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Guanxin Danshen Formulation improved the effect of mesenchymal stem cells transplantation for the treatment of myocardial infarction probably via enhancing the engraftment

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ABSTRACT

Although intravenous injection is the most convenient and feasible approach for mesenchymal stem cells (MSCs) delivery, the proportion of donor stem cells in the target myocardium after transplantation is small. It is believed that TCM enhances the effect of stem cell therapy by improving the hostile microenvironment and promoting the migration and survival of stem cells. Guanxin Danshen (GXDS) formulation is one of the main prescriptions for clinical treatment of ischemic heart diseases in China. The purpose of this study was to evaluate the effects of GXDS formulation administration combined with MSCs transplantation on cardiac function improvement, apoptosis, angiogenesis and survival of transplanted cells in an acute model of acute myocardial infarction (MI). After being labeled with GFP, MSCs were transplanted via intravenous injection. Meanwhile, GXDS dripping pills were given by intragastric administration for 4 weeks from 2 days before MI. Echocardiography showed moderate improvement in cardiac function after administration of GXDS formulation or intravenous transplantation of MSCs. However, GXDS formulation combined with MSCs transplantation significantly improved cardiac function after MI. The myocardial infarct size in rats treated with MSCs was similar to that in rats treated with GXDS formulation. However, GXDS formulation combined with MSCs transplantation significantly reduced infarction area. In addition, GXDS formulation combined with MSCs transplantation not only decreased cell apoptosis according to the TUNEL staining, but also enhanced angiogenesis in the peri-infarction and infarction area. Interestingly, the use of GXDS formulation increased the number of injected MSCs in the infarct area. Furthermore, GXDS formulation combined with MSCs transplantation increased SDF-1 levels in the infarcted area, but did not affect the expression of YAP. Our study provided a more feasible and accessible strategy to enhance the migration of stem cells after intravenous injection by oral administration of GXDS formulation. The combination of GXDS formulation and stem cell therapy has practical significance and application prospects in the treatment of ischemic cardiomyopathy such as MI.

1. Introduction

The cardiovascular disease, especially myocardial infarction (MI), has become the leading cause of death in the world. After MI, damaged cardiomyocytes are gradually replaced by fibroblasts, which induce ventricular remodeling and thinning of the ventricular wall, eventually leading to heart failure and even sudden death. Stem cells are multipotent cells that can differentiate into a variety functional cells under certain inducing conditions. The role of stem cell transplantation in promoting necrotic myocardial repair and improving cardiac function has been demonstrated in animal experiments and clinical studies [1–3]. Our previous studies have also showed that mesenchymal stem cells (MSCs) can survive in the host heart and improve impaired cardiac function in rats after transplantation into the border zone of the infarct zone [4,5]. Recently, we found that MSCs transplantation in self-assembling peptide modified with the peptide QHREDGS reduced scar size and improved heart function probably via exosome-mediated miR-21 in rats with MI [6].

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So far there are still many problems with stem cell transplantation, such as the approach of stem cell delivery, migration to the infarcted area after transplantation, survival after transplantation and efficiency of differentiation to functional cardiomyocytes, normal excitation-contraction coupling even after differentiation, and immunological rejection. First and foremost, the appropriate approach of stem cell delivery is an important thing worth considering in clinical application of stem cells therapy of MI. As the most convenient and feasible method for stem cell delivery, only a small proportion of stem cells engrafted in the target myocardium after intravenous transplantation. Nevertheless, adverse remodeling and progressive deterioration of left ventricle (LV) function were suppressed in mice with MI [7]. Therefore, strategies for improving the migration of stem cells into the injured area after intravenous injection warrant further investigation.

Traditional Chinese medicine (TCM) has been used for the treatment and prevention of diseases for thousands of years and has been proved to be effective and safe for the treatment of cardiovascular diseases [8,9]. Among them, the traditional Chinese herbal recipe Guanxin Danshen (GXDS) formulation, which has been recorded in the Chinese Pharmacopeia since 1995 edition, is one of the main prescriptions for clinical treatment of ischemic heart diseases in China. GXDS formulation is composed of three components: Salvia miltiorrhiza (Danshen), Panax notoginseng (Sanqi), and Dalbergia odorifera (Jiangxiang). Recent evidence showed that GXDS formulation protected against myocardial ischemia reperfusion injury-induced LV remodeling by upregulating estrogen receptor β via PI3K/Akt signaling [10].

Through the integration of traditional Chinese and western medicine, it is expected to provide new strategy to address the problems faced by stem cells transplantation in the treatment of ischemic diseases such as MI. Combination therapy with Compound Danshen Dripping Pills (CDDP) and human umbilical cord blood mononuclear cell transplantation significantly increased the survival of transplanted cells, inhibited cardiac cell apoptosis, decreased oxidative stress and inflammatory response, and improved cardiac function [11]. Tanshinone IIA- and astragaloside IV enhanced capacities of injected MSCs to home to ischemic myocardium sites at least partially by modulating C-X-C chemokine receptor type 4 (CXCR4) expression [12]. Another study indicated that tanshinone IIA not only promoted the expression of stromal cell-derived factor-1 (SDF-1), a major chemotactic factor involved in the homing of stem cells after transplantation [13], and the number of transplanted MSCs in the infarct area, but also enhanced the CXCR4 expression of MSCs in vitro. Based on these data, they proposed that Tanshinone IIA could increase the MSCs migration via up-regulating SDF-1/CXCR4 axis [14]. Therefore, the Chinese medicine is considered to improve the effect of stem cell therapy by improving microenvironment after transplantation and promoting stem cell migration and survival, but its specific mechanisms are not yet clear and worth further exploration.

The purpose of this study was to assess the effects of GXDS formulation administration combined with MSCs transplantation via intravenous injection on cardiac function improvement, cell apoptosis, angiogenesis and survival of transplanted cells in a model of acute MI. The expression of SDF-1 was further detected in damaged myocardial tissues to elucidate the possible mechanism underlying the enhanced engraftment of MSCs by administration of GXDS formulation.

2. Materials and methods

2.1. Animals

SD rats aged 3–5 days and SPF grade healthy male SD rats (200 ± 20 g) were provided by the Animal Experiment Center of Shanghai University of Traditional Chinese Medicine. The animals were kept in a room with 70% humidity and a constant temperature of 23 °C and free access to standard water and food. This study was approved by the animal ethics committee of Shanghai University of TCM and the Animal Research Committee of Shanghai. All experimental procedures and protocols were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health (USA) and efforts were made to minimize the number of rats used and any discomfort experienced.

