Therapeutic approach for global myocardial injury using bone marrow-derived mesenchymal stem cells by cardiac support device in rats

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 doi:10.20944/preprints202004.0227.v3

Abstract

Bone marrow-derived mesenchymal stem cells (BMSCs) have been considered a promising therapeutic approach to cardiovascular disease. This study intends to compare the effect of BMSCs through a standard active cardiac support device (ASD) and intravenous injection on global myocardial injury induced by isoproterenol. BMSCs were cultured *in vitro*, and the transplanted cells were labeled with a fluorescent dye CM-Dil. Isoproterenol (ISO) was injected into the rats; two weeks later, the labeled cells were transplanted into ISO-induced heart-injury rats through the tail vein or ASD device for five days. The rats were sacrificed on the first day, the third day, and the fifth day after transplantation to observe the distribution of cells in the myocardium by fluorescence microscopy. The hemodynamic indexes of the left ventricle were measured before sacrificing. H&E staining and Masson's trichrome staining were used to evaluate the cardiac histopathology. In the ASD groups, after three days of transplantation, there were many BMSCs on the epicardial surface, and after five days of transplantation, BMSCs were widely distributed in the ventricular muscle. But in the intravenous injection group, there were no labeled-BMSCs distributed. In the ASD+BMSCs-three days treated group and ASD+BMSCs -five days-treated group, left ventricular systolic pressure (LVSP), the maximum rate of left ventricular pressure rise (+dP/dt), the maximum rate of left ventricular pressure decline (-dP/dt) increased compared with model group and intravenous injection group ($P<0.05$). By giving BMSCs through ASD device, cells can rapidly and widely distribute in the myocardium and significantly improve heart function.

Keywords: Global myocardial injury, BMSCs, Active cardiac support device (ASD), Stem cell treatment, Epicardial delivery.

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1. Introduction

Cardiovascular diseases (CVD) have the highest mortality rate throughout the world. Myocardial hypertrophy and fibrosis can cause the formation of cicatricial tissue in the heart, leading to the death of myocardium, which can affect heart function and finally result in heart failure [2]. Therefore, the prevention of myocardial fibrosis may slow the progression of heart failure. Recent studies have shown that mesenchymal stem cells (MSCs) can promote angiogenesis, inhibit cell apoptosis, and promote tissue regeneration [3, 4]. Thus, MSC transplantation has become a promising alternative treatment to heart transplantation [1, 5]. There are many kinds of cells used in transplanting, among these are bone marrow mesenchymal stem cells (BMSCs) which show superiority to other cells for they can differentiate into multiple cells and released the growth factors into the damaged myocardium which will improve the symptoms of heart failure and increase the blood flow of the infarcted area, thereby promoting myocardial regeneration [6-9]. The safety and feasibility of BMSCs transplantation have been studied and documented by some clinical trials [10, 11].

As a right donor cell for myocardial transplantation, BMSCs are mostly injected through epicardium, endocardium with a catheter, intracoronary or intravenous, but they have some shortcoming that can't be avoided [12, 13]. This study aims to investigate whether an innovative epicardial delivery method, active hydraulic ventricular attaching support system (ASD), can increase the storage and engraftment of stem cells and repair global myocardial injury.

The primary techniques for BMSC administration include 1. Intravenous infusion: This is the simplest way to transplant cells with the ability of multiple infusions. 2. Epicardial injection: BMSCs are injected into the myocardial infarction area when the thoracic surgery is performed.

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3. Endocardial injection: Cells are inserted into the endocardium through a catheter. 4. Intracoronary injection: This method needs the application of the OTW balloon catheter to the infarcted coronary artery, then cells are sent through it to the ischemic myocardium [14-16]. However, current studies focus more on local delivery of stem cells in myocardial infarcted areas, but these local delivery methods will not be effective in global myocardium injury. Our research provided a novel approach to deliver BMSCs into the epicardial using ASD, a hollow net cover made of silicon covering both ventricles of the heart. ASD can provide sufficient contact time between BMSCs and epicardium and can widely spread the cells. In this research, we investigate whether the delivery of BMSCs by ASD can improve cardiac function after ISO-induced global myocardial injury. Compared with intravenous injection, the systemic delivery method can also deliver cells multiple times.

2. Materials and methods

2.1. Ethical Statement

All procedures and experiments used in this study comply with guidelines of the local institutional ethical committee of the China Pharmaceutical University (Jiangsu Province, Nanjing, China) regarding the care and welfare of animals under study (T/CPU, 235-2018, 2018-09-09).

