Original Article

Left Ventricular Geometry and Angiogenesis Improvement in Rat Chronic Ischemic Cardiomyopathy following Injection of Encapsulated Mesenchymal Stem Cells

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Received: 25/January/2022, Accepted: 14/June/2022

Abstract

Objective: Injection of hydrogel and cells into myocardial infarction (MI) patients is one of the emerging treatment techniques, however, it has some limitations such as a lack of electromechanical properties and neovascularization. We investigated the therapeutic potential of new electroactive hydrogel [reduced graphene oxide (rGO)/Alginate (ALG)] encapsulated human bone marrow mesenchymal stem cells (BMSCs).

Materials and Methods: The experimental study involved ligating the left anterior descending coronary artery (LAD) in rat models of chronic ischemic cardiomyopathy. Echocardiograms were analyzed at 4 and 8 weeks after MI treatment. In the eighth week after injection in the heart, the rats were sacrificed. Histological and immunohistochemical analyses were performed using Hematoxylin and Eosin (H&E) staining, Masson's trichrome staining and anti-CD31 antibody to analyze tissue structure and detect neovascularization.

Results: In comparison to the control and other treatment groups, MSCs encapsulated in rGO-ALG showed significant improvements in fractional shortening (FS), ejection fraction (EF), wall thickness and internal diameters ($P<0.05$). The morphological observation showed several small blood vessels formed around the transplantation site in all treated groups especially in the MSC-ALG-rGO group 8 weeks after the transplantation. Also, Masson's trichrome staining indicated an increased amount of collagen fibers in rGO-ALG-MSC. Microvessel density was significantly higher using MSC-ALG-rGO compared to controls ($P<0.01$).

Conclusion: This study demonstrates that intramyocardial injection of rGO/ALG, a bio-electroactive hydrogel, is safe for increasing LV function, neovascularization, and adjusting electrical characteristics following MI. The results confirm ALG promising capability as a natural therapeutic for cardiac regeneration.

Keywords: Alginates, Cell Therapy, Encapsulation, Graphene Oxide, Mesenchymal Stem Cells

Cell Journal(Yakhteh), Vol 24, No 12, December 2022, Pages: 741-747

Citation: Karimi Hajishoreh N, Baheiraei N, Naderi N, Salehnia M, Razavi M. Left ventricular geometry and angiogenesis improvement in rat chronic ischemic cardiomyopathy following injection of encapsulated mesenchymal stem cells. Cell J. 2022; 24(12): 741-747. doi: 10.22074/CELLJ.2022.557257.1040.

Introduction

Cardiovascular disease remains the main cause of death worldwide. In the United States, 86.2 million individuals had some type of cardiac disease in 2008, which is predicted to increase to 40.5 percent of the American population by 2030 (1).

Myocardial infarction (MI), made by blood flow blockage of the cardiac coronary arteries, is one of the most serious diseases with a high death ratio around the world (2). MI results in wall thinning, fibrosis, left ventricular (LV) dilation, and reduced cardiac function (3).The adult heart's inherent capacity to self-regenerate following myocardial damage is a major constraint in the treatment of cardiovascular disease. In the course of LV remodeling, structural and functional weakening of the LV occurs, leading to deteriorating clinical symptoms, exercise intolerance, and eventually death of the afflicted patient (4).

Despite advancements in pharmacological and interventional therapies, MI-related morbidity and mortality continue to increase (5). Transplantation of mesenchymal stem cells (MSCs) is a promising approach to repair damaged heart tissue after MI. Particularly, paracrine impacts of the transplanted MSCs play important roles in heart regeneration through the secretion of many growth factors and immune-modulatory cytokines.

Although cardiac cell injection has been shown to enhance heart function, there are still certain limitations that need to be addressed before their clinical application (6). Since the transplanted cells are subjected to high shear stress, caused by the injection and the harsh post-infarction

environment with high oxidative stress, their viability and efficacy remain low (7). One of the most significant issues associated with cell injection is the low engraftment rate as most of the cells are lost to the vasculature or leaking out of the injection site (8, 9). To overcome this problem, injected cells can be supported by being delivered with a biomaterial matrix. It has been stated that delivery of a proper material with cells or growth factors can be a more effective method to restore cardiac function compared with injecting materials or cells alone (6, 9, 10). In fact, injectable biopolymers allow the host body to perform as a bioreactor and recreate the injured tissue in situ (3).