2.2. Mesenchymal stem cell culture

MSCs were isolated from SD rats aged 3–5 days by adherent culture. After being sterilized in 75% ethanol for 5–10 min, the tibia and femur were separated, and then both ends of the bones were cut off to expose the bone marrow cavity. The bone marrow was rinsed with 10 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco, San Diego, USA). The flushing fluid was collected and centrifuged at 1000 rpm for 5 min. Afterwards, the supernatant was discarded and the cell pellet was resuspended with 5 ml of DMEM containing 15% fetal bovine serum (FBS, Gibco) in a 25 ml sterile culture flask and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were digested to conventional passage when culture flasks became around 80% semi-confluence. The experiments were performed with the cells of passage number 3–5. The characterization of MSCs was detected through immunofluorescence staining of cell surface markers including CD29, CD44 and CD45. MSCs were incubated with the primary antibodies (Abcam, USA) overnight at 4 °C after being fixed with 4% paraformaldehyde and then were reacted with Alexa Flour 488 secondary antibody (1:200; Invitrogen) for 2 h at room temperature.

2.3. Labeling with GFP

To trace the cells after injection in vivo, MSCs were labeled with GFP using lentivirus carrying GFP reporter (Genechem Ltd., Shanghai, China). MSCs were infected with GFP lentivirus according to the protocol manufacturer suggested.

2.4. Myocardial infarction model and treatment

The SD rats weighing 180–220 were anesthetized by 1% pentobarbital sodium (40 mg/kg, i.p.), and fixed on the surgical plate in a supine position on a warm heating pad (World Precision Instruments Inc., FL, USA). After tracheal cannulation, the rats were ventilated with room air using a rodent ventilator (Harvard Apparatus, USA) 1-cm incision was made at a distance of about 0.3 cm from the top of the xiphisternal synchondrosis. The muscle was bluntly dissected and the pericardium was cut open to expose the starting point of the left coronary artery (between the pulmonary arterial cone and the left atrial appendage). The left anterior descending coronary artery was then ligated at 2 to 3 mm below the starting point. Acute MI was confirmed with elevated ST segment on electrocardiograms. After the model was successfully established, the muscles and skin were sutured.

Rats were randomly divided into 4 groups as follows: (1) saline group; (2) MSCs group; (3) GXDS formulation group; (4) MSCs + GXDS formulation group. Saline (0.4 ml) or MSCs (10^6/0.4 ml) was injected by tail vein in the saline group and MSCs group respectively, 1 d after MI. In the GXDS formulation group, GXDS dripping pills (100 mg/kg/d, Yerui Pharmaceutical Ltd., Beijing, China) were given by intragastric administration from 2 d before MI for 4 w. The dosage of was GXDS dripping pills was determined based on the conversion of human dosage to rat dose on the basis of body surface area. In the MSCs + GXDS formulation group, the rats were administrated with GXDS dripping pills (100 mg/kg/d) from 2 d before MI for 4 w and an injection of MSCs (10^6/0.4 ml) intravenously at 1 d after MI.

2.5. Echocardiography

All rats were anesthetized with 2% isoflurane and echocardiographic assessment was conducted after the last GXDS treatment by a

commercially available echocardiography system (Vevo Visualsonics 2100; Visualsonics, Toronto, ON, Canada). M-mode tracing of the left ventricle was obtained from the parasternal long-axis view to calculate LV ejection fraction (EF) and LV fractional shortening (FS). All measurements were averaged over three consecutive cardiac cycles. Left-ventricular end-systolic (LVESV) and end-diastolic volume (LVEDV) were measured and recorded. The data of EF and FS was presented as percentage. The echocardiographic assessment was also conducted before cell injection to rule out the rats with EF higher than 60%, which as the sign of unsuccessful modeling.

2.6. Histopathologic examination

After echocardiographic assessment, the hearts were rapidly excised, and the wet weight was obtained. The tissues were embedded in Tissue-Tek OCT (Sakura, USA) after fixation with 4% paraformaldehyde, and then cut into 10-μm sections with freezing microtome. The slides were stained with Masson's trichrome to detect the fibrosis in cardiac muscle. The infarcted area was defined as the percentage of blue region in the cross-sectional area of whole myocardium in the LV wall and calculated with Image-Pro Plus software (Bethesda, USA).

2.7. Immunofluorescence staining

The slides with heart samples harvested at 4 weeks after cell injection were permeabilized with 0.1% Triton X-100 and then blocked with 5% normal goat serum for 1 h at room temperature. After that, the samples were incubated with mouse monoclonal CD31 antibody (1:200; Abcam, USA) overnight at 4 °C followed by incubation with goat anti-mouse secondary antibody coupled with Alexa Fluor 488 (1:200; Invitrogen, USA) for 2 h at room temperature. The nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). Vascular density was determined by randomly selecting at least five fields in each sample and counting the number of CD31-positive microvessels per HPF.

2.8. Terminal dUTP nick-end labeling assay

To investigate the role of GXDS formulation combined with MSCs transplantation on cytoprotection after MI, the number of apoptotic cells at 3 days after cell transplantation was determined by the terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) staining kit (Roche, Mannheim, Germany) according to the manufacturer's instructions followed by the immunofluorescence staining of cTnT (1:200; Abcam, USA). The nuclei were counter-stained with DAPI. The total number of TUNEL-positive nuclei was counted in at least five high-power fields (HPF).

2.9. Engraftment of MSCs in the infarcted region

To examine the presence of GFP-labeled MSCs in the heart at 3 days after injection, the samples were immunostained with GFP (Thermo Fisher Scientific, Waltham, MA, USA) and the images were acquired with a fluorescence microscope (IX53, Olympus). The number of GFP-labeled MSCs in the infarcted region was counted for the MSCs and MSCs + GXDS group.

2.10. Western Blot

Samples from the infarcted area were homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) and kept on ice for 30 min. Then the lysate was centrifuged at 13,000 *g* at 4 °C for 15 min. Protein concentration was determined by the BCA Protein Assay Kit (Pierce, USA). After being boiled for 5 min, 40 μg of protein lysates from heart samples were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, USA). The membranes were blocked in 5% non-fat milk for 1 h and probed with primary antibodies of SDF-1 (1:1000, Abcam) and Yes-associated protein (YAP; 1:1000, CST) overnight at 4 °C. Membranes were washed in Tris-buffered saline with Tween 20 and then incubated with appropriate secondary antibodies (1:5000, Abcam) for 2 h at room temperature. Subsequently, the formed immunocomplex was visualized by enhanced chemi-luminescence (Pierce Biotechnology, USA). The densities of bands were quantified using ImageJ software (NIH, Bethesda, MD, USA) and expressed as ratios to GAPDH.

