Int J Clin Exp Med 2017;10(2):2590-2597
www.ijcem.com /ISSN:1940-5901/IJCEM0041994

Original Article

Mesenchymal stem cells joint simvastatin therapy improves oleic acid induced acute lung injury in rats

Yao Yang, Yaobin Zhu, Xiangming Fan

Department of Pediatric Heart Center, Beijing Anzhen Hospital, Capital Medical University, Beijing 100029, China

Received July 29, 2016; Accepted November 23, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Acute lung injury (ALI) is still a complex problem that has no special method to deal with. Mesenchymal stem cells (MSCs) treatment is reported to be beneficial in ALI model. Though simvastatin has no significant effect in ALI model, it also has some mechanisms such as suppressing inflammation in theory. We hypothesized that the joint use of MSCs and Simvastatin has a better effect on ALI treatment. The rats were assigned to the following groups: Blank group as the vehicle; other animals received intravenous oleic acid (OA) treatment to induce ALI and were allocated to groups as follow: Control group, received intravenous PBS; Simvastatin group, received oral simvastatin treatment; MSCs group, received intravenous MSCs; Joint group received both simvastatin and MSCs treatment. All treatments were given at 4 h and 24 h after ALI was established. 48 h after the OA-ALI model was established, injury score of morphology of lung tissue, wet/dry ratio were calculated. Arterial blood pressure (PaO_2), the level of IL-6, IL-10 and TNF-α in serum were assessed. In order to find the possible mechanism, an in vitro experiment was designed as follow: There are two groups involved, Simvastatin group (n=12), 1×10^5 MSCs were cultured with 7.25 ng simvastatin in 500 μl serum-free MSC condition medium (MSC-CM); Control group (n=12), 1×10^5 MSCs were cultured in 500 μl serum-free MSC-CM alone. 24 hours later, the level of Keratinocyte growth factor (KGF) in MSC-CM was examined. Rats in joint group decreased the level of IL-6 (P=0.02) and TNF-α (P=0.03) in serum and increased IL-10 (P=0.02), and decreased the injury score (P=0.1) of lung tissue. The in vitro experiments showed that, co-culture with simvastatin, the level of KGF in Simvastatin group was significantly higher (P=0.02) than that in control group. Joint use of MSCs and simvastatin can significantly improve OA-ALI, and simvastatin's ability to stimulate MSCs to secret more KGF may be the probable mechanism.

Keywords: Acute lung injury, mesenchymal stem cells, simvastatin, oleic acid, keratinocyte growth factor

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), were first described in 1967 [1] in patients with acute onset of tachypnea and hypoxia and the loss of compliance after a variety of stimuli [2]. ALI is a life-threatening syndrome that causes high morbidity and mortality [3].

Recently, mesenchymal stem cells are widely reported to be beneficial in acute lung injury through its ability to balance pro-inflammatory/anti-inflammatory cytokines, anti-apoptosis, Maintenance of alveolar epithelial structure [4-6], etc. Besides its ability to differentiate into multiple function cells, some secretioncytokines suck as Keratinocyte growth factor (KGF) are believed to be the most effective pathway to improve cells function [7-9].

KGF protein was purified as a 26-28 kDa monomeric polypeptide [10], which contains 194-amino acid, including a classic signal peptide for secretion and one potential N-linked glycosylation site [11]. Intratracheal administration of KGF was shown to provide significant protection from a variety of toxic exposures, including hyperopia, acid instillation, napthylthiourea (ANTU, model of increased permeability pulmonary edema), radiation and bleomycin. Similar results were observed when KGF was administered intravenously. The results from preclinical models suggested that KGF should be tested in clinical trials involving lung injury [12].

Statins are also used to treat ALI, they are 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, prescribed to treat elevated levels of cholesterol and cardiovascu-

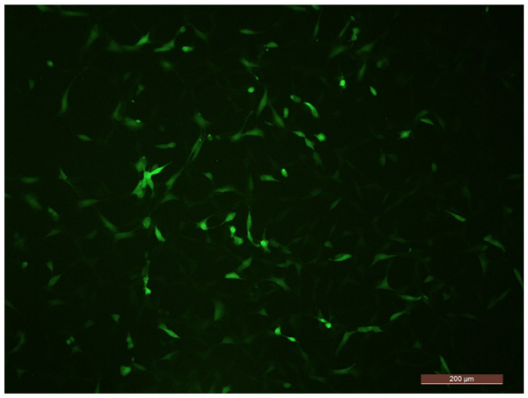

Figure 1. Mesenchymal stem cells with GFP fluorescence (by Leica Microscope).

