and time of hospitalization.

Preparation of MSCs

Clinical grade MSCs were supplied, by Cruilife Stem Cell co. LTD. The number of MSCs were calculated based on the weight of the patient (1×10^6 MSCs / kg body weight). We used passage 4–6 MSCs for collection of exosomes. MSCs were analyzed using flow cytometry for the appropriate markers before use (CD73, CD90, CD105, CD14, CD19, CD34, CD45 and HLA-DR). All procedures performed in this study involving human samples were in accordance with the ethical standards of the institutional research committee and the guidelines set by the Declaration of Helsinki.

Isolation and characterization of exosomes isolated from MSCs

Exosomes were collected and purified from secretion of MSCs using multiple ultrafiltration (Fig. 2A). The secretion was first centrifuged at 4 °C at 3000 g for 20 min, and filtered through a 0.22 µm filter to remove any cells or cell debris. The filtered secretomes were then placed in a new sterile EP tube, followed by addition of 0.2 ml exosomes separation and purification solution (Shanghai Gefan Biotechnology Co., Ltd. Product No.: ex010). The contents were mixed well at 4 °C overnight, and centrifuged at 4 °C at 3000 g for 20 min, the following day. After the supernatant was aspirated, it was centrifuged at 4 °C for 1500 g 5 min to remove the residual solution. After adding 1000 µl sterile PBS to re suspend and precipitate exosomes, the solution was centrifuged for 1 hour at 100 000 g on a high-speed centrifuge, and this was repeated three times. Exosome samples were analyzed for proper size using nanoparticle tracking analysis (NTA; NanoSight NS300, Malvern), and for morphology using transmission electron microscopy (TEM; Tecnai G2 Spirit Bio TWIN). Additionally, successful exosome isolation was confirmed using immunoblotting for known exosome markers CD9 (ab92726, abcam), CD81 (ab109201, abcam), and Flotillin 2 (ab181988, abcam) (Fig. 2).

After nebulization, the MSCs exosomes were sprayed on a sterile glass plate, and a dish was placed under the glass plate for collecting the liquid containing exosomes. After nebulization, the size and markers of MSCs exosomes were evaluated again. No significant difference was observed in the size and markers of MSC-derived exosomes before and after nebulization (Fig. 2C and D).

Nebulization of MSC-derived exosomes

After obtaining ethical approval, all patients diagnosed with COVID-19 pneumonia who provided an informed consent was treated with nebulization of MSC-derived exosomes. The extracted exosomes from MSCs were diluted to 5 ml with 0.9% sodium chloride, and added to the atomizer (Emedical, Excellentcare Medical Ltd. China). The nebulization was performed twice a day (am 8:30, pm 16:00), for 10 minutes each. The patients were assessed by the investigators after receiving the nebulization treatment.

Treatment procedure for MSC-derived exosomes and general patient information

This study was conducted from Feb 26, 2020 to Sep 4, 2020. Seven patients diagnosed with COVID-19 pneumonia, including 2 severe cases (patient 2 and 4) and 5 minor cases (patient 1, 3, 5, 6 and 7) were enrolled in the study (Table 1). Patient 1, 2, 3 and 4 received nebulization of MSC-derived exosomes at the end of the after antiviral treatment for a period of time. Patient 1 was a minor case of COVID-19 and did not have any underlying disease conditions. Patient 2 was a severe case of COVID-19 with liver damage. Patient 3 was a minor case and patient 4 was a severe case, both without any underlying disease. Patient 5, 6 and 7 received nebulization of MSC-derived exosomes from the beginning of treatment. The information about all the treatment modalities of the patients were collected. The timepoint of the delivery of MSC-derived exosomes nebulization treatment for each patient is shown in Fig. 1.

Table 1
The general information of the enrolled patients.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Gender	F	M	F	F	M	M	M
Age (years)	62	53	23	61	43	19	57
Underlying diseases	No	Liver damage	No	No	Fatty liver	No	Diabetes mellitus
COVID-19 type	Common	Severe	Common	Severe	Common	Common	Common
Fever (°C, base line)	38.5	37.4	37.6	38.9	37.7	37.4	37.6
Cough	Yes	Yes	No	Yes	No	No	Yes
Weak	Yes	No	No	Yes	No	No	No
Diarrhea	No	Yes	No	No	No	No	No
Shortness of breath	No	No	No	Yes	No	No	No
Chest tightness	Yes	Yes	No	No	No	No	No
Oxygen saturation at rest state (%)	87	74	93	61	97	97	98
Date of diagnosed	Feb 9	Feb 9	Feb 8	Jan 31	Mar 28	Mar 27	Aug 20
Date of MSCs exosomes treatment	Feb 27	Feb 27	Feb 27	Feb 27	Apr 1	Apr 4	Aug 23
Date of recovery	Mar 2	Mar 2	Mar 11	Mar 9	Apr 11	Apr 13	Sep 4
Hospital day	22	22	31	38	14	17	15
Yes : Presence of relevant clinical symptoms, such as cough, weak, diarrhea, shortness of breath and chest tightness.							
No : No relevant clinical symptoms.							