2.2. Experimental animals

Zhejiang Laboratory Animal Center provided a total of 40 male Sprague-Dawley (SD) rats weighing from 220-250g. During the experiment, animal handling and disposal conform to the criteria of the China Pharmaceutical University Institutional Animal Care and Use Committee. All animals were placed in the environment of free food and water for 1 week before the

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experiment. Isoproterenol was used to induce global myocardial injury, as described before, with little modification [17]. Briefly, isoproterenol was administered with saline to a solution of 85 mg/ml and injected into the rats at 170mg/kg for two consecutive days. The control group was administered subcutaneously with 0.5 ml of saline. Forty rats were randomly divided into two groups, 8 rats were selected randomly as the (ⅰ) Control group, and the other rats were modeled with isoproterenol according to the method mentioned above. Two weeks later, the survived 30 rats were divided into 5 groups randomly, (ⅱ) ISO group (model group), (ⅲ) BMSC+ASD-one day treated group (ⅳ) BMSC+ASD-three days treated group (ⅴ) BMSC+ASD-five days treated group, (ⅵ) BMSC+IV treated group. BMSCs were given through the tail vein in IV-treated group and were delivered through the outside tube of ASD in the ASD-treated groups, once a day for 5 days.

2.3. Isolation of BMSCs

3 to 4 weeks old rats with the weight of 60-80g were sacrificed. BMSCs were isolated from tibia and femur under sterile conditions, and cultured in low glucose DMEM (Glenview, USA) containing 10% FBS (Hyclone, USA) and 1% penicillin-streptomycin (Gibico, USA) in plastic culture flasks with the concentration of 1×10^9 at 37℃ in 5% CO_2 saturated wet incubator. Four days later, the whole culture solution was changed, the unattached cells were washed off, and the adherent cells propagated for 3 to 4 passages before transplantation. Then the culture solution was changed every two days. The cells grow to 80%-90% confluence, trypsinized (0.25% trypsin/1Mm EDTA, Solarbio, China), and expanded in a ratio of 1:2.

2.4. Identification of BMSCs

Harvesting the third generation BMSCs, and analyzing them by flow cytometry. Trypsinize and

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prepare the single-cell suspension in phosphate-buffered saline (PBS) per 1×10^6 cells, 2.5μg/ml, 2 antibodies, including FITC anti-rat CD45 antibody, APC anti-mouse/rat CD29 antibody (Biolegend, USA) were added and incubated on ice for 30 min in the dark. Cells were washed with 2ml PBS twice and centrifugated at 350 r/min for 5 minutes. Flow cytometric analysis was performed, and the quantitative analysis results were obtained.

2.5. Labeling of BMSCs

To track the BMSCs after transplantation, cells were labeled with cell tracker CM-Dil (Invitrogen, USA). Cells were suspended at a density of 1×10^6/ml in the serum-free culture medium, then 5μl cell labeling solution was added into the suspension with very gentle mixing. The labeled cells were incubated at 37 ℃ for 20 minutes, then washed twice with 2ml PBS at 1000 r/min for 5 minutes. The labeled cells were observed under a fluorescence microscope and analyzed by flow cytometry, then cultured in expansion medium for transplantation.

2.6. Designing and Manufacturing of ASD

Active hydraulic ventricular attaching support system (ASD) is a net cover made of silicon, a biocompatible material with a heart-shaped configuration consisting of flexible interconnected hollow tubules; it is surgically fixed to the outer wall of the heart and covers both ventricles of the heart providing physical support [18]. ASD is manufactured as a previously described method [19, 20]. Briefly, the 3D printed wax model of ASD was made at first, and covered it with silicone evenly, then heated it at 100˚C for one hour to melt the wax and obtained the pure silicone ASD **(Fig. 1a, b,c)**.

2.7. Implantation of ASD

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Two weeks after the last ISO injection, rats were sedated with 10% chloral hydrate by intraperitoneal injection (0.03ml/kg). The small animal ventilator was used to keep breathing. After confirming the ventricular position, the pericardium was separated, and the heart was exposed. ASD was fixed on the surface of the heart with suture and covered both ventricles, and with a connection of silicon tube entered the chest wall from the second intercostal space, it pierced the skin from the back of the ear **(Fig. 1d, e)**.