lar disease. While statins can reduce plasma cholesterol by as much as 30-55%, statins also have potent anti-inflammatory and immunomodulatory properties that may be beneficial against certain infectious diseases in particular community-acquired pneumonia (CAP) [13]. Statins' ability above is dose-dependent, when animals take 6 times as much daily dosage, the effect appears obviously, otherwise, the differences is not significant [14].

The purpose of this study is to find whether a common dosage of simvastatin combined with MSCs will be beneficial to ALI, in clinic, no patient will take such a high dosage of simvastatin to deal with high blood cholesterol after all.

Material and methods

All procedures were carried out according to a protocol approved by the animal care and use committee of Anzhen Hospital, Capital Medical University. Animals were maintained and received care in Laboratory Animal Care Center of Anzhen Hospital. The study protocol was approved by the Laboratory Animal Ethics Committee of Capital Medical University.

MSCs culture and conditioned medium (CM) preparation

Frozen vial of passage 1 OriCellTM Sprague Dawley (SD) Rat MSCs with GFP (SD MSCs/GFP) and dermal fibroblasts (DF) was purchased from Cyagen Biosciences (CA, USA). The MSCs were thawed and expanded as previously described [7] and then generated using a protocol described. The MSCsconstantly differentiated into bone, fat, and cartilage in culture and were negative for hematopoietic markers (CD34, CD36, CD117, and CD45) and positive for CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD59 (>99%), positive for CD90 (>99%), CD105 (>99%), and CD166 (>99%). MSCs (2×10^6) and DF (2×10^6) were washed and cultured without serum for 24 h. The cells were again washed and the subsequent serum-free medium for the next 24 h was used as the conditioned medium (CM). For in vivo experiments, 15 ml of this medium was concentrated using a 3000 Da centrifugal concentrating filter (Amicon, Billerica, Massachusetts, USA) to give 500 µl. The MScs' image wasshown in **Figure 1**.

Oleic acid induced acute lung injury model and experiment design

Male SD rats weighing 180-225 g, 2-3 weeks old from the National Animal Center (Beijing, China) were used. Oleic acid (OA) induced ALI (OA-ALI) model was built up as previously described [15]. Briefly, oleic acid and 95% ethanol were mixed at a solvent ratio of 1:1. Then injected at a dose of 0.1 ml/kg through the tail vein of the experiment animal to build up ALI model after the animal was anesthetized with 3% pentobarbital through peritoneal cavity.

There were 50 rats involved, rats were randomly allocated into Blank group, Control group, Simvastatin group, MSCs group and joint group (10 rats per group). At the first time point, rats in Blank group received intravenous ethanol at a dose of 0.1 ml/kg, rats in another 4 groups received intravenous OA/ethanol solution at a dose of 0.1 ml/kg to induce ALI. 4 hours after the first time point (the second time point), the rats in Blank and Control group received 0.5 ml normal saline via stomach tube and intravenous injection of 500 µl phosphate buffered saline (PBS), rats in MSCs group received 0.5 ml normal saline via stomach tube and intravenous injection of 2×10^6 MSCs in 500 µl PBS, rats in Simvastatin group received simvastatin at a dose of 12 mg/kg in 0.5 ml normal saline via stomach tube [14] and intravenous injection of 500 µl PBS, rats in joint group received both MSCs and simvastatin treatment. 24 hours after the first time point (the third time point), rat in all groups received the same treatments that were given at the second time point. All the intravenous injections were through the tail vein.

48-hour after the first time point (the fourth time point), all the rats were anesthesia with intraperitoneal pentobarbital (10 mg/kg), 15% KCl solution 10 ml was injected through inferior vena cava to kill experiment animals, Lungs and hearts were harvested, blood samples were drawn from the abdominal aortas. After that, rats were killed by intravenous injection of 15% KCl (5 ml).