Among biopolymers, which have been utilized for MI treatment, alginate (ALG) has been shown to improve LV function in animal models (3, 11). For example, it has been shown that intramyocardial injection of ALG in dogs with chronic heart failure could increase LV thickness and recover LV structure and function (11). Apart from decreasing wall stress that underlies the benefits of ALG therapy, an increase in LV wall thickness and reduction of LV size are other mechanisms, which can lead to a reduced end-diastolic length of the contractile element. Besides, ALG therapy can lessen cardiomyocyte hypertrophy induced by continuous LV enlargement and consequent augmentation of mechanical stretch (3). In chronic HF dogs, also, an ALG hydrogel implant enhanced LV function and halted progressive remodeling (11).

Electrical integration of the insulated hydrogels with the infarct myocardium can be delayed leading to arrhythmias. However, electroactive hydrogels could improve cell-cell electrical coupling and synchronous contractions of the cardiac cells; which is necessary for the effective integration with the host tissue (12, 13). Graphene-based nanomaterials, especially graphene oxide (GO) and its derivatives such as reduced GO (rGO), are likely to effectively reinforce materials to restore the ischemia in heart tissue due to their outstanding high mechanical and electrical properties. Also, rGO flakes could increase cell-ECM interactions by adsorption of ECM proteins such as fibronectin (FN) from cell culture serum (14). The potential of rGO application for MI treatment has been previously studied. It has been revealed that the combination of rGO flakes with MSC spheroids implanted into mouse-infarcted myocardium can successfully increase the expression of angiogenic growth factors and connexin 43, and improve MSC efficacy for MI treatment (15). Our group has previously demonstrated the in vitro assessment of ALG-rGO electroactive hydrogel for cardiac application. The presence of rGO, as an electroactive moiety, improved the physicochemical and biological properties of the ALG hydrogel (16). Here, we have further evaluated the therapeutic outcomes of ALG injection combined with human MSCs (hMSCs) and rGO in a rat model of chronic ischemic MI to improve cardiac function and induce neovascularization.

Materials and Methods

Experimental groups

Animal models were randomly categorized into seven experimental groups containing a sham group (surgical operation without any treatment) and the control group [receiving phosphate buffered saline (PBS) as treatment] (n=7/group).

Intramyocardial injection of hydrogel in Rat MI model

Twenty-eight days after left anterior descending artery (LAD) ligation, animals were anesthetized (isoflurane 1%) and underwent thoracotomy surgery. Then, intubation was done via mouth and inhaled anesthesia was continued and placed on mechanical ventilation. Identification of the infarct zone was detected via visual observation showing a darker area compared to other LV border zones near the anterior apex of the LV wall. Animals received an intramyocardial injection (28G insulin needle, 20 µl) in five parts of the infarct border zone. Each group that needed to get hBMSCs received 5×10^6 cells/µl. Simultaneously, a cross-linker ($CaCl_2$ 102 mM) was injected in the same site, in a separate syringe. The consent of each participant was aqcuired by filling out the informed Consent form and the code of National Committee of Ethics in Biomedicine Researches was approved in Tarbiat Modares University ID Number of Ethical Committee (IR.TMU.REC.1396.700).

Evaluation of cardiac function

Four and eight weeks after treatment, assessment of cardiac function was performed via transthoracic echocardiography in all animals by an echocardiologist with expertise in small animal echocardiography who was blinded to the study group allocations. Rats were positioned supine after the chest wall was shaved. Transthoracic two-dimensional (2D) echocardiography was performed using a 10-MHz linear array transducer connected to a Vivid 7 expert ultrasound system at a speed of 100 mm/s (General Electric-Vingmed Ultrasound, Horten, Norway), under low dose ketamine (10-20 mg/kg i.p) anesthesia. Parasternal M-mode and 2D short-axis views at the level of the papillary muscle were used to measure left ventricular internal diameter in systole (LVIDs), left ventricular internal diameter in diastole (LVIDd), left ventricular posterior wall thickness in diastole (LVPWd), and left ventricular posterior wall thickness in systole (LVPWs). Left ventricular ejection fraction percentage (LVEF %) was calculated using the following formula (Eq. 1):

$$\text{LVEF \%: } (LVIDd^2 - LVIDs^2)/LVIDd^2 \quad \text{(Eq. 1)}$$

To calculate the percentage of fractional shortening (FS%), the following formula (Eq. 2) was used (3, 17):