Statistical analysis

Data which were suitable for statistical analysis were analyzed using SPSS software (SPSS 22.0). Differences between two groups were assessed using unpaired two-tailed *t* tests or chi square test based on the type of the data. Data involving more than two groups were assessed using analysis of variance (ANOVA). P values < 0.05 indicated statistical significance.

Results

The primary safety outcome

No acute allergic reactions, such as itchy rash, swelling of the throat or tongue, shortness of breath, vomiting, lightheadedness, and low blood pressure were observed within two hours after the nebulization treatment. Secondary allergic reaction was also not observed post treatment. No adverse events were reported.

MSCs therapy is considered safe for lung diseases, such as chronic obstructive pulmonary disease (COPD), Acute respiratory distress syndrome (ARDS), and Idiopathic pulmonary fibrosis (IPF) (20, 21). Numerous completed phase I trials have demonstrated that no serious, acute, adverse events were reported in MSC therapy (22, 23). Several studies have been conducted on the safety of MSCs exosomes therapy. The findings of these studies suggested that MSC-derived exosomes could be safely and easily used in the treatment of lung diseases (24). However, the route of administration of MSC-derived exosomes in most of studies was through intravenous injection. Our results demonstrated that nebulization of MSC-derived exosomes was safe and could be employed in the treatment of lung diseases.

The efficacy outcome

The plasma C-reaction protein (CRP) levels decreased from 88.4 mg/L (Feb 17) to 4.3 mg/L (Feb 28) and 0.4 mg/L (Mar 1) in patient 1. In patient 2 (severe type), it decreased from 30.8 mg/L (Feb 23) to 18.9 mg/L (Feb 28) and 3.5 mg/L (Mar 2). In patient 3, it decreased from 5.7 mg/L (Feb 15) to 0.5 mg/L (Mar 3). In patient 4 (severe type), it decreased from 31.8 mg/L (Feb 25) to 11.4 mg/L (Mar 9). The nebulization treatment in patients 1, 2, 3, and 4 started from Feb 27. In patient 5, the CRP levels were found to be 0.5 mg/L (Mar 28) and 0.5 mg/L (April 4), without any change in its level. In patient 6, the CRP levels decreased from 2.2 mg/L (Mar 30) to 0.5 mg/L (April 8). The nebulization treatment for patients 5 and 6 started

from April 1 and April 4, respectively. In patient 7, the CRP levels were found to be 1.9 mg/L (Aug 21), 5.4 mg/L (Aug 25) and 3 mg/L (Aug 29) (Table 2). Although the CRP value before the nebulization treatment (23.00 ± 31.87) was comparatively higher than that after the treatment (5.92 ± 6.92), there was no significant difference between the two values ($p = 0.178$).

Table 2

The efficacy outcomes (C-reactive protein, total white blood cell count, total lymphocyte count, the respiratory rate, the fever and shortness of breath) before and after the nebulization treatment.

Laboratory index	Patient 1 (common type)		Patient 2 (severe type)		Patient 3 (common type)		Patient 4 (severe type)		Patient 5 (common type)		Patient 6 (common type)		Patient 7 (common type)	
	Before	After												
C-reactive protein (mg/L)	88.4	0.4	30.8	3.5	5.7	0.5	31.8	11.4	0.5	0.5	2.2	0.5	1.9	5.4
White cell count (*10 ⁹ per liter)	5.28	4.96	5.99	5.02	7.28	6.87	6.38	3.95	8.16	5.26	6.02	8.41	4.34	5.63
Lymphoma count (*10 ⁹ per liter)	3.6	2.91	1.36	2.03	2.87	2.62	1.17	1.1	2.77	2.36	2.08	2.08	3.08	2.95
Respiratory rate (/min)	12	13	32	25	15	14	22	20	14	15	16	15	13	14
Fever (°C)	36.5	36.6	36.7	36.5	36.3	36.4	37.1	37	36.5	36.6	36.4	36.5	37.6	36.5
Shortness of breath	No	No	Yes	No	No	No								
Before : Before nebulization treatment.														
After : After nebulization treatment.														
No: No shortness of breath.														
Yes: Presence of shortness of breath.														