Histological assessment

Animals were killed at the fourth time point and lungs and hearts were removed, 30 ml 0.9% NaCl solution were injected through main pulmonary to wash blood cells in lung vascular. Histological lung damage was assessed. For microscopy studies, lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Lung injury score=[(alveolar hemorrhage points/no. of fields) + 2×(alveolar infiltrate points/no. of fields) + 3×(fibrin points/no. of fields) + (alveolar septal congestion/no. of fields)]/total number of alveoli counted [16].

Wet/dry ratio and and PaO_2

The middle lobe of the right lung of each animal was excised and weighed immediately after excision. Lung tissues were dried in a drying oven at 55°C for 24 hours and weighed again. The lung wet-to-dry ratio was calculated as $weight_{wet}$ /$weight_{dry}$×100%. Gases were determined using a gas analyzer (i-STAT, Yueda Company, China).

Concentration of cytokines in plasma

The concentration of TNF-α, IL-6 and IL-10 in plasma supernatants were measured in duplicate by ELISA kits (TNF-α, IL-6, IL-10 kits were from R&D Systems, Minneapolis) according to the manufacturer's instructions. The OD was read using a spectrophotometer set at a wavelength of 450 nm within 30 min.

Mesenchymal stem cellscultured with simvastatin

An invitro experiment was designed to evaluate whether cultured with simvastatin will have effect on MSCs' paracrine activation. There are two groups involved, Simvastatin group (n=12), $1×10^5$ MSCs was culture with 7.25 ng simvastatin in 500 μl serum-free MSC-CM (Cyagen Biosciences, CA, USA); control group (n=12), $1×10^5$ MSCs was cultured in 500 μl serum-free MSC-CM alone. 24 hours later, KGF, HGF, TGF-β level in MSC-CM were evaluated with ELISA kits. The concentrations of MSCs and simvastatin to prepare MSC-CM were calculated as follows:

MSCs' concentration: Rats weight 200 g, blood is calculated as 20 ml, and the plasma was 12 ml according to the plasma's volume percent in blood [17]. There were $2×10^6$ MSCs in total plasma, then the concentration of MSCs in plasma was $2×10^5$/ml, then, in 500 μl MSC-CM, $1×10^5$ MSCs is the choice of the concentration.

Simvastatin concentration: we choose the concentration max: 14.5 ng/ml in plasma according to the previous study [18] to prepare the MSC-CM.

Paracrine cytokine

KGF was not only detected in serum but also in MSCs culture medium by ELISA (R&D Systems). After exposure to experimental conditions, the supernatants of MSCs culture medium were harvested and immediately centrifuged. The concentrations of soluble mediators including HGF, TGF-β and KGF were measured by ELISA (R&D Systems, USA) according to the manufacturer's instructions [19].

Statistical analysis

Data were analyzed using SPSS 16.0 (Chicago, USA). The distribution of all data was tested for normality using Kolmogorov-Smirnov tests. Data were analyzed by one-way ANOVA. Comparisons between two groups were performed using unpaired two-tailed Student *t* tests. A two-tailed *P* value <0.05 was considered significant.

Results

There was no significant difference in body weight among the groups (P>0.05) and no early death before the fourth time point.

Mesenchymal stem cells joint simvastatin conserved lung structure

MSCs and joint groups showed a significant lower alveolar thickness as evidenced by