2.11. Statistical analysis

All data were reported as means ± standard deviation. Data analyses were carried out with statistical software (IBM SPSS Statistics for Windows, Version 23.0, IBM Corp., NY, USA). The student's *t*-test and one-way analysis of variance (ANOVA) with Scheffe's post hoc multiple-comparison analysis were performed for statistical analyses. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MSCs morphology and GFP labeling

Most of the MSCs we cultured expressed CD29 and CD44 and were negative for CD45 (Fig. 1A), which indicated that the MSCs have a high purity. It was observed that most of the cells presented with the shape of fusiform or polygon (Fig. 1A). Cells were subcultured or harvested to carry out experiments by trypsinization when they reached 80–90% confluence. To trace the fate of transplanted cells, MSCs were labeled with GFP by lentivirus with high infection efficiency (Fig. 1B).

3.2. GXDS formulation combined with MSCs transplantation improved cardiac function after myocardial infarction

The heart function was examined by echocardiogram at 4 weeks after transplantation. There was no significant difference in EF and FS among the saline group, MSCs group and GXDS formulation group, which indicated that MSCs transplantation through intravenous injection or oral administration of GXDS formulation had no significant effect on recovery of EF and FS values after MI. However, EF and FS values were much higher in MSCs + GXDS formulation combination group than the saline group, MSCs group and GXDS formulation group, respectively (Fig. 2A, B). EF values of individual rats before cell injection and after the last GXDS treatment were shown in Table S1 in the supplemental materials. There was no statistically significant difference in LVEDV among the four groups (Fig. 2C). LVESV value in MSCs + GXDS formulation group was significantly lower than that of the saline group and GXDS formulation group (Fig. 2D).

3.3. GXDS formulation combined with MSCs transplantation decreased infarction area

Masson's trichrome staining of heart tissues was performed to detect the infarction size in each group at 4 weeks after transplantation. The red color observed under microscope indicated normal myocardium and blue indicated collagen fibers after staining. As can be seen in Fig. 3A, the myocardium was replaced by collagen robustly after MI in saline-injected rats. The infarction areas in the MSCs group and the GXDS formulation group were smaller than that in the saline group, but the difference was not statistically significant. The rats treated with MSCs appeared a similar infarction area to the ones administrated with GXDS formulation. However, GXDS formulation combined with MSCs transplantation significantly reduced the infarction area, suggesting that MSCs and GXDS formulation played a synergistic role in reducing and preventing the myocardial fibrosis, and promoting myocardial repair (Fig. 3B). The heart weight and body weight of the rats were

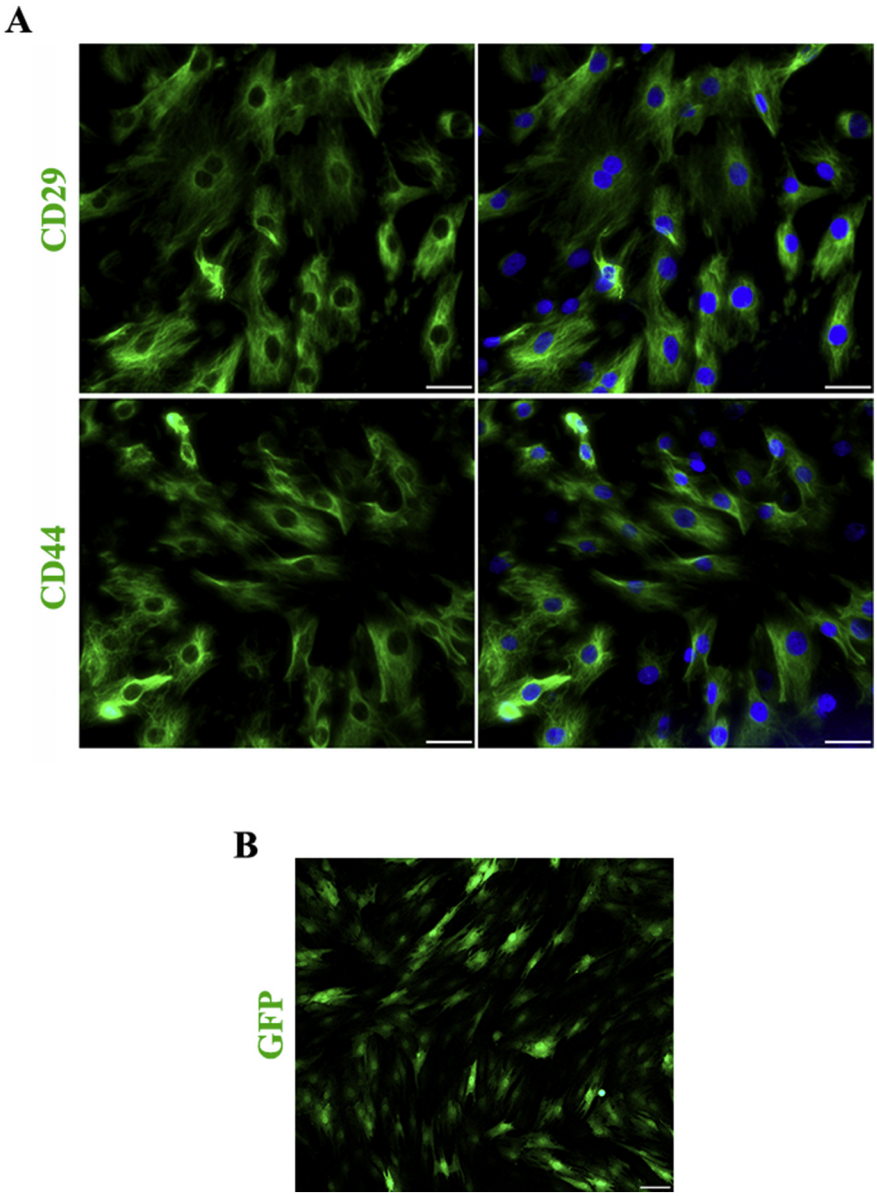

Fig. 1. The characteristic of cultured MSCs and GFP labeling. (A) Most of the MSCs we cultured expressed CD29 and CD44. Scale bar: 25 μm. (B) MSCs were labeled with GFP by lentivirus with high infection efficiency. Scale bar: 50 μm.

weighed respectively to calculate the ratio of heart weight to body weight (HW/BW). The data in the group of MSCs or GXDS formulation was much lower than that in the group of saline. There was no significant difference between MSCs + GXDS group and MSCs or GXDS formulation alone (Fig. 3C).

3.4. GXDS formulation combined with MSCs transplantation reduced cell apoptosis

Three days after MSCs transplantation, cell apoptosis in the infarcted area was detected by TUNEL staining (Fig. 4A). The number of apoptotic cells was significantly reduced after MSCs transplantation or GXDS formulation treatment. Moreover, the number of apoptotic cells in the MSCs + GXDS formulation group was significantly less than that of the MSCs transplantation group or GXDS formulation group, suggesting that the MSCs group or the GXDS formulation group had significantly lower anti-apoptotic effect than the MSCs + GXDS formulation combination group (Fig. 4B).