In patient 1, oxygen saturation without supplementation rose from 95% (Feb 26) to 98% (Mar 2). In patient 2 (severe type), oxygen saturation increased from 95% (Feb 26) to 98% (Mar 3). In patient 4 (severe type), oxygen saturation rose from 95% (Feb 26) to 100% (Mar 1). In patient 3, 5, 6 and 7, there was no significant change in oxygen saturation post nebulization treatment. Therefore, we found that there was no significant difference in oxygen saturation before and after nebulization MSCs exosomes ($p > 0.05$).

Additionally there was no significant difference in the total white blood cell count, total lymphocyte count, fever and shortness of breath before and after the nebulization treatment ($p > 0.05$). Alanine aminotransferase (ALT) in Patient 2, a severe case with liver function damage, decreased from 168 u/l (Feb 26) to 92 u/l (Feb 28), and 52 u/l (Mar 2).

Computed tomography (CT) of the chest revealed that the nebulization of MSC-derived exosomes was beneficial to the absorption of pulmonary lesions. On Apr 3, the first CT scan of Patient 6 (minor case of COVID-19 pneumonia, received nebulization of MSC-derived exosomes from Apr 4) showed an isolated nodule outside the left lower lobe of the lung. On April 10, the second CT examination showed that the density of the left inferior lobe nodule was significantly lower with a narrow range. On April 21, the third CT examination showed that the lesions in the lower left lung were completely absorbed. The time taken for complete absorption of pulmonary lesions in patient 6 was 18 days. The absorption time of similar lung lesions in another patient (a minor case of COVID-19 pneumonia, that did not receive the nebulization treatment) was 27 days. There was significant difference in time of complete absorption of pulmonary lesions (16.00 ± 5.23 vs 20.85 ± 3.57) between the patients received nebulization of exosomes from the beginning of treatment and other patients (did not received nebulization or received nebulization at the end of treatment) in minor cases of COVID-19 pneumonia ($p = 0.033$). Patients with severe cases of COVID-19 pneumonia received nebulization treatment at the later stage of treatment. Compared to the patients who did not receive the nebulization treatment, patient 2 showed obvious absorption of pulmonary lesions. In patients who did not receive the nebulization treatment, there were presence of fibrous shadows in the lung lesions (Fig. 3).

Several studies have demonstrated that bone marrow-derived exosomes can reduce lung inflammation, alleviate pulmonary edema and post-inflammatory complications in animal models of acute lung injury, ARDS, asthma and other inflammatory diseases. (25–27). MSC-derived

exosomes usually contain bioactive substances such as mRNA, miRNA and protein (28). These substances have been shown to effectively reduce inflammatory processes and modulate airway remodeling (13, 29). We also demonstrated that MSC-derived exosomes can reduce the CRP level in different degrees of patients with COVID-19 pneumonia, which was similar to the findings of a previous study (18). Although there was a decrease in CRP level after the nebulization treatment, it did not achieve statistical significance due to the small number of cases and large standard deviation. The patients who received nebulization treatment at an earlier stage showed more beneficial effects in terms of the absorption of pulmonary inflammation. Our results showed that nebulization of MSC-derived exosomes is also beneficial to the absorption of pulmonary lesions in minor cases of COVID-19 pneumonia and the reduction of cellulose residues in severe cases of COVID-19.

Time of hospitalization

The average time of hospitalization was 18.74 ± 4.72 days for all the COVID-19 patients, 18.29 ± 4.60 days for minor cases of COVID-19, and 22.6 ± 4.3 days for severe cases of COVID-19. The time of hospitalization for patients 5, 6 and 7, with minor cases of COVID-19 (received nebulization of exosomes from the beginning of treatment) were 15.3 ± 1.33 days (14, 17 and 15 days, respectively). The time of hospitalization for patients 1 and 3, with minor cases of COVID-19 (received nebulization of exosomes at the end of treatment) were 22 and 31 days, respectively. There was significant difference in time of hospitalization between the patients received nebulization of exosomes from the beginning of treatment and other patients (did not received nebulization or received nebulization at the end of treatment) in minor cases of COVID-19 pneumonia ($p = 0.035$). The time of hospitalization for patients 2 and 4, with severe cases of COVID-19 (received nebulization of exosomes at the end of treatment) were 22 and 38 days, respectively. The patients who received nebulization treatment at an earlier stage showed more beneficial effects in terms of the time of hospitalization.