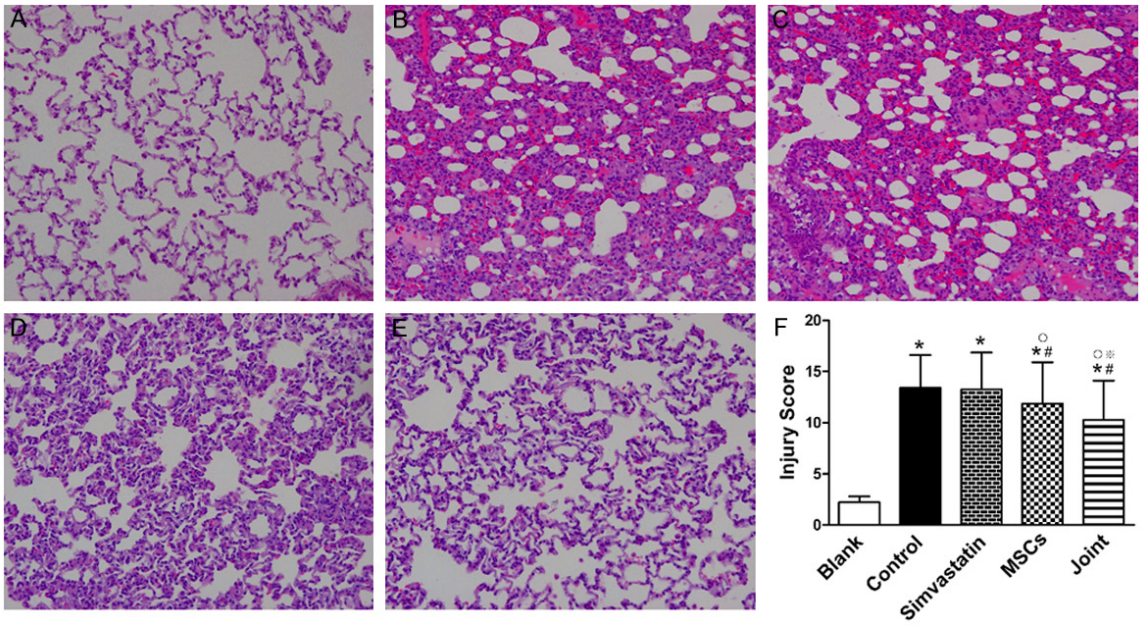

Figure 2. Representative micrograph of histopathology in each experimental group (HE stain ×200). A. Normal lung tissue in Blank group; B. Control group, OA-ALI model was established, there was alveolar congestion, edema and infiltration of polymorph nuclear neutrophils in lung tissue; C. Simvastatin group, OA-ALI after treated with oral simvastatin, the extent of lung damage was not significantly different from that in Control group; D. MSCs group, after treated with intravenous MSCs, the extent of damage is significantly different from that in OA and Simvastatin group; E. Joint group, the extent of damage is significantly lower than that in OA, Simvastatin and MSCs group; F. Injury score of each group. *P<0.05 vs Blank group, ◦P<0.05 vs Control group, #P<0.05 vs Simvastatin group, ※P<0.05 vs MSCs group.

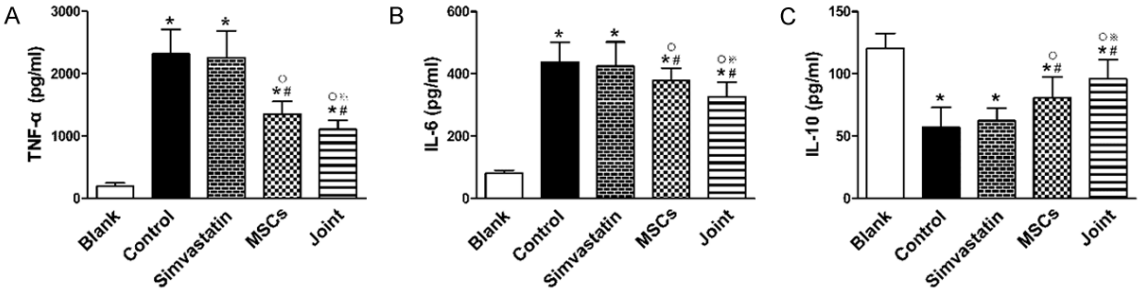

Figure 3. Inflammatory cytokines in serum in each group. A. TNF-α; B. IL-6; C. IL-10. *P<0.05 vs Blank group, ◦P<0.05 vs Control group, #P<0.05 vs Simvastatin group, ※P<0.05 vs MSCs group.

reduced alveolar tissue volume fraction and increased recovery of airspace volume as evidenced by increased alveolar airspace volume fraction, while joint group had a more significant better result versus all other groups (P<0.05). Simvastatin group did not show an ideal result. The injury score of each group was as follows: Blank group 2.23±0.56, Control group 13.41±3.19, Simvastatin group 13.25±3.60, MSCs group 11.88±4.03, and joint group 10.27±3.85.

Representative histological sections of lung (A-E) and the injury score (F) of each group was shown in **Figure 2**.