$$\text{FS\%: } [(LVIDd-LVIDs)/LVIDd] \times 100 \quad \text{(Eq. 2)}$$

Histology assessment

Eight weeks after MI treatments, rats were sacrificed with pentobarbital overdose (200 mg/kg). Their chest was opened and the hearts were removed and transferred immediately to paraformaldehyde 4% (Merck, Germany) for fixation. The paraffin-embedded blocks were sectioned at 5 µm thickness. Two sets of tissue sections (n=10) for each sample were collected and stained with hematoxylin and eosin (H&E) and Masson's trichrome (Merck, Germany) for morphological and collagen observation, respectively (18).

To evaluate neovascularization, immunohistochemistry was performed. After fixation with 4% paraformaldehyde for 24 hours, samples were permeabilized with 0.4% Triton X100 (Sigma-Aldrich, Germany) followed by being blocked in 10% goat serum (Sigma-Aldrich, Germany). Antigen retrieval processes were performed by sodium citrate buffer (10 mM for 20 minutes), then samples were blocked in 10% goat serum (Sigma-Aldrich, Germany). To quench endogenous peroxidase activity in samples, hydrogen peroxide (3wt.%) was applied for 10 minutes. The sections were then incubated with mouse monoclonal CD31 antibody (SC-376764, 1:100, Santa Cruz Biotechnology) for 2 hours at 37˚C followed by washing with PBS. Subsequently, samples were incubated with the secondary antibody (goat anti-mouse-HRP, 1:200, Sigma-Aldrich) for 3 hours. Hematoxylin was also used for counter-staining. Photomicrographs were taken with an Olympus microscope (Olympus Center Valy, PA) and Images were further quantified by ImageJ software (1.46r version, USA). The results were expressed as the mean number of vessels ± SDM. Negative control sections were obtained by omitting the primary antibody for CD31. Based on the acquired results on echocardiography, this evaluation was performed for MSC-ALG and MSC-ALG -rGO groups, and the results were compared with the control group.

Statistical analysis

Results were expressed as the mean ± standard deviation. We used SPSS (version 16.1, IBM, USA). One-way ANOVA analysis of variance was used to compare the means from multiple experimental groups, followed by a post hoc Tukey test. $P<0.05$ was considered statistically significant.

Results

Echocardiographic findings

Table 1 shows comparison of echocardiographic parameters in different groups at 4 and 8 weeks after the treatment. Compared to the control and other treatment groups, myocardial injection of rGO-ALG-MSC significantly improved ejection fraction, fractional shortening, wall thickness, and internal diameter ($P<0.05$). For example, 4 weeks after treatment, LVFS percentage in the MSC-ALG-rGO group increased from around 11 to 30 percent, whereas this value in ALG, ALG-MSC, and hBMSC was about 24, 25, and 27 percent respectively, compared to the control group.

Table 1: Echocardiographic data of experimental groups at 4 and 8 weeks post-treatment

Groups	Sham	MI (control)	ALG	ALG-MSC	hBMSC	MSC-ALG-rGO
			4 week after treatment			
LVPWs (mm)	2.6 ± 0.03	2 ± 0.01*†	2 ± 0.02	2.4 ± 0.05*	2.2 ± 0.02	2.6 ± 0.0†
LVPWd (mm)	1.6 ± 0.02	1.4 ± 0.03*†‡	1.4 ± 0.01	1.5 ± 0.03*	1.6 ± 0.03‡	1.6 ± 0.01†
LVIDs (mm)	3.2 ± 0.01	6.3 ± 0.11*†	5.2 ± 0.09	4.5 ± 0.08*	4.8 ± 0.07*	6.4 ± 0.1†
LVIDd (mm)	6.2 ± 0.050	7.4 ± 0.1*†	6.9 ± 0.12	6 ± 0.04*	6.6 ± 0.09	6.2 ± 0.07†
LVFS%	47.74 ± 3.24	11.56 ± 7.61*†	24.65 ± 2.59*	25.72 ± 8.81*	27.15 ± 7.43	30.13 ± 7.30†
LVEF%	84.05 ± 2.78	27.9 ± 16.53†*	54.59 ± 4.18*	55.45 ± 15.6*	58.05 ± 11.15*	62/6010/87 †
			8 weeks after treatment			
LVPws (mm)	2.6 ± 0.03	2 ± 0.01	2.6 ± 0.04	2.5 ± 0.05	2.3 ± 0.02	2.6 ± 0.01
LVPwd (mm)	1.6 ± 0.02	1.4 ± 0.03	1.6 ± 0.02	1.6 ± 0.02	1.5 ± 0.01	1.6 ± 0.01
LVIDs (mm)	3.2 ± 0.01	6.3 ± 0.11	4.6 ± 0.05*	4.8 ± 0.05*	4.3 ± 0.1*	6.3 ± 0.1
LVIDd (mm)	6.2 ± 0.050	7.4 ± 0.1	7.3 ± 0.08	6.8 ± 0.06*	6.5 ± 0.12	6.2 ± 0.07
LVFS%	47.74 ± 3.24	11.56 ± 7.61	33.0 ± 3.65*	29.4 ± 4.60	33.29 ± 0.54*	32.95 ± 6.12*
LVEF%	84.05 ± 2.78	26.9 ± 16.53*	63.33 ± 4.83*	60.06 ± 6.91*	62.59 ± 7.76*	67.85 ± 8.93*