COVID-19 nucleic acid detection

RT-PCR was performed using the genomic DNA of COVID-19 pneumonia patients. In all patients, the nucleic acid has changed from positive to negative before the treatment of exosomes nebulization.

Serum immune Factor Analysis

Patient 6 was tested for immune factors (IL-2, IL-4, IL-6, IL-10, TNF α , IFN- γ , IL-17A, CD3, CD4, CD8, CD4/CD8, TH19 and NK) (Table 4). The largest difference was observed in case of IFN - γ post nebulization treatment. There was a two-fold increase in IFN - γ , IL-17A and TH19 after the nebulization treatment. However, NK cells showed a two-fold decrease after the treatment (Table 3).

Table 3
Analysis of serum immune factors (IL-2, IL-4, IL-6, IL-10, TNF α , IFN- γ , IL-17A, CD3, CD4, CD8, CD4/CD8, TH19 and NK) in Patients 6.
The time of nebulization was April 4.

Date	IL-2	IL-4	IL-6	IL-10	TNF- α	IFN- γ	IL-17A	CD3	CD4	CD8	CD4/CD8	TH19	NK
Before (Mar 30)	2.19	2.05	3.25	2.76	1.94	0.63	0	55.5	25.88	23.13	1.12	7.56	31.04
After (April 8)	No	No	3.54	0.83	No	No	No	67.62	33.52	27.64	1.21	14.40	14.68
After (April 13)	0.51	3.65	4.57	2.13	1.01	41.23	4.73	64.91	33.98	25.72	1.35	15.15	15.20
Before: Before nebulization treatment.													
After: After nebulization treatment.													
No: No data.													

Although several therapeutic approaches have been proposed for COVID-19 pneumonia, only few of them are effective. At present, it is believed that reducing the lung injury may be the key to save patients with COVID-19 pneumonia. Our results suggested that nebulization of MSC-derived exosomes can promote the absorption of pulmonary lesions, and reduce the time of hospitalization for minor cases of COVID-19 pneumonia. Nebulization of MSC-derived exosomes from the beginning of the treatment could be more beneficial to the patients.

Drug nebulization is an effective way to treat lung diseases. In case of nebulization, exosomes can act directly on the lungs and show a faster effect. However, there are only a few reports on the nebulization of MSC-derived exosomes. Our results indicate that nebulization of MSC-derived exosomes is safe and feasible therapeutic approach for the treatment of patients with COVID-19 pneumonia. After the extraction of exosomes, they can be stored up to a week in a 4 °C refrigerator and could be added to the existing atomizer, as and when required. The convenience of drug storage and its use is very crucial for COVID-19 pneumonia therapy, especially in countries and regions with lacking advanced health-care facilities.

However, our research has obvious shortcomings. Due to the short duration of the outbreak in China, only seven COVID-19 pneumonia patients were included in this study. Additionally, only 3 patients underwent the nebulization of MSC-derived exosomes from the beginning of treatment. It also should be noted that we have not compared the exosomes nebulization treatment to various other administration methods in this study, such as intravenous injection.

In conclusion, nebulization MSCs exosomes is a novel method that could be used in the treatment of COVID-19 pneumonia. Our clinical study involving a small sample size, shows that this method is safe, effective and simple especially for minor cases of COVID-19 pneumonia. Nebulization of MSC-derived exosomes from the beginning of the treatment may benefit patients more effectively.

Abbreviations

World Health Organization (WHO)

Mesenchymal stem cells (MSCs)

Declarations

Author Contributions

Conceptualization, Yigang Chen and Hao Wang; Methodology, Chu mei-ping and Hao Wang; Software, Linjie Bian; Formal Analysis, Danping Wu and Linjie Bian; Investigation, Chu mei-ping and Huang jie-hui; Resources, Chu mei-ping and Huang jie-hui; Writing – Original Draft, Yigang Chen; Writing – Review & Editing, Hao Wang, Supervision, Jiazeng Xia; Project Administration, Jiazeng Xia; Funding Acquisition, Yigang Chen and Chu mei-ping.