Mesenchymal stem cellsjoint simvastatinimproved lung edema and raised PaO_2

MSCs and joint groups showed a significant lower wet/dry ratio, which indicates a lower extent of lung edema. As a result, PaO_2 raised significantly in MSCs and joint group. The wet/

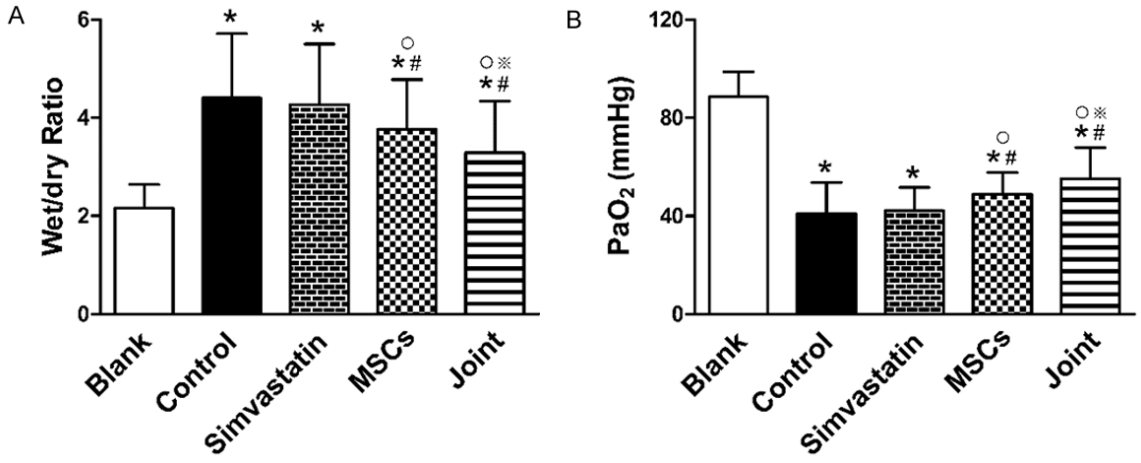

Figure 4. Extent of lung edema and PaO_2. A. Wet/dry ratio in each group; B. PaO_2 in each group. *P<0.05 vs Blank group, ◦P<0.05 vs Control group, #P<0.05 vs Simvastatin group, ※P<0.05 vs MSCs group.

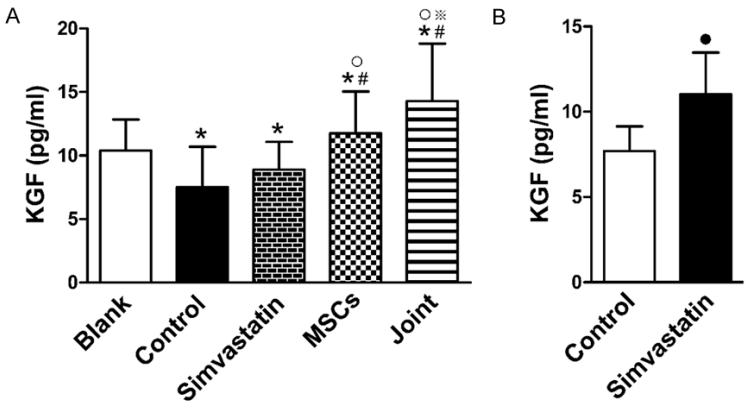

Figure 5. Keratinocyte growth factor concentration. A. Concentration of KGF in serum; B. concentration of KGF in in vitro. *P<0.05 vs Blank group, ◦P<0.05 vs Control group, #P<0.05 vs Simvastatin group, ※P<0.05 vs MSCs group, •P<0.05 vs Control group.

dry ratio and PaO_2 (mmHg) were as follows: Blank group (2.16±0.48, 88.55±10.26), Control group (4.40±1.31, 41.07±12.63), Simvastatin group (4.27±1.23, 42.29±9.33), MSCs group (3.77±1.01, 48.96±8.75), and joint group (3.29±1.05, 55.19±12.71). Versus all other groups, joint group has a significant result in the two indicators above (P<0.05).

The wet/dry ratio statistic (A) and PaO_2 (B) are shown in **Figure 3**.

Mesenchymal stem cellsjoint simvastatin balanced pro/anti-inflammatory cytokines

MSCs and joint groups showed a significant lower concentration in serum of pro-inflammatory (IL-6, TNF-α) and higher anti-inflammatory (IL-10) cytokines. Versus all other groups, joint group has a significant different result in the three indicators above (P<0.05).

The concentration of TNF-α (A), IL-6 (B) and IL-10 (C) in serum statistic figures are shown in **Figure 4**.

