AMLS Archives of Medical Laboratory Sciences

Original Article

Effects of Mesenchymal Stem Cell Transplantation in Combination with Heparin on Acute Myocardial Infarction in a Rabbit Model

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Received: May 1, 2021; Accepted: May 22, 2021

Abstract

Background and Aim: Background: Stem cell transplantation, combined with some bioactive substances, has revealed promising outcomes in treating cardiac tissue damage caused by myocardial infarction (MI). In the present study, we evaluated the beneficial consequence of mesenchymal stem cells (MSCs) transplantation combined with heparin on heart damage within infarcted rabbits.

Methods: Twenty-eight male New Zealand white rabbits were randomly distributed into four groups: control, MI, MI+ MSCs, and MI+MSCs+ heparin. Functional parameters of the left ventricle through echocardiography, lesion area through Macro trichrome evaluation, and angiogenesis through Masson's trichrome staining were compared between groups.

Results: Ejection fraction and fractional shortening were improved in MI +MSCs and MI+ MSCs + heparin group compared to the MI group ($P<0.05$). The lesion area was significantly reduced, and angiogenesis was markedly increased in MI +MSC + heparin treated animals compared to MI and MI +MSCs groups.

Conclusion: Although MSCs injection to infarcted area restored normal heart function, we concluded that in the infarcted region of animals, MSCs injection combined with exogenous heparin could have more effects on the left ventricle functional parameters, cardiac lesions, and new vessel formation.

Keywords: Mesenchymal Stem Cells; Angiogenesis; Heparin; Myocardial Infarction.

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Please cite this article as: Hajigol S, Moradi Gardeshi T, Safizadeh F, Khosh Ravesh R, Abbasi M. Effects of Mesenchymal Stem Cell Transplantation in Combination with Heparin on Acute Myocardial Infarction in a Rabbit Model. Arch Med Lab Sci. 2021;7:1-9 (e19). https://doi.org/10.22037/amls.v7.34932

Introduction

Cardiovascular diseases (CVDs) constitute the main cause of global premature deaths and are a prominent contributor to decreased quality of life. The World Health Organization (WHO) has reported that CVD led to approximately 17.8 million deaths worldwide in 2017 (1). According to the global burden of diseases (GBD), CVDs were responsible for 46% of all Iranian deaths (2). CVDs are mentioned to a group of disorders such as myocardial infarction (MI), heart failure, and several vascular conditions that affect the heart and vasculature (3, 4). After birth, myocardial cells lose their ability to proliferation, and in MI, these cells are loosed and replaced with scar tissue, which results in irreversible damage to the structure and electromechanical function of the heart (5). Despite therapeutic approaches in cardiac repair following MI, including bypass surgery, the treatment and

management remain a major challenge to public health. Since both vasculature and myocardial tissue are excessively damaged during MI, novel therapeutic advances should simultaneously target both (5-7).

The last two decades have dramatically developed modern regenerative medicine strategies for cardiac regeneration (8-11). Growing evidence shows that stem cell-based therapy has emerged as a promising strategy to repair damaged myocardial tissue in animal models and clinical trials following an acute infarction (12-15).

Various stem cells, including embryonic stem cells and adult stem cells, have been studied in heart disease and have shown recovering effects. Although they act their roles through several mechanisms, cell therapy's main objectives are vascularization, prevention of cardiomyocyte apoptosis, and improved heart electromechanical function (16-18).

One of the most widely engaged stem cells is mesenchymal cells originated from bone marrow. These cells can migrate and differentiate into different cell types under special circumstances, including cardiomyocytes, endothelial cells, and smooth muscle cells (19-21).

Some clinical studies have illustrated that stem cell therapy, combined with various bioactive compounds, has affirmative impacts in restoring the normal structure and function of damaged cardiac tissue (22, 23).

Heparin sulfate is a ubiquitous component of the extracellular matrix and basement membrane and has a wide range of structures and functions. The heparin sulfate chain has been shown to participate in angiogenesis's biological processes (24-27). Heparin-binding vascular endothelial growth factor (VEGF) cooperates in angiogenesis prompted by hypoxia, inflammation, and tumor growth. Studies have also shown that heparin administration during left ventricular hypertension increases fibroblast growth factor (FGF)2 and VEGF in the left ventricle and increases blood flow capacity and capillary density (28-30).