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Ethics approval and consent to participate

The study was conducted in Wuxi No.5 people's Hospital, China, and approved by the ethics committee of the hospital (No. 2020- 003-1). The safety and scientific validity of this study "have been issued in Chinese Clinical Trial Registry (ChiCTR2000030261).

Clinical data from patients were obtained after acquiring consent of patients in accordance with the protocol approved by the Ethics Committee of the ethics committee of Wuxi No.5 people's Hospital, China.

Consent for publication

All presentations of case reports have consent for publication.

Availability of Data and Materials

All supporting data are included in the article.

Competing interests

The authors declare that they have no conflicts of interest related to the studies described.

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Figures

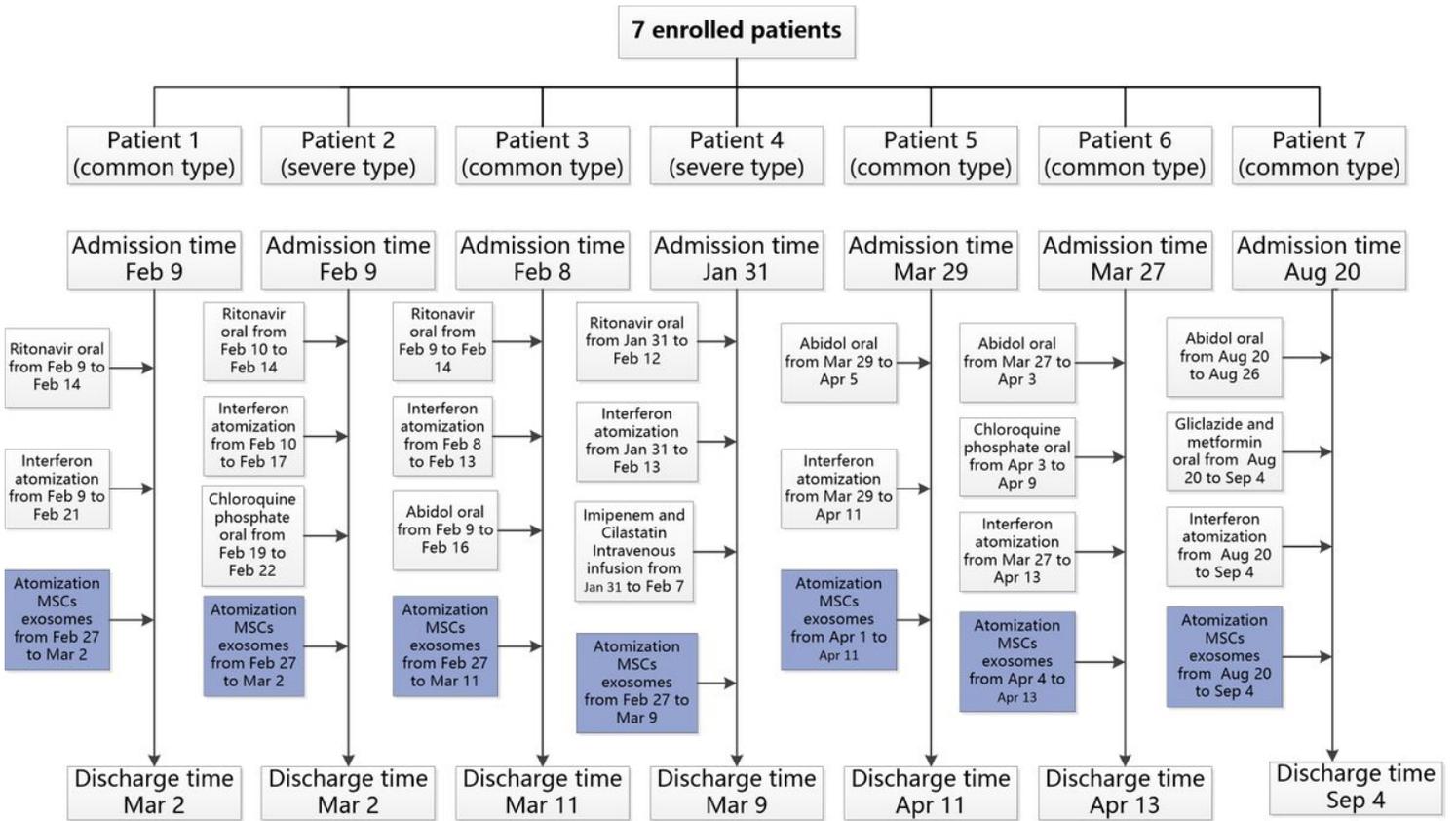


Figure 1

The flow chart showing the timeline of MSC-derived exosomes nebulization treatment.