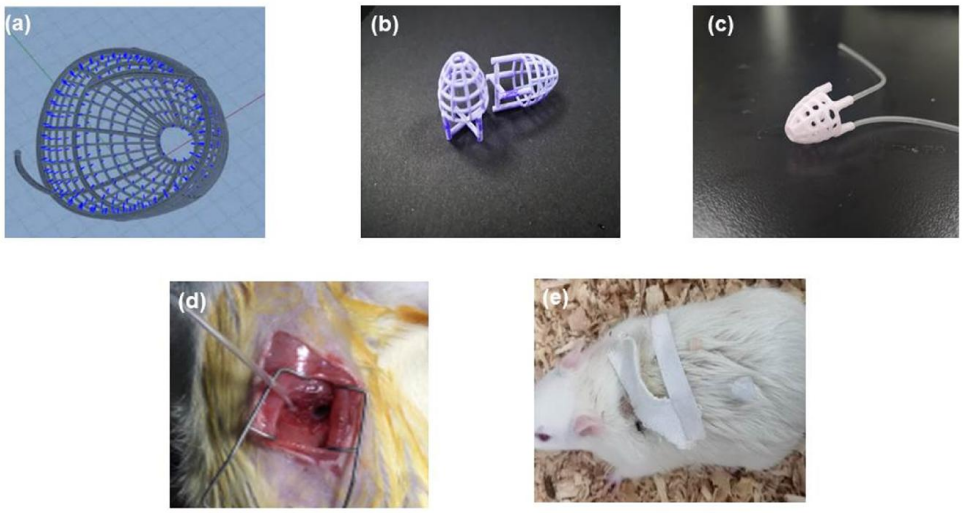

Figure 1. (a): 3D structure of ASD, **(b)**: 3D printed wax model of ASD, **(c)**: Pure silicone ASD, **(d):** Implantation of ASD, **(e):** Catheter back of the ear for cell delivering.

2.8. Implantation of BMSCs

The BMSCs labeled by CM-Dil were harvested and suspended at a density of 2×10^5/ml with PBS. Rats in intravenous injection group and ASD groups received 2×10^5 cells once a day for 5 days**.** In this study, the rats in the ASD group were sacrificed on 1, 3, and 5 days and the IV injection group was sacrificed on 5 days.

2.9. Cardiac performance measurement

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Before sacrificing, the rats were anesthetized with 10% chloral hydrate (0.3g/kg) by intraperitoneal injection. II electrocardiograph was used to monitor ECG continuously. In order to evaluate the left ventricular function, the right carotid cannula method was used, generally speaking, one end of the heparin saline (500U/ml)-filled polyethylene tube was inserted into the left ventricle and the other end connected to the pressure sensor and then connected to the BL-420S biologic function experiment system, and the hemodynamic indexes of the left ventricle were measured including heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), the maximum rate of LV pressure rise ($+dP/dt_{max}$) and a maximum speed of LV pressure decline ($-dP/dt_{max}$).

2.10. Cardiac histopathology

After hemodynamic evaluation, the animals were sacrificed, and the heart was quickly removed. Washed in saline and cut into two transverse segments 2-3mm thin paralleling to the atrioventricular groove, then one was placed in 10% formalin for 24 hours, and the other was stored at -80℃ in OCT. All the segments in 10% formalin were dehydrated with ascending order of ethanol and fixed with paraffin blocks separately using an automatic tissue processor. The paraffin block was cut into 5μm tissue sections using the microtome and then stained accordingly. Masson's trichrome staining was performed to distinguish collagen and muscle fiber. After staining, digital pathology was used, and each section was randomly taken from five different visual fields for measurement. The percentage of collagen in each visual field was calculated by ImageJ software, and the average was taken to represent the percentage of collagen fiber in the myocardium. The other part was made into a frozen section with a thickness of 5μm, observing labeled cells by fluorescence microscope.

2.11. Statistical analysis

All experimental data were presented as mean ±SD, and analyzed by statistical package for the social sciences (SPSS21.0). The LSD-q test to compare the values between the groups and the one-way analysis of variance (ANOVA) test was used between groups. *P* values <0.05 were considered statistically significant.

3. Results

In this experiment, three rats died after the injection of ISO in 24h; one died after the ASD implant surgery. Therefore there were six rats left in each group.

3.1. Morphology and characterization of BMSCs

After the cells were inoculated into the culture flask, there were different kinds of cells, most of the cells were round and with different sizes, suspended them in culture medium (**Fig. 2a**). After 24h of culture, only a few cells grew adherently; the spindle cells were scattered growth. After 72h of the extraction, the number of adhered cells increased, growing into small pieces(**Fig. 2b**). At last, after 14 days of culture, the cells increased in a large number, which was uniform spindle-shaped (**Fig. 2c**).

BMSCs were labeled with CM-Dil at 3 passages and detected by a fluorescence microscope before delivery (**Fig. 2d**). After the first passage of the labeled cells, there was no change in the cell morphology and the fluorescence intensity (**Fig. 2e**). The flow cytometry results showed CM-Dil labeling rate was >90% (**Fig. 2f)**, and the isolated cells expressed the MSC marker CD29 but didn't have the pan leukocyte marker CD45 (**Fig. 2g**).