In line with the studies, we decided to use heparin family drugs to increase the angiogenic capacity of bone marrow MSCs in MI treatment.

Methods

Animal model

This investigation follows the "Guide for the Care and Use of Laboratory Animals," published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011). All human sample collections, animal care, and experimental protocols handled in this study were under ethical permissions approved by the Iran University of Medical Sciences' ethical committee. In this study, twenty-eight New Zealand white rabbits with a weight ranging from 2500 to 3000 g were purchased from Razi Vaccine and Serum Research Institute. All animals were housed in controlled temperature conditions (22±2°C) and 12 hours' dark-light cycles. Animals had free access to standard laboratory feed temperature conditions randomly distributed into four groups, with seven animals in each group.

1) control group (normal rabbits): 150 µl culture media were injected into animals intramyocardially.

2) MI-induced rabbits: MI was induced, and animals did not receive any injection.

3) MI+MSCs group: 150 µl MSCs were injected intramyocardially into the infarcted area.

4) MI+MSCs + heparin group: 150 µl MSCs were injected intramyocardially into the infarcted area, followed by subcutaneous heparin injection (200 U/Kg for 28 days).

Induction of MI

Animals were anesthetized with 35 mg/kg ketamine and 5 mg/ kg xylazine intramuscularly. Under the sterile condition, a left intercostal thoracotomy was performed between two and three intercostal spaces. Then, the left anterior descending (LAD) was ligated between the first and second diagonal branches of the coronary artery with a 6.0 chromic suture. Following coronary blood flow occlusion, a pale color in the distal myocardium was observed after ligation. This color change confirmed that a heart attack has occurred.

Human mesenchymal stem cell (hMSCs) isolation and culture

Human bone marrow samples were obtained from Shariati Hospital (Tehran University of Medical Sciences, Iran). The cells were cultured and grown in a culture medium with 20% fetal bovine serum

(FBS) and 1% penicillin + streptomycin and incubated in an incubator at 37 0C with 5% CO2. After 24 hours, the mesenchymal cells adhered to the bottom of the flasks. After 60% of confluency, adhered cells were trypsinized and harvested for subculture. 150 µl of cell culture medium containing 106 MSCs cells was injected intramyocardially to rabbits' infarcted area in MSCs and MSCs + heparin groups.

Flow cytometry assay

After 80% confluency, human MSCs were trypsinized and detached from flasks, centrifuged (at 900 ×g for 7 min). The pellets were resuspended in 500 µl of PBS and incubated with appropriate antibodies (1 hour in the dark) for reading with a flow cytometer (Partec, Germany). The applied MSC-specific antibodies were included anti-CD105, anti-CD90, anti-166. Also, anti-CD44 and anti-CD45, and anti-CD34 were used as negative markers.

Measurement of the left ventricle (LV) functional activity

Functional parameters of LV, including ejection fraction (EF), Left-ventricular end-diastolic dimension (LVEDD), and fraction shortening (FS), were analyzed by echocardiography.

Histological evaluation

The isolated heart tissues were kept in 4% formaldehyde solution for seven days. Then the paraffin block is prepared according to the protocol by Tissue Processor. Tissue sections were made with a microtome, 7 microns thick, and were placed on a slide. For Masson's trichrome staining, the prepared slides were stained with Masson's trichrome for angiogenesis evaluation.

Statistical analysis

All results were analyzed using the SPSS version 15.0 (SPSS Inc. Chicago, IL, USA). ANOVA test and Tukey (post hoc) were performed to compare case results with the control group. Kruskal-Wallis and Mann-Whitney tests were used to compare MSCs+heparin results with MSCs results. All study data were displayed as mean ± standard deviation (SD), and $P<0.05$ was used as statistically significant.

Results

Bone marrow MSCs were firstly prepared and cultured (Figure 1). After obtaining the MSCs, heart surgery was implemented to create a MI model in rabbits, and 150 µl cultured cells followed with heparin (200 U/Kg) were used for treatment.