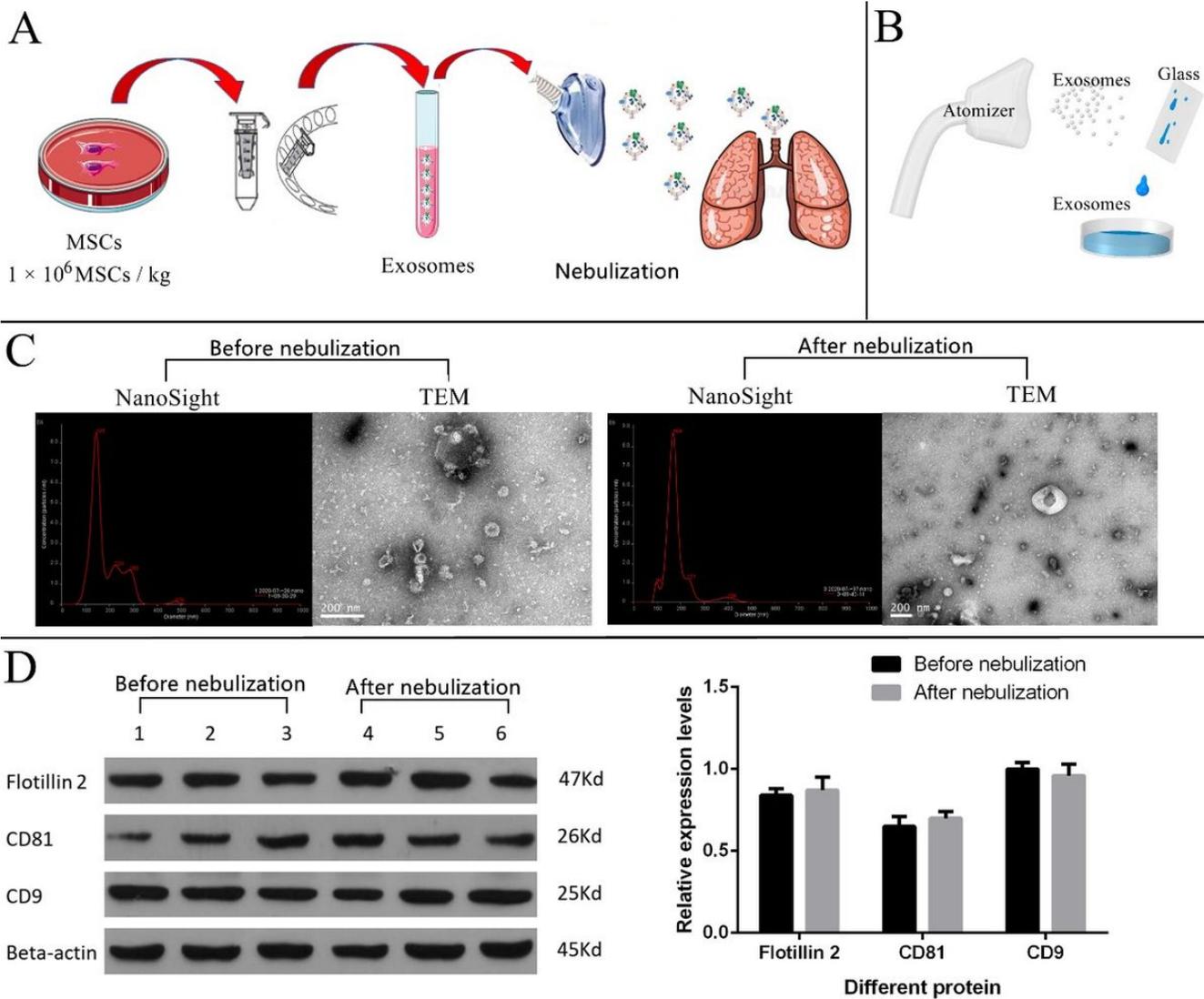


Figure 2

Isolation and characterization of exosomes from MSCs. (A) Schematic diagram of nebulization treatment of MSC-derived exosome exosomes. (B) Schematic diagram of the collecting device of exosome after atomization. (C) Exosome samples were analyzed by nanoparticle tracking analysis and transmission electron microscopy. (D) Exosome samples were confirmed using immunoblotting for known exosome markers CD9, CD81 and Flotillin 2.