3.5. GXDS formulation combined with MSCs transplantation promoted angiogenesis

Improved microvascular perfusion of the lesion after MI was of great benefit to the survival of cardiomyocytes and improvement of cardiac function. The angiogenesis in the peri-infarction and infarction area of rat hearts was detected by immunofluorescence staining for CD31 at 4 weeks after transplantation (Fig. 5A). CD31 is a specific marker of vascular endothelial cells, and the detection of CD31 expression could reveal angiogenesis after treated with MSCs and GXDS formulation. As shown in Fig. 5B, the number of CD31-positive microvessels in both MSCs and GXDS formulation treated rats was significantly higher than that in the saline group. The number of microvessels in the rats received MSCs + GXDS formulation treatment was prominently elevated compared to that in the MSCs group or the GXDS formulation group, suggesting that the combined treatment of MSCs + GXDS formula is more beneficial for angiogenesis after MI.

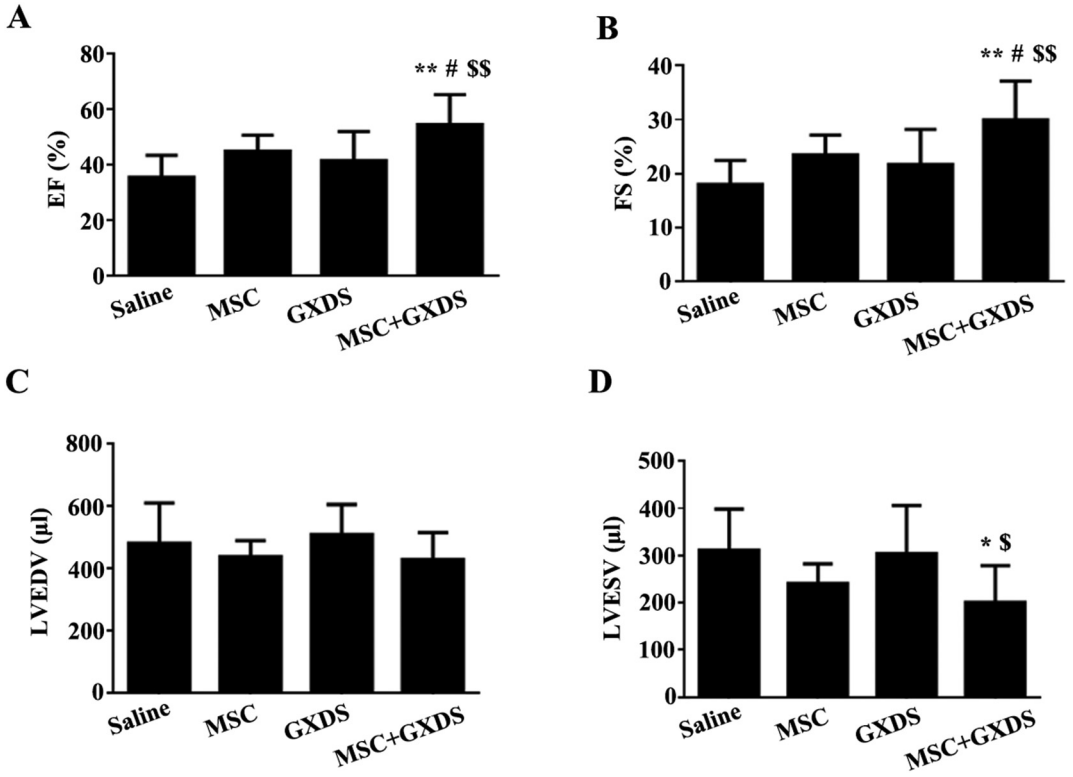

Fig. 2. GXDS formulation combined with MSCs transplantation improved cardiac function at 4 weeks after cell injection. (A-D) The parameters of EF, FS, LVEDV, and LVESV were compared among the different treatments. $^{*}P < 0.05$ and $^{**}P < 0.01$versus the saline group. $^{\#}P < 0.05$ versus the MSC group. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus the GXDS group. $n = 10$.

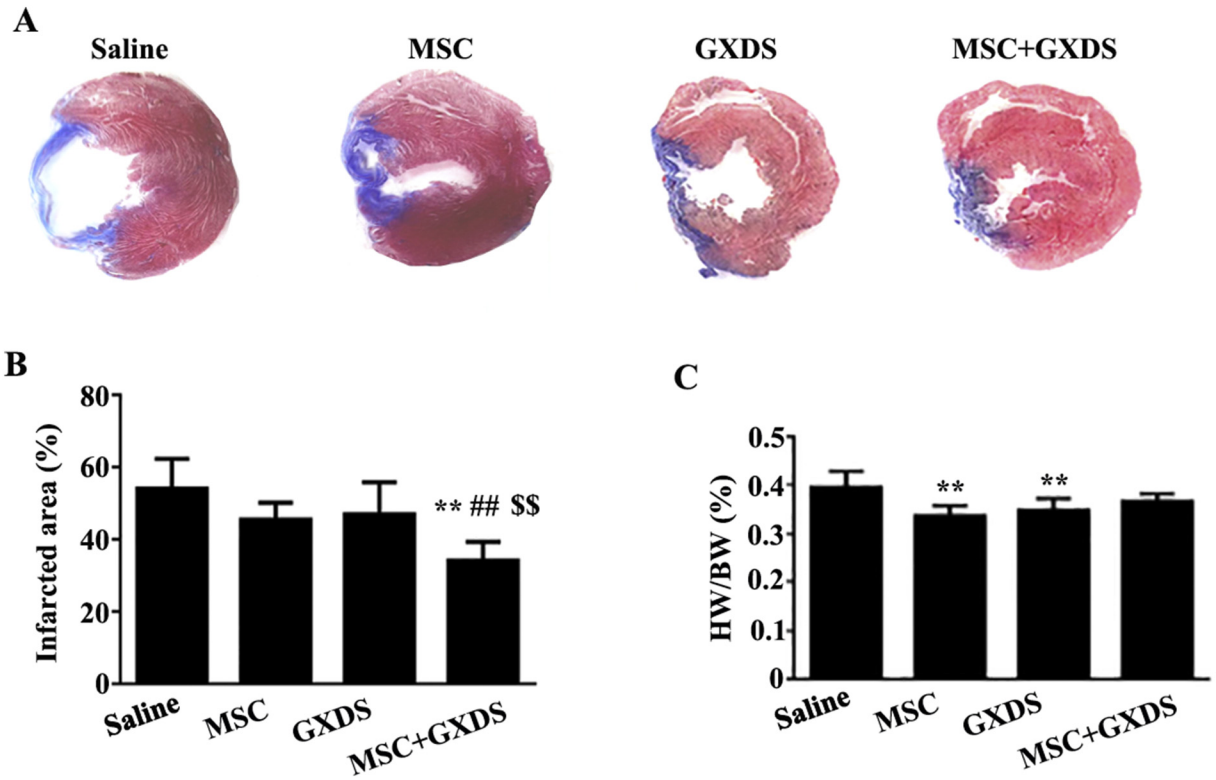

Fig. 3. GXDS formulation combined with MSCs transplantation reduced infarction area at 4 weeks after cell injection. (A) Masson's trichrome staining was performed to detect the infarction area in each group. (B) Quantitative analysis of myocardial infarct area. (C) Quantitative analysis of HW/BW. $^{**}P < 0.01$versus the saline group. $^{\#\#}P < 0.01$ versus the MSC group. $^{\$\$}P < 0.01$ versus the GXDS group. n = 10.