LVPWs; Left ventricular posterior wall thickness at end-systole, LVPWd; Left ventricular posterior wall thickness at end-diastole, LVIDs; Left ventricular internal diameter end systole, LVIDd; Left ventricular internal diameter end diastole, LVFS; Left ventricular fractional shortening, LVEF; Left ventricular ejection fraction, MI; Myocardium infraction, ALG; Alginate, MSC; Mesenchymal stem cell, hBMSC; Human bone marrow-derived mesenchymal stromal cells, rGO; Reduced graphene oxide. One way ANOVA test showed MSC-rGO-ALG significantly improved echocardiographic parameters in the 4th and 8th weeks after treatment, and *, †, ‡, #; $P<0.05$.

Over the follow-up duration (from the 4th week to the 8th week of treatment), there was no change in echocardiographic parameters for the control group. However, in all treatment groups, LVEF parameters were significantly improved (P<0.05, Fig.1). Eight weeks after the treatment, in the MSC-ALG-rGO group, LVEF% was significantly higher when compared to the control group; at 26% and 66%, respectively (P<0.05).

Histological findings

The representative H&E staining images are presented in Figure 2. The morphological observation showed several small blood vessels formed around the transplantation site in all treated groups especially in the MSC-ALG-rGO group 8 weeks after the transplantation. Also, Masson's trichrome staining indicated an increased amount of collagen fibers in rGO-ALG-MSC.

Anti CD31 antibody was applied to detect neovascularization in three experimental groups (control, MSC-ALG, and MSC –ALG- rGO) and the results were further quantified by ImageJ software (Fig.3). More vessels were detected in the MSC –ALG- rGO group. The microvessel density in control, MSC-ALG, and MSC –ALG- rGO groups were 4.2 ± 1.3, 8.6 ± 2.0, and 19.6 ± 3.2 respectively per microscopic field, 8 weeks post-treatment. This value was significantly higher in the MSC-ALG-rGO treatment group compared with other groups (P<0.01). The angiogenesis index was also considerably higher in the MSC-ALG group compared to the control group (P<0.05).