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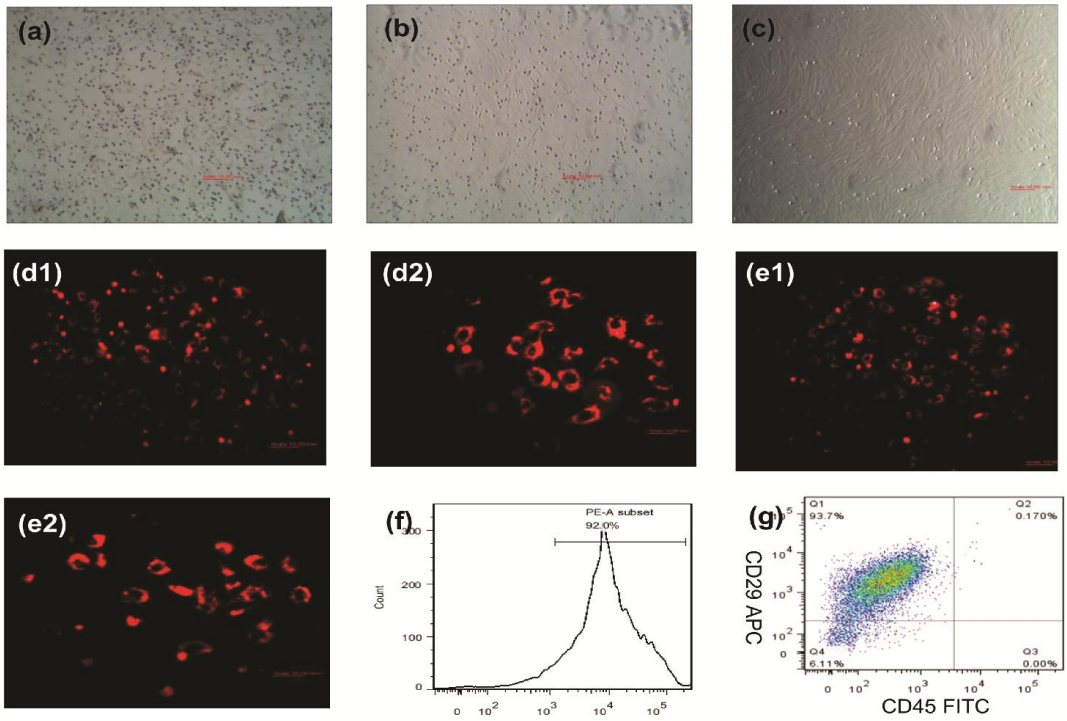

Figure 2. Cell morphology and characterization. **a, b, c:** Morphology of cultured cells at different stages. **(a)**: The first day of cell inoculation, **(b)**: 4 days after initial plating, **(c)**: 14 days after initial plating. BMSCs were collected from rat femurs and tibias and expanded in culture with an irregular shape, three or four days later, the cells appeared fibroblast-like in morphology, 14 days later, the cells formed a confluent monolayer**. (d):** BMSCs were labeled with Chloromethylbenzamido-1,1′-Dioctadecyl-3,3,3′3′Tetramethylindocarb cyanine Perchlorate (CM-Dil). **(e):** Labeled cells after passage **(f):** Flow cytometric analysis showed the labeling rate of CM-Dil was >90%. **(g):** Flow cytometric analysis of adherent cells. Most cultured BMSCs expressed CD29. In contrast, the majority of MSCs CD45 negative. a, b, c 40× magnification, d1,e1 100× magnification, d2 e2 200× magnification

3.2. Effects of BMSCs transplantation on heart function

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In this experiment, there was no significant difference in heart rate. Two weeks after the last ISO injection. Compared with the ISO group, LVSP, LV+dP/dt_{max}, and LV-dP/dt_{max} (P<0.05) were significantly increased in ASD-three days treated group and ASD-five days treated group. As compared to the BMSCs+iv group, the ASD-five days treated group also showed significantly increased LVSP and LV+dP/dt_{max} (P<0.05) (**Table 1, Fig. 3**).