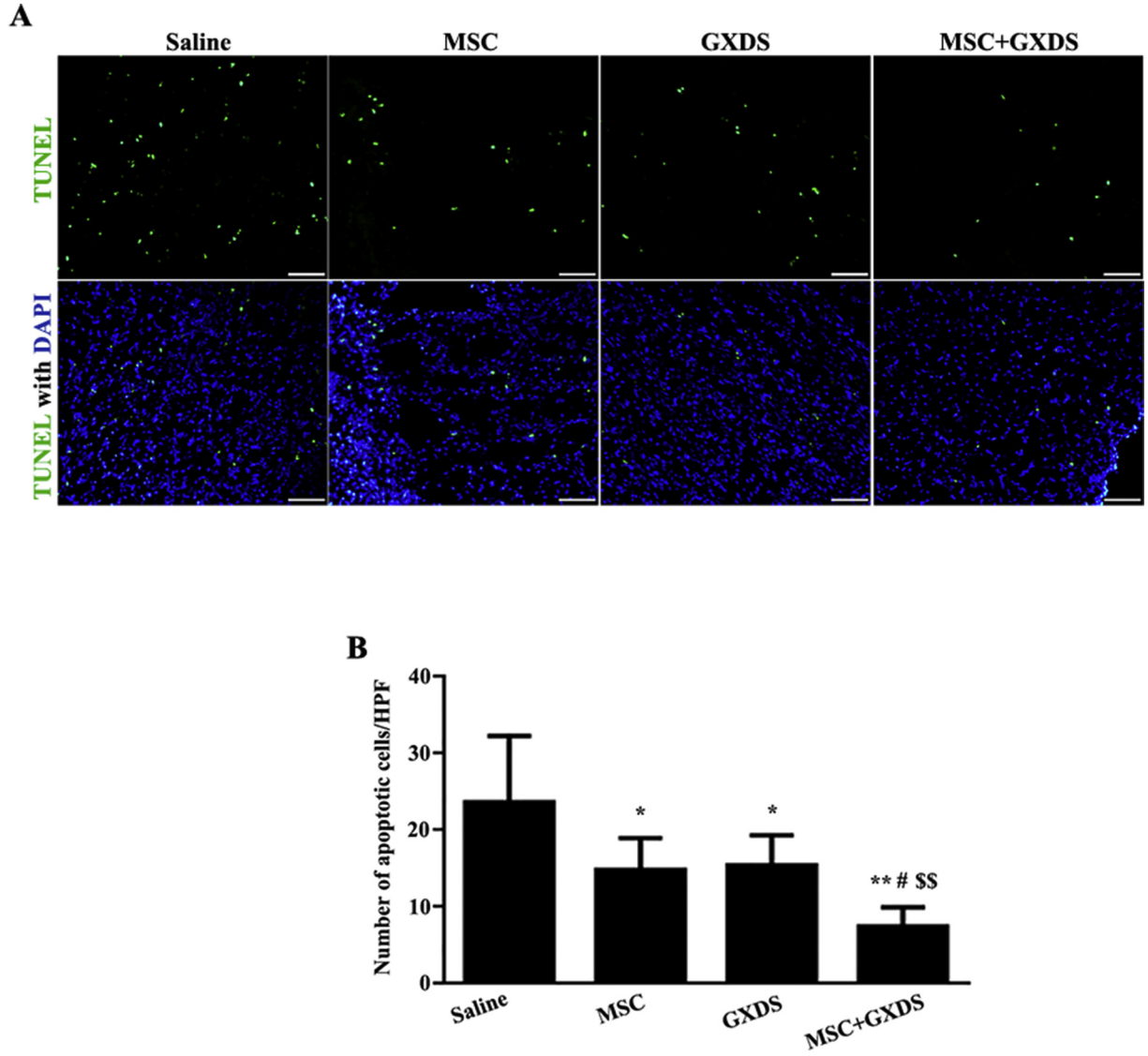

Fig. 4. GXDS formulation combined with MSCs transplantation alleviated cell apoptosis at 3 days after cell injection. (A) The number of apoptotic cells was detected by TUNEL staining. Scale bar: 50 μm. (B) The number of TUNEL-positive nuclei was calculated and analysed. $^{*}P < 0.05$ and $^{**}P < 0.01$versus the saline group. $^{\#}P < 0.05$ versus the MSC group. $^{\$\$}P < 0.01$ versus the GXDS group. $n = 3$.

3.6. GXDS formulation administration enhanced the engraftment of MSCs

Due to the lower number of transplanted cells in the heart after intravenous injection, we asked whether GXDS formulation could enhance the migration of MSCs towards the infarcted area. The presence of injected stem cells in the peri-infarction and infarction area were traced by GFP labeling at 3 days after injection. The number of transplanted MSCs that could be detected in the BMSCs group was very limited (Fig. 6A). Interestingly, the number of GFP-labeled stem cells in the MSCs + GXDS formulation group was significantly larger than that in the MSCs group (Fig. 6B), indicating that GXDS formulation promoted the engraftment of transplanted MSCs to the heart after intravenous administration.

3.7. GXDS formulation combined with MSCs transplantation increased the expression of SDF-1 in the infarcted area

To further elucidate the possible mechanism underlying the roles of GXDS formulation combined with MSCs transplantation for the treatment of MI and enhanced engraftment of MSCs, the expression of SDF-1 in the infarcted myocardium tissues was detected by Western blot at 4 weeks after transplantation (Fig. 7A). The expression of SDF-1 in the MSCs group was upregulated compared to that in the control saline group, but the difference was not statistically significant. Compared with the saline group, administration of GXDS or MSCs + GXDS obviously increased the level of SDF-1. Moreover, the MSCs + GXDS formulation combination group showed better promoted expression of SDF-1 than the GXDS formulation group (Fig. 7B). Since YAP is involved in cell proliferation and migration, the expression of YAP in the infarcted myocardium tissues was detected by Western blot. However, there was no significant difference in YAP expression between the four groups at 4 weeks after transplantation (Fig. 7C, D).