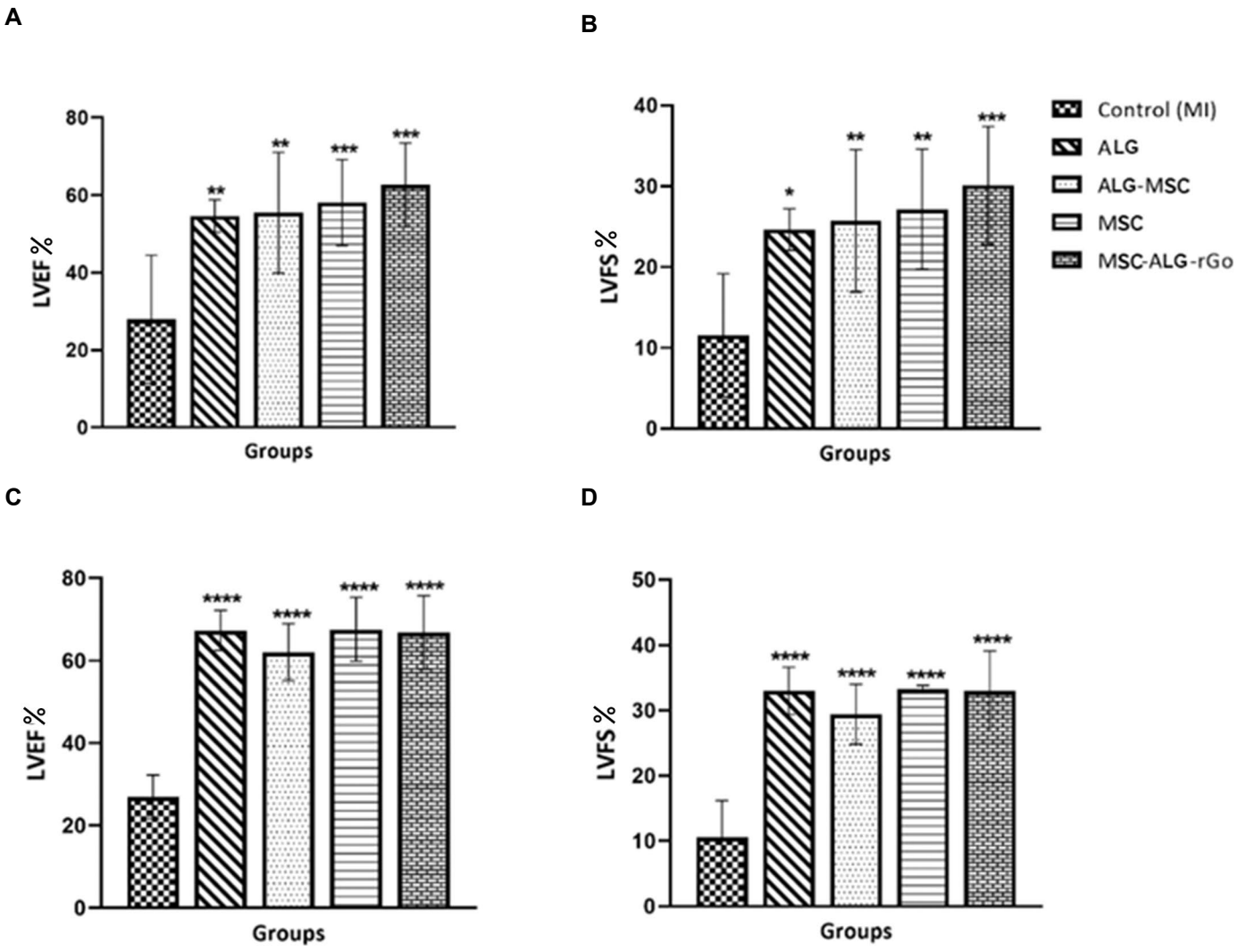

Fig.1: Effect of experimental groups on LV functions after treatment. **A.** LVEF% 4 weeks after treatment, **B.** LVFS% 4weeks after treatment, **C.** LVEF% 8 weeks after treatment, **D.** LVFS% 8 weeks after treatment. All of the treated groups showed significant increase in LVEF% and LVFS% compared to the control group (n=7/group, *; P<0.05, **; P<0.001, ***; P<0.0001, ****; P<0.00001). LVEF; Left ventricular ejection fraction, LVFS; Left ventricular fractional shortening, ALG; Alginate, MSC; Mesenchymal stem cell, and rGO; Reduced graphene oxide.