Flow cytometry finding

For the characterization of MSCs extracted from bone marrow, we employ a flow cytometry technique to investigate specific markers of mesenchymal cells compared to hematopoietic cells. The flow cytometric findings displayed that many cultured cell amounts expressed mesenchymal markers, including CD105 (99.5%), CD90 (98.8%), CD44 (96.8%), and CD166 (93%). In contrast, hematopoietic markers' expression rate, including CD45 and CD34, was 3.8% and 2.8%, respectively. These results confirm MSCs quality and hematopoietic cell removal after MSCs isolation from bone marrow (Figure 2).

Effect of heparin and MSCs on hemodynamic parameters

FS, EF, and LVEDD were compared after induction of MI and at 4 and 8 weeks after treatment (Table 1). After MI, LVEDD did not change between groups. The difference between any of the groups was not significant. (Table 1). MSCs transplantation significantly improved the EF and FS in 4 and 8 weeks after treatment. All these beneficial impacts of MSCs were significantly increased in combination with heparin injection.

Histological analysis

Angiogenesis in the heart tissue was measured 8 weeks after treatment by Masson's trichrome staining. These findings revealed that angiogenesis was significantly higher in MI+MSCs and MI+MSCs+heparin-treated rabbits than MI group. The histological evaluation also showed that the amount of angiogenesis was significantly higher in MI+MSCs+heparin than in the MI+MSCs group (Figure 3). Angiogenesis was measured in a unit of surface (µm2). The mean number of counted new vessels in the MI group and MSCs and MSCs+heparin receiving groups were 5.8±0.67, 19.6±1.34, and 26±0.71, respectively (Figure 4).

Macro-trichrome studies for measuring the lesion's level

Macro-trichrome studies were examined under a microscope using white light. Lesion levels were assessed in the study groups. The lesion level in the macro-trichrome study in the studied groups showed that the MI group's lesion level was significantly different from MSCs and MSC+heparin groups. The lesion level in the sham group and MSCs and MSCs+heparin groups was 25047.2±911.42, 14509.4±588.08, 11065.6 ± 756.68, respectively.

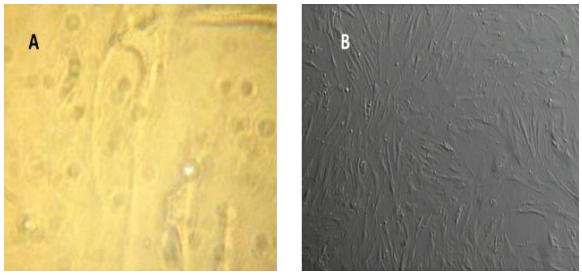

Figure 1. (A) Bone marrow stem cells. (B) Human bone marrow-derived cells in the fifth passage.

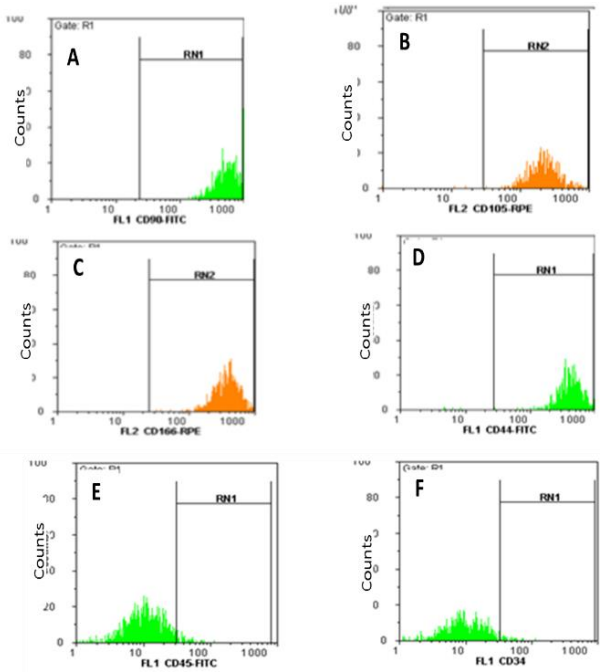

Figure 2. Flow cytometry diagram of A) CD90, B) CD105, C) CD166, and CD44 mesenchymal antibodies and E) CD45 and CD CD34 hematopoietic antibodies.