4. Discussion

Although stem cell-based therapy has emerged as a potential therapeutic strategy for MI patients, it still faces numerous problems and difficulties, and painstaking efforts are required to resolve these hurdles before translation into clinical application. In this study, we investigated the effect of GXDS formulation combined with MSCs transplantation against cardiac injury after MI. The findings demonstrated that administration of GXDS formulation promoted the migration of the

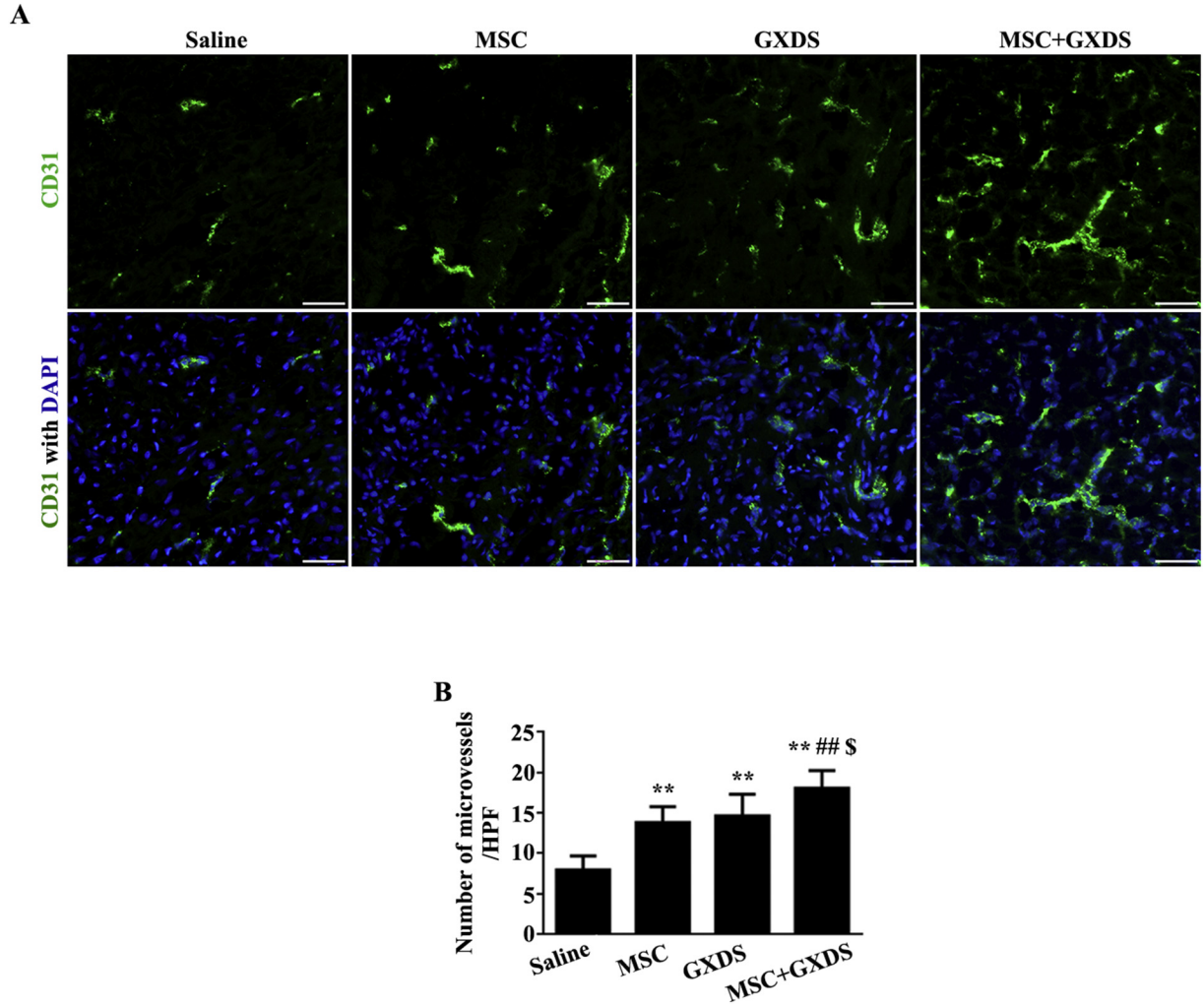

Fig. 5. GXDS formulation combined with MSCs transplantation promoted angiogenesis at 4 weeks after cell injection. (A) The blood vessels in the peri-infarction and infarction area of rat hearts was examined by immunofluorescence staining for CD31. Scale bar: 25 μm. (B) Quantification of microvessel density. $^{**}P < 0.01$versus the saline group. $^{\#\#}P < 0.01$ versus the MSC group. $^{\$}P < 0.05$ versus the GXDS group. n = 10.

injected MSCs into the infarcted region. GXDS formulation may also increase the survival of MSCs in the hostile microenvironment after engrafted into the heart as the number of MSCs in the group of MSCs + GXDS was profoundly higher than that in the MSCs group. However, labeling cells using dual reporter system such as Luciferase-GFP allows not only imaging in vivo, but also tracing transplanted cells in situ by immunohistochemistry.

It has been revealed that CDDP could improve the cardiac microenvironment by suppressing oxidative stress and inflammatory response after acute MI [11,15]. Since Salvia miltiorrhiza and Panax notoginseng are two key components of CDDP and GXDS formulation, we assumed that GXDS formulation could reduce oxidative stress and inflammatory response. However, superoxide dismutase activity, malondialdehyde content and inflammatory cytokines reflecting oxidative stress and inflammatory response warrant further investigation. This study demonstrated that GXDS formulation combined with MSCs transplantation not only reduced cell apoptosis, but also enhanced the angiogenesis. Moreover, the combined MSCs + GXDS formulation group had better cardiac function improvement than the MSCs group or the GXDS formulation group. Therefore, the use of GXDS to achieve synergistic therapy with MSCs transplantation will provide a potential solution for cardiac repair. However, 4-week assessment in vivo post MI is not enough to prove the functional efficacy since left ventricular remodeling can go on until 3–6 months post MI. Thus, it's important to examine the cardiac function for a longer duration after MI to further substantiate our hypothesis in the future, for example, at least 3 months.