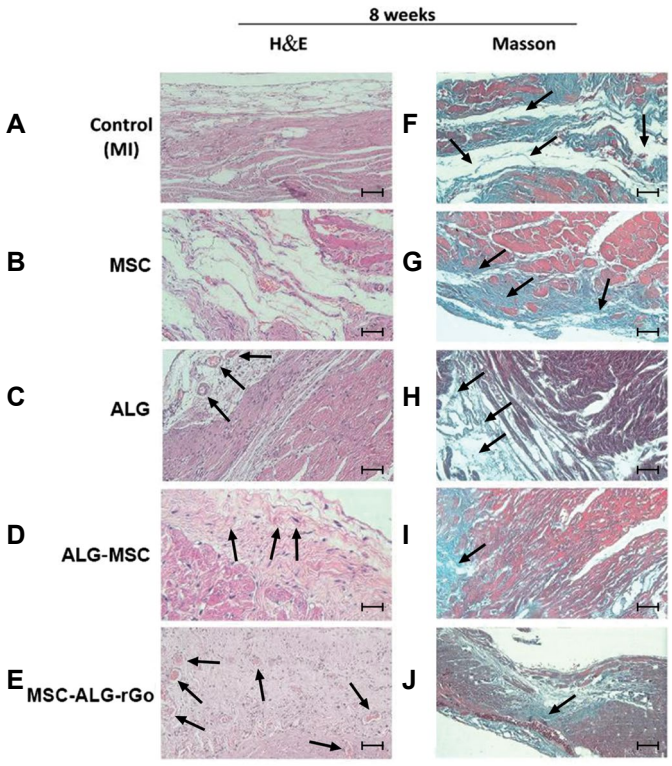

Fig.2: Histologic assessments 8 weeks after post-treatment. **A-E.** Effect of treatment on cardiac tissue remodeling 8 weeks after treatment. H&E staining to visualize myocyte and neovascularization. **F-J.** Masson's trichrome staining to distinguish collagen fibrils and scar tissues in the treated rats (magnification: 50x). MI; Myocardium infraction, ALG; Alginate, MSC; Mesenchymal stromal cell, and rGO; Reduced graphene oxide.

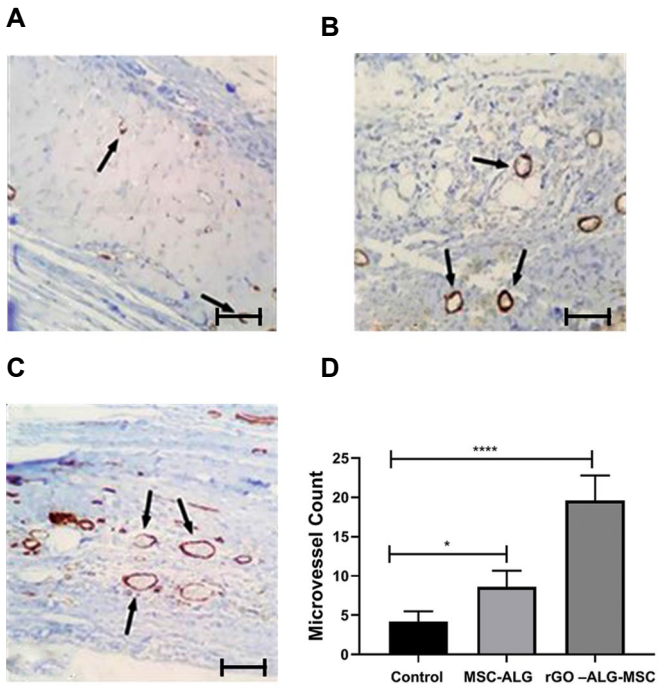

Fig.3: The immunohistochemistry images for CD31 marker to determine angiogenesis. The positive reaction for CD31 markers is shown with brown color and the arrows indicate several blood vessels. **A.** Control, **B.** MSC-ALG, **C.** MSC-ALG -rGO ($P<0.05$, $P<0.01$) (200x magnification). **D.** The results were further quantified by microvessel density defined by counting the positive staining for CD31 in five fields. MI; Myocardium infraction, ALG; Alginate, MSC; Mesenchymal stromal cells, and rGO; Reduced graphene oxide.

Discussion

Despite recent advances in treatment methods, the mortality rate from cardiovascular illnesses has increased significantly (19). Administration of stem cells with electroactive hydrogel could be a promising approach for recovery of effective signal transition and a cell-cell connection in the infarct area. Our experiment showed that injections of ALG hydrogel containing rGO/hMSCs in the free wall of the failing heart can improve LV function and promote the preservation of LV wall thickness. Our team previously demonstrated that the inclusion of rGO, an electroactive moiety, within ALG could improve hBM-MSC viability and offer a viable substrate for upregulation of cardiomyocyte gene expression, even without *in vitro* electrical stimulation (16). To assess the effect of rGO and/or hMSCs addition to ALG hydrogel on tissue regeneration, LV function was evaluated by echocardiography and dissected hearts were assessed for scarring and ventricular remodeling. A significant increase in LVPWs, LVPWd, LVIDs, and LVIDd was observed, all of which confirmed improvement in cardiac function following injection of ALG encapsulated hMSCs/rGO at week 8.