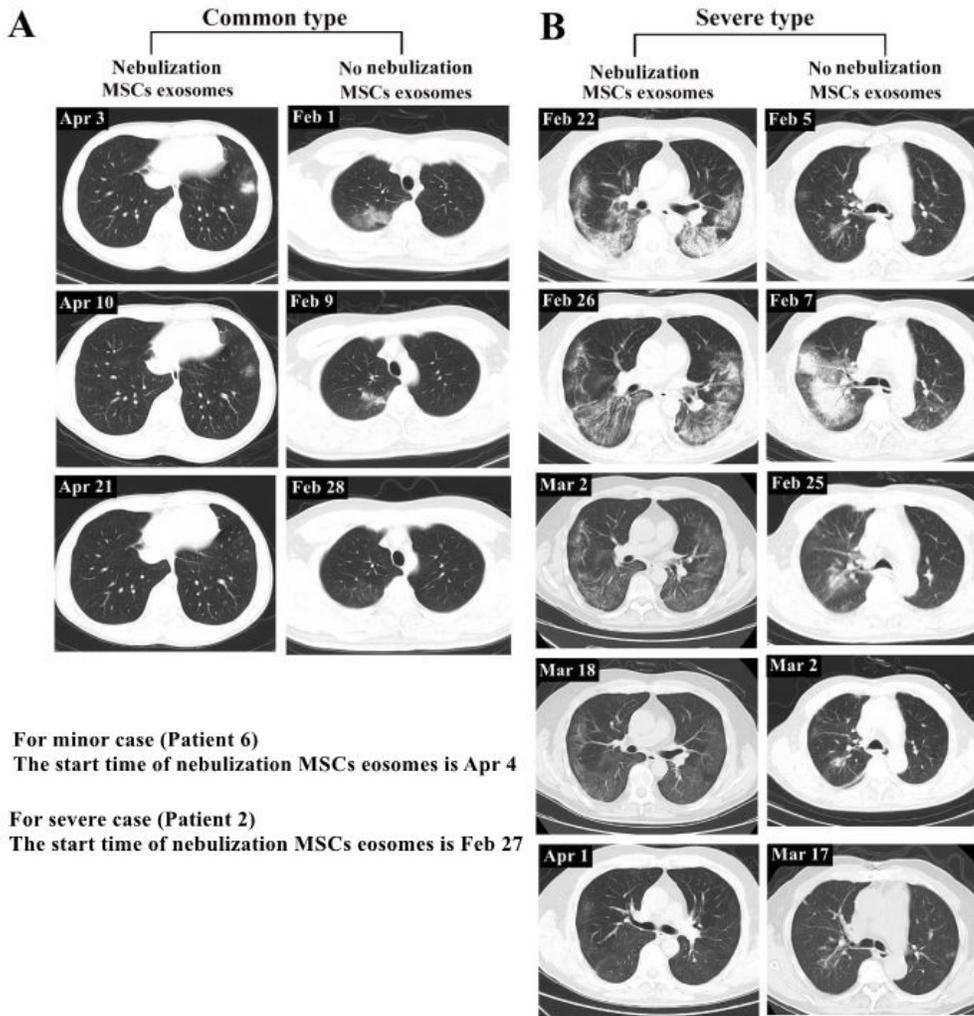


Figure 3

Chest computerized tomography (CT) images of minor and severe cases of COVID-19. (A) On Apr 3, the first CT scan of Patient 6 showed an isolated nodule outside the left lower lobe of the lung. On Apr 4, Patient 6 received nebulization treatment. On April 10, the second CT examination showed that the density of the left inferior lobe nodule to be significantly lower with a narrow range. On April 21, the third CT examination showed that the lesions in the lower left lung were completely absorbed. The absorption time of similar lung lesions in another patient, who did not receive nebulization of MSCs exosomes, was slower than that of patient 5. (B) From Feb 27, the patient received nebulization treatment. On Mar 18, the CT scan of patient 2 showed that there was obvious absorption of lesions in the two lungs, and the density of the lesions continued to fade. On Apr 1, the pulmonary lesions in patient 2 were absorbed completely. In the other patient who did not receive nebulization treatment, there was presence of a fibrous cord shadow after absorption of pulmonary focus.