There are two important approaches of stem cell transplantation: direct intramyocardial injection and intravascular injection, such as intravenous injection. Direct intramyocardial injection can cause secondary damage to the body, leading to arrhythmias, and the survival rate of stem cells injected into the myocardium is limited. Most stem cells died or were lost after transplanted into the myocardium, possibly due to cell loss caused by myocardial contraction, and may also be taken away by the blood circulation or flew out through pinhole [16]. Systemic delivery of stem cells provides a minimally invasive and clinically acceptable alternative. Intravenous transplantation of human umbilical cord blood MSCs improved cardiac function and protected infarcted myocardium [17]. Intravenously infused human multipotent stromal cells reduced inflammatory responses, reduced infarct size, and improved cardiac function [18,19]. However, some studies have shown that the effect of cardiac repair after MI by intravenous injection of stem cells is not significant. In animal experiments, intravenous infusion of adipose-derived stem cells or MSCs showed minor improvement in cardiac function [20–22]. Furthermore, a randomized, double-blind, phase I/II study of intravenous allogeneic MSCs in patients with acute MI showed that intravenous injection of allogeneic MSCs was safe and well tolerated. However, at 6 months, EF and infarct volume were not

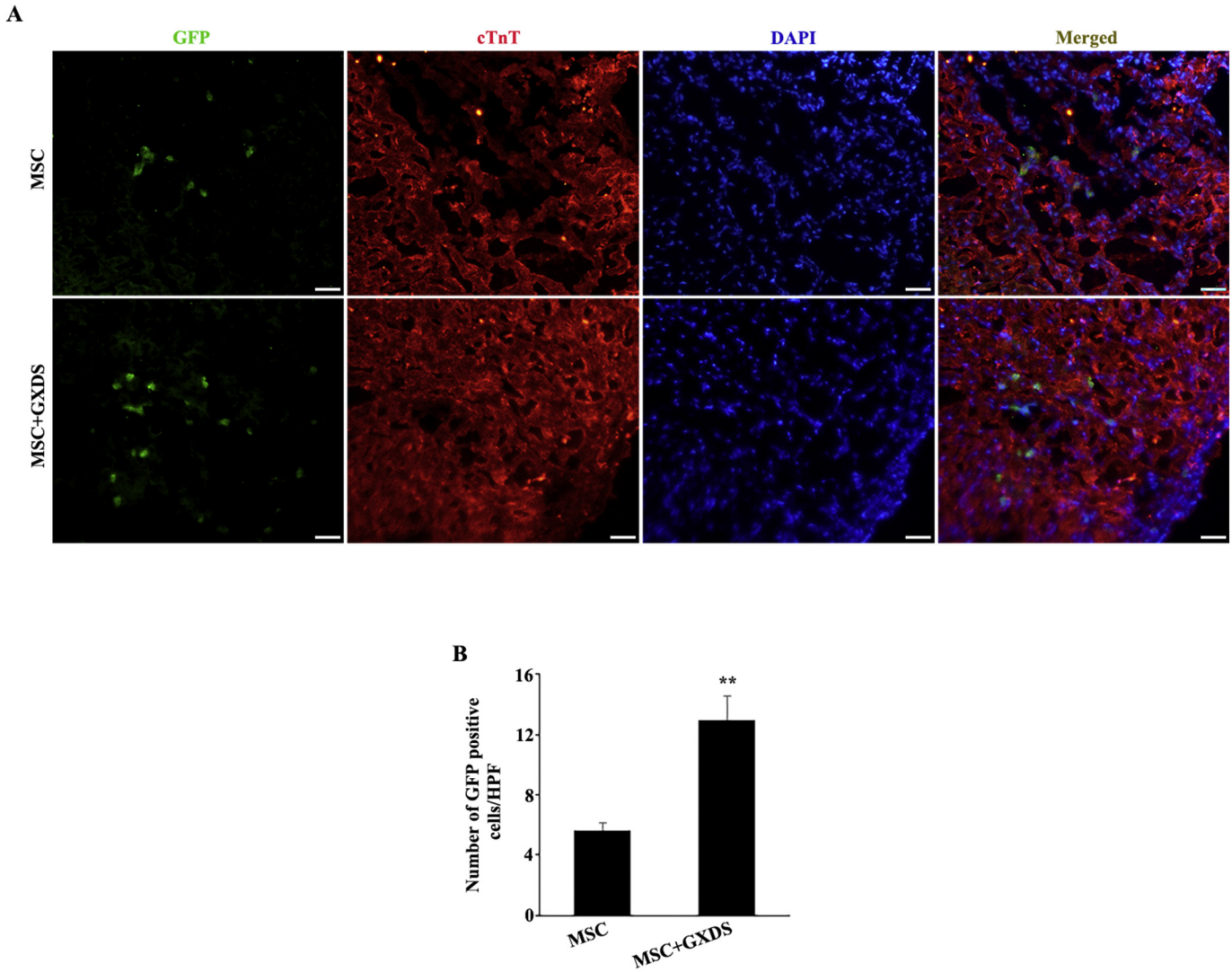

Fig. 6. GXDS formulation combined with MSCs transplantation promoted the engraftment of MSCs at 3 days after cell injection. (A) The engraftment of injected stem cells in the peri-infarction and infarction area was traced by GFP under fluorescence microscope. The GFP positive cells did not express cTnT and were located between or around the cardiomyocytes. Scale bar: 25 μm. (B) The number of GFP positive MSCs was counted and compared. $^{**}P < 0.01$versus the MSC group. n = 3.

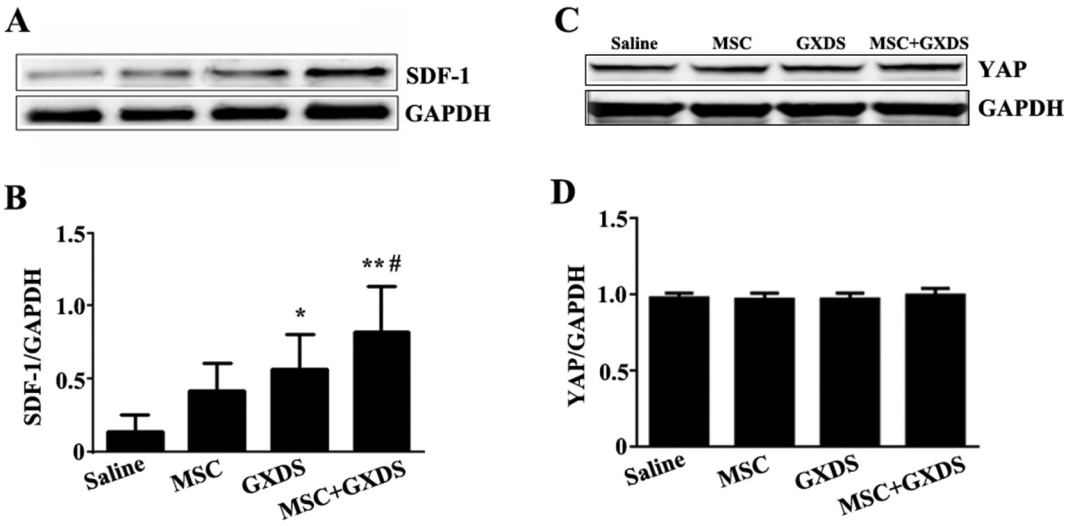

Fig. 7. GXDS formulation combined with MSCs transplantation increased the expression of SDF-1, but not YAP, in the infarcted region at 4 weeks after cell injection. (A) The expression of SDF-1 was detected by Western blot. (B) The semiquantitative data of Western blots for SDF-1. (C) The expression of YAP was detected by Western blot. (D) The semiquantitative data of Western blots for YAP. $^{*}P < 0.05$ and $^{**}P < 0.01$versus the saline group. $^{\#}P < 0.05$ versus the MSC group. n = 10.