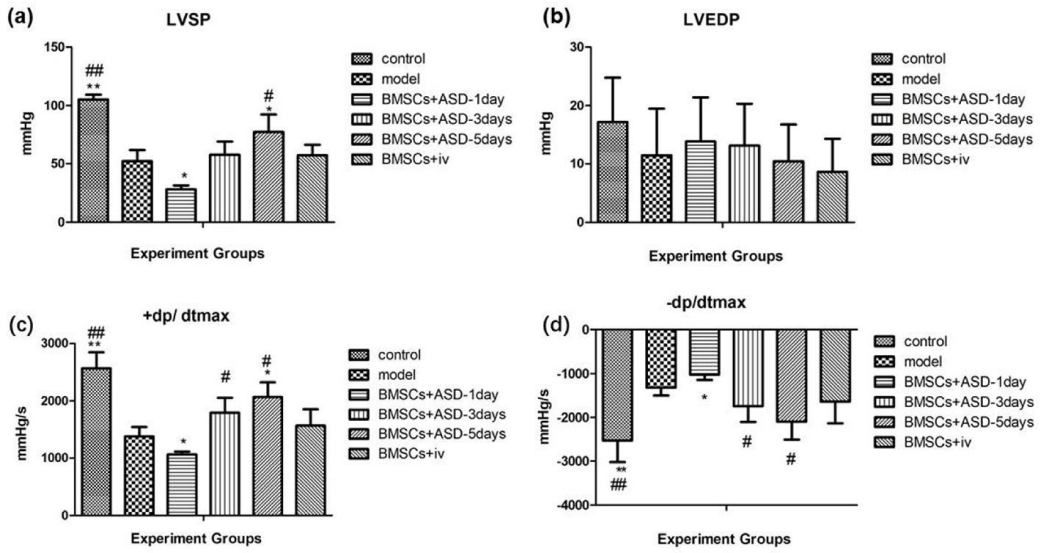

Figure 3. Effects of BMSCs transplantation on left ventricular (LV) hemodynamic parameters in the experiments. #significant difference vs. model group, ##P value <0.01, #P value <0.05, *significant difference vs. BMSCs+IV group, **P value <0.01, *P value <0.05 (Each group n = 6). HR, Heart rate; LVSP, Left ventricular systolic pressure; LVEDP, Left ventricular end-diastolic pressure; +dP/ dt_{max}, Maximum rate of left ventricular pressure rise; -dP/dt_{max}, Maximum rate of left ventricular pressure decline.

Table 1. The effects of BMSCs transplantation on HR, LVSP, LVEDP, and ±dP/dt_{max}

Groups	HR	LVSP	LVEDP	+dP/dt_{max}	-dP/dt_{max}

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		mmHg	mmHg	(mmHg/s)	(mmHg/s)
control	380.83±30.82	105.34±4.06 ##**	17.18±7.16	2566.94±279.73 ##**	-2528.8±495.23 ##**
model	423.67±11.41	52.12±9.56	11.45±8.04	1378.91±162.38	-1317.87±181.75
BMSCs+ASD-1day	421.17±21.65	29.58±4.96*	13.84±7.59	1062.22±52.23*	-1019.61±109.9*
BMSCs+ASD-3days	415.16±20.67	57.64±11.47	13.13±7.21	1794.03±261.66#	-1741.13±360.46#
BMSCs+ASD-5days	425.5±34.56	77.34±15.03#*	10.44±6.32	2082.03±283.48#*	2093.75±413.74#
BMSCs+iv	411.33±51.89	57.16±9.18	8.6±5.70	1566.77±287.43	1634.32±500.77

Data are presented as mean ±SD, #significant difference vs. model group, ##*P* value <0.01, #*P* value <0.05, *significant difference vs. BMSCs+IV group, **P value <0.01, *P value <0.05 (Each group n = 6). HR, Heart rate; LVSP, Left ventricular systolic pressure; LVEDP, Left ventricular end-diastolic pressure; $+dP/dt_{max}$, Maximum rate of left ventricular pressure rise; $-dP/dt_{max}$, Maximum rate of left ventricular pressure decline.

3.3. Distribution of CM-Dil labeled cells

After transplantation, labeled cells were observed in ASD+BMSCs -three days treated and ASD+BMSCs -five days treated groups, but not in other groups (**Fig.4**).

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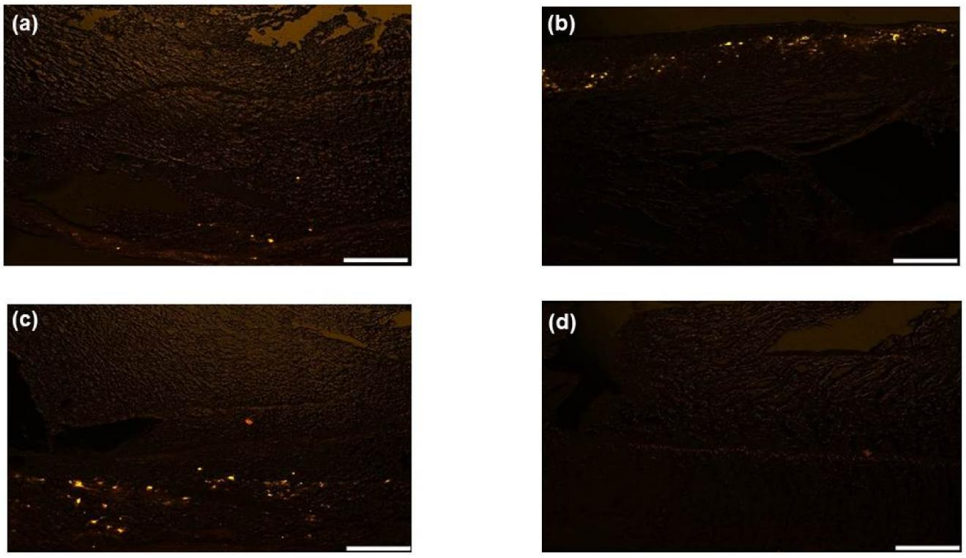