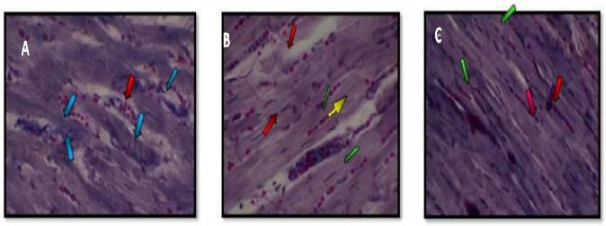

Figure 3. Comparison and evaluation of angiogenesis in the study groups by using Mason's trichrome staining after 8th week. (A) Fibrous tissue of the heart after ischemia. (B) Angiogenesis is well seen in the BMSC-treated group. (C) Angiogenesis is seen in the BMSC + heparin group. Yellow arrow: collagen; blue arrow: fibrous tissue; red arrow: mature blood cells; green arrow: blood vessel.

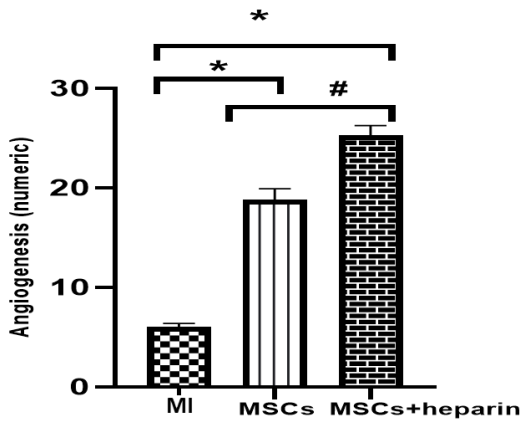

Figure 4. Comparison of angiogenesis in Masson's trichrome staining. *Significant differences with the MI group, #significant difference between MSC group and MSC + heparin. MSC: mesenchymal stem cell.

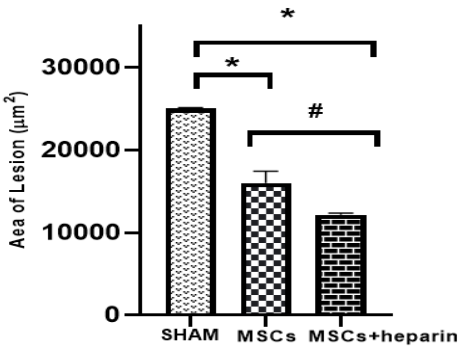

Figure 5. Comparison of lesion levels in macro-trichrome evaluation. *Significant differences with the MI group, # significant difference between MSC group and MSC+heparin. MSC: mesenchymal stem cell.

Table 1. Comparisons of cardiac functional parameters between study groups.

		Control	MI	MSCs	MSCs + heparin	p-value
EF%	After MI	69.05 ± 0.012	48.1 ± 0.01	53.2 ± 0.09	52 ± 0.016	< 0.05
	4 weeks	69.85 ± 0.09	49.89 ± 0.014	55 ± 0.05	54.9 ± 0.06	< 0.05
	8 weeks	71.02 ± 0.012	45.9 ± 0.03	55.6 ±0.04	55.3 ± 0.015	< 0.05
FS	After MI	44.23 ± 0.14	20.02 ± 0.001	25.23 ± 0.02	28.24 ± 0.03	< 0.05
	4 weeks	43.23 ± 0.07	22.09 ± 0.032	23.04 ± 0.04	26.12 ± 0.08	< 0.05
	8 weeks	43.86 ± 0.85	23.95 ± 0.04	27.4 ± 0.41	30.01 ± 0.07	< 0.05
LVEDD	After MI	13.36 ± 0.01	12.1 ± 0.12	11.9 ± 0.03	11.6 ± 0.31	> 0.05
	4 weeks	14.23 ± 0.14	13.8 ± 0.15	12 ± 0.09	12.1 ± 0.01	> 0.05
	8 weeks	13.36 ± 0.45	12.4 ± 0.02	13.8 ± 0.01	12.4 ± 0.25	> 0.05

Comparisons of cardiac functional parameters were evaluated by echocardiography between study groups after MI induction and at 4 and 8 weeks after treatment. A one-way ANOVA test was applied for data analysis. All results were presented as mean ± standard deviation (SD), and $P<0.05$ was considered statistically significant. Abbreviations: LVEDD: Left Ventricle End Diastolic Dimension, FS: fractional shortening, EF: ejection fraction, MI: myocardial infarction, MSCs: mesenchymal stem cells.