significantly different between BMSCs and placebo groups [23]. Our data also showed that MSCs transplantation through intravenous injection had no significant effect on recovery of heart function after MI. One of the major problems of stem cell therapy with intravascular injection is a lack of engraftment of sufficient stem cells at the site of injury. Technologies to improve the migration of stem cells to the infarcted heart include modification of a targeting antibody on the microbubbles, overexpression of CXCR4 or miR-211 [20–22]. Although GXDS were administered before inducing MI, the difference of EF values among the groups was not obvious before cell injection, which indicated that all of the rats were in the similar conditions of myocardial injury and the short period of GXDS administration before cell transplantation didn't affect the heart function significantly. Our study provided a more feasible and accessible strategy for promoting migration of stem cells after intravenous injection by oral administration of GXDS formulation.

GXDS formulation consisted of Salvia miltiorrhiza, Panax notoginseng and Lignum Dalbergiae Odoriferae. The composition of the prescription is reasonable, the clinical efficacy is exact, has been listed in the Chinese Pharmacopeia. Among them, Salvia miltiorrhiza has mainly two types of active ingredients: the water-soluble ingredient known as salvianolic acid B, and fat-soluble ingredient known as tanshinone IIA. Salvianolic acid B has strong anti-oxidant activity in vitro and is capable of removing superoxide anions, and tanshinone IIA in Salvia miltiorrhiza protects H_2O_2 and doxorubicin-induced cardiomyocyte injury in neonatal rats [24,25]. We also found that transplantation of salvianolic acid B pretreated MSCs improved cardiac function in rats with MI through angiogenesis and paracrine mechanisms [5]. The main active components of Panax notoginseng include Ginsenoside Rb1 (Rb1), ginsenoside Rg1 (Rg1), and notoginsenoside R1 (R1). Ginsenoside Rb1 was reported to exert the protective effect on injured cardiomyocytes by increasing the activity of SOD and inhibiting the levels of MDA and ROS [26]. A recent study indicated that Salvia miltiorrhiza and Lignum Dalbergiae Odoriferae exerted synergistic therapeutic efficacies to improve electrocardiogram results and heart rate, reduce the heart weight index and myocardial infarct size [27]. However, in our study, the improvement of heart function after administration of GXDS formulation was moderate, which may be attributed to the its dosage. In a previous study, the authors found that only rats treated with high doses of the GXDS formulation showed a significant increase in cardiac function, rather than low and moderate doses, according to echocardiography [10]. Another factor that should be taken into account is whether the absorption and metabolism of active ingredients mentioned above could be successfully achieved after oral administration of the compound GXDS formulation. Consequently, it is valuable to clarify the content levels of the different active ingredients in GXDS formulation after in vivo administration.

MSCs are capable of efficient migration to injured tissues after intravenous injection, suggesting that MSCs express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration to sites of injury [28]. SDF-1, a member of the secretory CXC chemotactic protein superfamily, a chemokine that plays a major role in cell trafficking and homing of stem cells [29]. CXCR4 is a G protein-coupled receptor with seven transmembrane structures consisted of 352 amino acids, and is expressed on the cell surface and is also detected in the cytoplasm [30]. The SDF-1/CXCR4 signaling pathway is involved in mediating the mobilization of stem cells from bone marrow, transportation across the intervascular endothelial space, and even stimulating the angiogenesis after tissue injury [13,31,32]. SDF-1/CXCR4 signaling plays a crucial role in the recruitment of bone marrow-derived cells to the heart after MI and can further increase homing in the presence of injury [33]. In mice with a lack of CXCR4 expression, the effect of bone marrow MSC transplantation on MI was significantly reduced [34]. However, the level of SDF-1 was decreased after 7 d in MI due to the degradation by matrix metalloproteinases [35]. The experimental results in the present study showed that the combination of GXDS formulation and MSCs transplantation further improved the expression level of SDF-1 in the infarcted tissues. Furthermore, the number of GFP-labeled MSCs in the MSCs + GXDS formulation group was significantly higher than the MSCs group, which might be related to higher expression of SDF-1. However, whether SDF-1/CXCR4 signaling pathway plays a role and what role it plays in the promotion of the transplantation of MSCs to treat MI with GXDS formulation remains to be studied.

A growing body of evidence suggests that vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were upregulated after MI and that these molecules have an important role in stem cell recruitment [28,36,37]. Therefore, it would be interesting to verify the expression of these chemoattractant cytokines after GXDS formulation combined with MSCs transplantation therapy. In addition, the transcriptional co-activator YAP is a key driver of cell proliferation and survival. We previously found that YAP expression in the heart was decreased with age [38]. In an experimental mouse MI model, cardiac-specific YAP activation improved cardiac function and increased survival [39]. However, GXDS combined with MSC transplantation had no significant effect on YAP levels after MI. Collectively, the exact mechanism of GXDS combined with MSCs transplantation for MI remains to be further elucidated.

In conclusion, the combination of GXDS formulation and MSCs transplantation can improve cardiac function, promote angiogenesis, and increase migration and survival of MSCs after MI. The effects of GXDS formulation combined with MSCs transplantation were superior to the MSCs or GXDS formulation alone. This study also suggested that the SDF-1/CXCR4 signaling pathway may play a role in promoting the migration and recruitment of MSCs after intravenous injection, and improving cardiac repair after treatment with GXDS formulation combined with MSCs transplantation may be attributed to the enhanced engraftment and survival of MSCs. The combination of traditional Chinese medicine GXDS formulation and stem cell therapy has practical significance and application prospects in the treatment of ischemic cardiomyopathy such as MI.

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Authors' contributions

FM and HG conceived and designed the research. XH performed most experiments and animal works. HL, CL, QW and ZL assisted some experiments and result analysis. XH and HG drafted and wrote the manuscript.

Declaration of competing interest

The authors have no conflicts of interest to disclose.

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