Figure 4. BMSCs engraftment in myocardium. The CM-Dil labeled BMSCs were detected under fluorescence microscopy. **(a):** BMSCs+ASD-1day group, **(b):** BMSCs+ASD-3days group, **(c):** BMSCs+ASD-5days group, **(d):** BMSCs+IV group, 40× magnifications, scale bar 100 μm.

The labeled cells in BMSCs+ASD-3 day's group were mainly distributed on the epicardium, BMSCs were observed near the middle layer of the myocardium in BMSCs+ASD-5days group. There were no BMSCs that could be found in the BMSCs+IV group.

3.4 Histopathological examination of heart tissues

We investigated the cardiac histopathology by H&E staining and Masson's trichrome staining. In the group of rats undergoing myocardial injury, H&E staining showed a significant loss of myocardial structure and the infiltration of inflammatory cells (**Fig. 5**). Cardiac muscle fibers of the model group degenerated in BMSCs+ASD-1day group, BMSCs+IV group (**Fig. 5c, 5f**). There were a large number of inflammatory cells infiltrated and distributed diffusely. Myofibrils were arranged neatly near endocardium on BMSCs+ASD-3days group (**Fig. 5d**). In BMSCs+ASD-5days group, myocardium was arranged tightly, and less inflammatory cells

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dispersed in the endocardium (**Fig. 5e**).

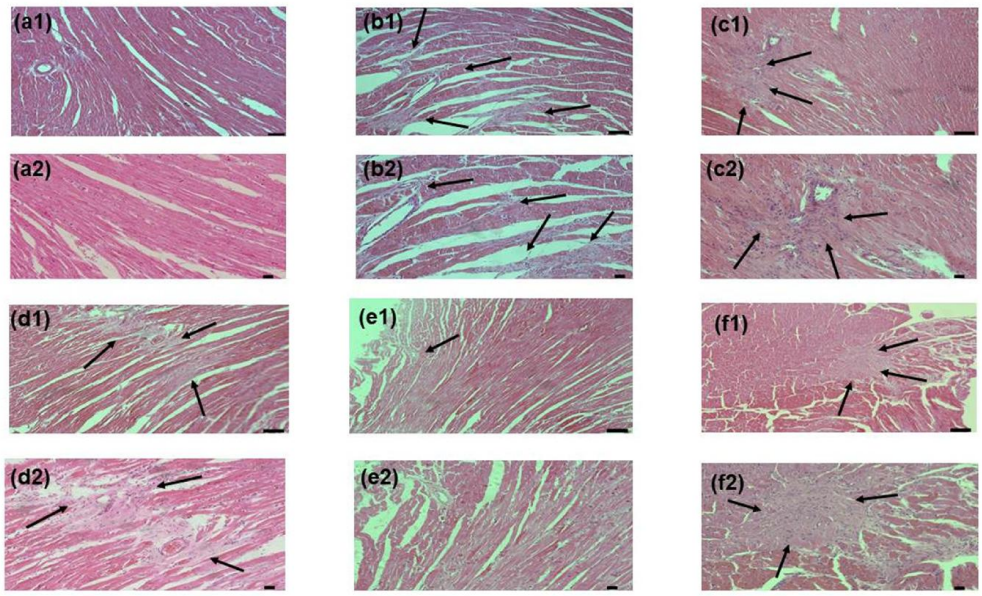

Figure 5. H&E staining of the ventricles **(a):** control group, **(b):** model group, **(c):** BMSCs+ASD-1day group, **(d):** BMSCs+ASD-3days group, **(e):** BMSCs+ASD-5days group, **(f):** BMSCs+IV group, a1, b1,c1, d1, f1 100× magnification, a2, b2, c2, d2, e2 200× magnification, scale bar 100 μm. H&E staining showed that the myocardial fibers from the ventricles in the model group, BMSCs+ASD-1day group, BMSCs+IV group were thicker, and myofibril ruptured. A large number of inflammatory cells (arrowheads) infiltrated and distributed diffusely. Myofibrils were arranged neatly near endocardium on BMSCs+ASD-3days group. Myocardium was arranged tightly on the BMSCs+ASD-5 day's group.