Discussion

In this study, MI was induced in a rabbit model, and then the effect of MSC infusion alone and with heparin on hemodynamic and histological parameters was investigated. Heparin significantly improved the beneficial effect of MSCs on angiogenesis induction and hemodynamic parameters improvement after MI.

Several studies have represented the beneficial impacts of originated-bone marrow MSCs therapy in MI, neurological, and kidney disorders (31-34). The susceptibility of MSCs to regenerate cardiac damage following ischemic myocardium in animal and clinical results is a step forward in ameliorating cardiac cell therapy as a relevant therapeutic approach. Further investigations will most likely have required to find more practical strategies to augment and facilitate stem cell impacts on myocardial regeneration (35-37). Most clinical trials have shown that 4 to 6 months after stem cell transplantation, LVEF increases about 7 to 9 percent, systolic end volume decreases, blood flow and angiogenesis improve in the infarcted area (11, 38-40). In a clinical investigation, Peter Dandruff and his colleagues showed that patients who underwent coronary artery bypass graft (CABG) surgery with MSCs injection had a better ventricular ejection index or EF than patients who had only extracellular bypass surgery without stem cell therapy (41). The present study results indicated that bone marrow MSCs transplantation for cardiac repair improved EF and FS in infarcted rabbits, in agreement with previous studies.

Bone marrow MSCs possess a high potential for regeneration of cardiac performance since engrafted MSCs secrete different biological factors, including platelet-derived growth factor (PDGF), stem cell-derived factor (SDF), VEGF, and matrix metalloproteinase (MMPs) in a paracrine manner. These factors contribute to angiogenesis induction, extracellular matrix remodeling, and stem cell recruitment (42, 43). The paracrine secretion of VEGF, a proangiogenic factor, promotes angiogenesis induction and vascular regeneration and also impairs the apoptotic pathway (44, 45). Tang YL et al. reported that cardiac performance improvement in MSCs therapy might be related to the self-renewal capacity of transplanted MSCs that can retain angiogenesis and cardiomyocyte recovery through VEGF protein secretion (14, 46). Our previous finding showed that heparin, combined with MSCs therapy, improves stem cells' actions in the VEGF secretion and cardiac tissue restoration after infarction (47). Heparin is an anticoagulant factor and can bind to VEGF and finally enhance this angiogenic factor's affinity to its receptor (48). So, it seems that heparin is a beneficial option to increase the effect of stem cell transplantation for angiogenesis induction and cardiac tissue regeneration. The combination of exogenous heparin with MSCs transplantation therapy into an infarcted rabbit model was investigated in the

current study. The histological analysis confirmed that new vessel formation was increased in the ventricular myocardium of animals who received MSCs + heparin compared to MSCs alone. Histological evaluations also highlighted increased angiogenesis amount and decreased lesion area in the MSCs group compared to MI and in the MSCs+ heparin group compared to MSCs alone. According to previous studies, the recovery velocity of the injured myocardium can be influenced by the MSCs differentiation into myocardiocytes, increased angiogenesis in the damaged heart tissue, and paracrine secretion of growth factors led to increased tissue repair at the injury area.

Conclusion

Overall, these data demonstrated that MSCs therapy combined with exogenous heparin administration could induce angiogenesis and reduce the lesion area after cardiac injury. Although this study examined the combined effects of MSCs and heparin on hemodynamic parameters and angiogenesis, it did not address other MSCs and heparin effects on damaged heart tissue. Therefore, in later studies, it is recommended that other functional aspects of MSCs and heparin be investigated to improve infected tissue.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this work.

Acknowledgment

The Department of Physiology of Iran University of Medical Sciences would be greatly acknowledged for their support.

Funding/Support

The present research was not supported by any funding organization of the commercial, nonprofit or public sectors.

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