Mesenchymal stem cellssecreted more keratinocyte growth factor though co-cultured with simvastatin

Concentration of KGF (pg/ml) in serum was as follows: Blank group (10.39±2.45), Control group (7.52±3.18), Simvastatin group (7.92±2.17), MSCs group (11.76±3.28), and Joint group (14.29±3.52). (P<0.05). In vitro experiment showed that, concentration of KGF (pg/ml) in MSC-CM was: Control group (7.69±1.45), Simvastatin group (11.03±2.44). Versus all other groups, the concentration of KGF has a significantly different result (P<0.05).

The concentration in MCS-CM was showed in **Figure 5**.

Discussion

This study produced three unique findings: (1) oral simvastatin treatment alone at a dose of 12 mg/kg does not have significant benefit on

OA-ALI; (2) intravenous MSCs joint oral simvastatin treatment lead to better results than MSCs alone; and (3) co-cultured with simvastatin, there is significantly higher concentration of KGF in MSC-CM.

Who does not suffer from Hyperlipidemia is not conventional taking simvastatin, so in the present study, oral simvastatin is not designed to administered before the injury. To simulate a real rescue course after acute lung injury, we choose four time points to administrate treatment. For patients with ALI, injury accord at the first time point, the period to the second time point is 4 hours, which is consumed to reach hospital and receive treatment. The third time point is 24 hours after the injury happened. The fourth time point is set to evaluate the treatment effect.

Simvastatin is believed to have anti-inflammatory effect, which has the ability to provide neuroprotection [20], reduce the chances of infection-induced preterm birth [21], attenuates ventilator-induced lung injury [22], etc. But in the present study, oral simvastatin treatment at a dose of 12 mg/kg does not show significant benefit on lung disease in rats, which is consistent with the previous study [21].

MSCs' beneficial effect is partly through its paracrine mechanism, which means MSCs can secret paracrine cytokines that can influence cells or tissues nearby. In the present study, we find intravenous MSCs administration can improve OA-ALI in rats, which showed at conserving lung tissue, alleviating lung edema and balancing pro/anti-inflammation. In joint group, the result is even more encouraging, which is better in the indicator above.

In order to find the mechanism of MSCs joint simvastatin led to a better result, we use simvastatin to co-culture with MSCs, and used ELISA to examine whether there is an increase of concentration of a kind of paracrine cytokines. And we found that, the concentration of KGF, which is proved to be beneficial to ALI [23], in simvastatin group was significantly higher than that in control group.

So, the present study showed that, in the case of oral simvastatin did not have a significant effect, intravenous MSCs joint oral simvastatin treatment leads to a significantly better results than intravenous MSCs treatment alone to OA-ALI, that indicate there is a synergy between them, or simvastatin may act as a catalyst, through stimulating MSCs to get better effect. Upon the widely accepted opinion, paracrine mechanism is the most effective way to improve injured tissue, we involved 3 paracrine cytokines in in vitro experiment, and find the concentration of KGF in MSC-CM with simvastatin is significantly higher than that without simvastatin. So we came to the conclusion that, simvastatin may acted as a catalyst, which stimulated MSCs to secret more KGF to improve OA-ALI.

Conclusion

It is intravenous MSCs treatment but not oral simvastatin treatment is significantly beneficial to OA-ALI, but the joint use of both of them can lead to an even better result than MSCs treatment alone. The probable mechanism may be that, when oral simvastatin was given it can stimulate MSCs to secret more KGF which is proved to be beneficial to ALI.

Acknowledgements

The present study was supported by National Natural Science Foundation (NO. 81400305); Beijing Natural Science Foundation (NO. 7122056, NO. 7142049, NO. 7142137, NO. 7152045); Beijing Talents Fund (NO. 2014-000021469G233); Beijing Municipal Health Bureau-level talent Fund (NO. 2014-3-043); Beijing Municipal Administration of Hospitals Incubating Program (PX2016046); Beijing Talents Fund (NO. 2014000021469G233). We appreciate Xin Yi and Taotao Li who works in Beijing Institute of Heart, Lung and Blood Vessel Diseases. The present study cannot be accomplished without her kindly help.

Disclosure of conflict of interest

None.

Address correspondence to: Xiangming Fan, Department of Pediatric Heart Center, Beijing Anzhen Hospital, Capital Medical University, Anzhen Road 2, Beijing 100029, China. Tel: 18611918073; E-mail: dr_fanxiangming@outlook.com

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