Masson's trichrome staining showed a massive increase in the collagen fibers among the degenerating muscle fibers in the model group, BMSCs+ASD-1day group, BMSCs+IV group (**Fig. 6**). After 5 days' treatment, the collagen fibers decreased (P <0.05), especially in the myocardium's medium and endocardium.

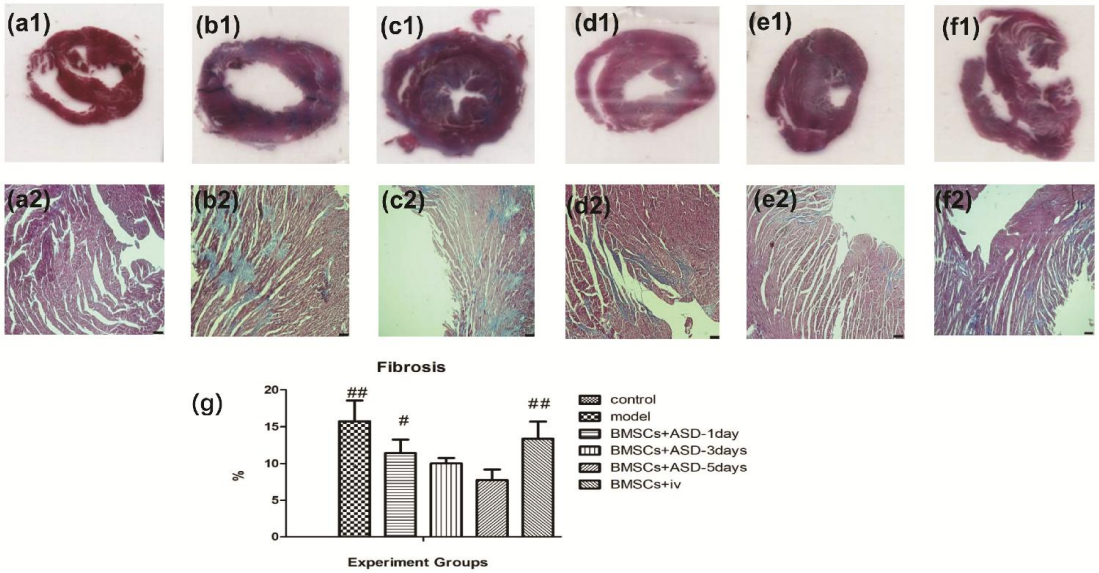

Figure 6. Masson's trichrome of ventricles **(a):** control group, **(b):** model group, **(c):** BMSCs+ASD-1day group, **(d):** BMSCs+ASD-3days group, **(e):** BMSCs+ASD-5days group, **(f):** BMSCs+IV group, **(g):** quantification of fibrosis of heart tissues. # significant difference vs. BMSCs+ASD-5 days group, ##*P* value <0.01, #*P* value <0.05 (Each group n = 6)); a1, b1, c1, d1, f1 image scanning by digital pathology; a2, b2, c2, d2, e2, f2 in 100× magnifications, scale bar 100 μm. Masson's trichrome staining showed that large collagen fibers appeared in the model group, ASD-1 day group, and IV group. But in the group of ASD-3 days and ASD-5 days, the area of degenerating muscle fibers reduced.

4. Discussion

Teerlink *et al.,* [21] have confirmed that subcutaneous injection of ISO (> 85 mg/kg) can cause diffuse myocardial fibrosis after 2 weeks, and gradually lead to heart failure. Compared with ischemic myocardial infarction, this method can reflect the natural process of HF, similar to that in patients [22]. In this study, we established the rat model of extensive myocardial injury by subcutaneous injection of ISO at a dose of 170 mg/kg for 2 consecutive days. The changes in

hemodynamic parameters often used to evaluate heart function [23, 24]. Two weeks later, our results showed that compared with the control group, the left ventricular function reduced in the groups received ISO injection (P <0.05).

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stem cells that exist in the bone marrow and can differentiate into various mesodermal cells, such as bone cells, fat, and matrix tissue cells. BMSCs were cells that can secrete a variety of hematopoietic factors to support hematopoiesis and immune regulation ability [25].

According to the international society for cellular therapy (ISCT) criteria, MSCs should adhere to plastic and have specific surface antigen measured by flow cytometry [26]. BMSCs can express a variety of surface antigens, but no particular surface antigens. According to reports, CD29, CD44, CD59 are positive for surface antigen expression, while hematopoietic stem cell markers CD45, CD14, and CD117 are negative. In this experiment, CD29 and CD45 were selected, and it was found that adherent cells express $CD29^{+}CD45^{-}$, combining with the results of morphology, we confirmed that the isolated and cultured cells were BMSCs.

BMSC therapy is a viable option for heart renovation. There are many ways to deliver BMSCs, but traditional methods of transportation always have some problems such as low storage rate, low cell survival rate, and inability to be administered multiple times [27]. Intravenous injection of BMSCs has been thought to be useful for diffuse myocardial disease, before engrafting in the infarcted myocardium, cells will go through the systemic circulation and receive the local signal of injured tissue [28]. In recent studies, epicardium delivery of BMSCs has been considered a promising method [29, 30], which can not only avoid myocardial injury and inflammation caused by intravenous injection. ASD is a kind of device that can deliver the drug or cells into

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the epicardium improving delivery efficiency [31, 32]. Besides, it can provide physical support to prevent ventricular enlargement. Our objective is to compare the effectiveness of multiple BMSC deliveries through veins and ASD.

In this experiment, the increase of LVSP, and $\pm dP/dt_{max}$ in BMSCs+ASD-3days group and BMSCs+ASD-5days group indicated that the cardiac contraction improved. In our previous research, ASD can not only deliver drugs but also provide pressure. For the BMSCs+ASD-1day group, the decrease of these indicators may be caused by the thoracotomy. There was no difference in these indicators in the BMSCs+IV group compared with the model group. The results indicated that although IV injection was a more convenient delivery method, the treatment effect was not evident. According to another study, the cells would be caught by other organs such as the liver, lung, or spleen [33].

CM-Dil is a kind of lipophilic non-toxic live-cell dye that can steadily track cells *in vivo* or *vitro* [34]. The labeled cells could be injected into *vivo* to show the migration and differentiation of transplanted cells effectively. In this experiment, CM-Dil marked BMSCs were observed under a fluorescence microscope in the BMSCs+ASD group. In ASD+BMSCs groups, as the time and the frequency of transplantation increased, the cells were more widely distributed and gradually migrated to the endocardial as the transplants increased, which will be beneficial for extensive myocardial damage.

In many cases of heart failure, increased content and cardiac fibrosis can trigger a series of changes in ventricular function [35, 36]. The New York heart association (NYHA) reported that myocardial apoptosis in patients with heart failure of cardiac service III and IV accounted for 0.12%-0.70% of myocardial tissue, while that in normal myocardium accounted for only

0.001%-0.01% [37]. Masson's trichrome is one of the primary methods for displaying fiber staining in tissues. It is mainly used for the differential analysis of collagen and smooth muscle and one of the classic techniques for collagen fiber staining, dyed blue. In our experiment, the collagen deposition area increased significantly after two weeks of ISO injection. We also observed that compared with the model group and IV injection group, the myocardial collagen area and the perivascular collagen area of the left ventricular endocardium in the BMSCs+ASD-treated groups were significantly decreased (P <0.01), indicating that the BMSCs grafted by the ASD can repair myocardial fibrosis effectively.

The cell sheet approach is one of the latest strategies for epicardial delivery of the heart utilizing innovative biomedical engineering, which can minimize systemic side effects by targeting delivery. ASD has a similar advantage as a cell sheet, but it can also spread the stem cells or drugs all over the ventricles and deliver cells continuously [38]. Our previous study has confirmed the delivery of lidocaine by ASD was beneficial to reversing calcium chloride-induced ventricular fibrillation [19]. Similarly, ASD delivery of nitroglycerine has improved cardiac function and has a protective effect on the ischemic injury of the heart [39]. Moreover, delivering traditional drug *Salvia* by ASD also showed benefits in reversing ventricular remodeling and improving heart function [31]. The combination of cells and therapeutic substances has become a reliable method. As a novel device, ASD can not only provide BMSCs with a favorable environment to promote retention but also increases the contact area and many cells recruited in the myocardium. There are also some limitations to this study. Further study also needed for the specific mechanism of this technology. This study just initially confirmed that cells reached the myocardium more efficiently through ASD than IV delivery

which has been considered effective for global myocardial injury, it remains to determine the BMSC differentiation in the myocardial, and the proper paracrine factors released by BMSC that are beneficial for myocardial repair.

5. Conclusions

In this experiment, we firstly provide a kind of cell delivery method for global myocardial injury. This study demonstrated ASD increased BMSCs distribution area and significant improvement in cardiac function compared with the IV injection group. The results of this research indicate that multiple times the delivery of BMSCs by ASD is more effective for global injured myocardium. Further study should focus on integrating engrafted cells with the myocardium and enhancing cell survival by delivering with other substances.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant nos.30973003 and 30901993), Administration of TCM Jiangsu Province China (No. LZ11093) Qing Lan Project, National Found for Fostering Talents of Basic Science of PR China (No. J1030830). Furthermore, all the authors thank and acknowledge their respective Universities and Institutes.

Conflict of Interest

All authors declare no conflict of interest.

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