Over the past decade, ALG as a polysaccharide biomaterial has been considered the main candidate for cardiac regeneration (20). ALG implants might reduce LV dilatation while improving LV geometry, preventing electrical dispersion in HF patients (4). A recent study also demonstrated that an injectable hydrogel (Alginate/Dextran/β-Glycerophosphate) loaded with hMSCs is a promising strategy to improve the heart regeneration after MI. The authors achieved acceptable findings from ejection fraction, fibrosis, and vessel density with decreasing infraction size (21). The existence of injectable ALG in the infarct area created physical support for scar tissue by decreasing the stress of myofibers and inflammatory response in the microenvironment. Also, ALG assisted in inhibiting myocardial remodeling after MI (22).

The H&E staining and immunohistochemistry results showed the higher neovascularization and lower collagen fibrosis formation (indicating reduced scarring/collagen deposition) in rGO/ALG encapsulated hMSCs group compared to control groups. In a study performed on a model of MI in dogs, the alginate-treated group showed a significant increase in LV systolic and diastolic function and wall thickness compared to the control group; whereas LVEF and LV thickness in the control group decreased. Therefore, this investigation indicated that ALG could be considered as a promising biomaterial for the treatment of patients with advanced heart failure (11). According to our investigation, parameters such as EF and FS in the 4th and 8th weeks after treatments had a significant increase in the MSC-ALG-rGO group compared to the control group at 62, 67, and 30, 32%, respectively. These results indicate that MSC-ALG-rGO compositions have preventional effects on LV geometry and cardiac dysfunctionality after MI.

Graphene-based nanomaterials can effectively scavenge reactive oxygen species(ROS) providing antioxidant activities to the transplanted cells and MI tissues (23). The antioxidant activity of rGO has been previously considered as the reason to increase the survival and therapeutic efficacy of hMSCs delivered for MI treatment (7). In addition, the presence of rGO can improve the adhesion of MSC in the damaged area and consequently increase the paracrine action of angiogenic growth factors such as VEGF and FGF2. Ultimately, it increases the regeneration rate of damaged heart tissue (15) .The efficacy of ALG and hMSCs in preventing or reversing cardiac remodeling has been proved before (3, 24), but the injection of hMSCs along with rGO/ALG is a novel method to improve cardiac function and reverse cardiac remodeling.

As reported by a study, cardiomyocytes cultured on rGO-GelMA hydrogel sheets showed a stronger contraction, faster heartbeat rate, as well as improved electrical conductivity and mechanical properties, compared to that of cells cultured on GelMA hydrogels only. Therefore, rGO hybrid with a biocompatible hydrogel is an applicable strategy for the treatment of cardiac tissue engineering (25) .In a study by Choe et al. (7), the effect of rGO/ALG encapsulated human umbilical cord-derived MSCs was investigated. The results of Masson's Trichrome staining showed rGO/ALG containing MSCs have less fibrous tissue than the control group and alginate/GO, indicating a reduction in scar tissue and the presence of more collagen.

Based on the obtained results, we are assuming that the injected composition of rGO/hMSCs into the infarcted area could improve cardiac repair via the electrical conductivity of rGO as well as the ability to interact with ECM protein like FN which promoted the expression level of connexin 43 and angiogenic paracrine factors as has been previously proven (15). A recent study demonstrated that impaired propagation of electrical signals of the heart after MI could be improved by an applied anisotropic cardiac patch of rGO/silk fibroin composition. Increased pumping function, reduced susceptibility to arrhythmias, thickened LV walls, promoted angiogenesis of capillaries and better survival of cardiomyocytes were all considered the results of adding rGO to the silk patch via restoring the anisotropic electrical microenvironment in the infarcted myocardium (26). In summary, a combination of rGO, alginate, and hMSCs might be a viable approach for treating patients with MI.

Conclusion

Our experiments confirmed that the presence of rGO, as an electro-active moiety within ALG appears safe for intramyocardial injection, improving (LV) function and neovascularization. Applying this strategy provides a desirable electroactive hydrogel for stem cell therapy in patients with ischemic heart disease. However, more investigations are required to assess the safety of using rGO with ALG for the treatment of MI.

Acknowledgments

The authors acknowledge Dr. Behshid Gadrdoost for her technical assistance. The research was partially financially supported by Tarbiat Modares University, Tehran, Iran. There is no conflict of interest in this study.

Authors' Contributions

N.K.H.; Performed the experiments and wrote the main manuscript text, and performed data analysis. N.B.; Designed and supervised all experiments and manuscript writing. N.N.; Participated in the Eco cardiology investigation and participated in manuscript writing. M.S.; Data analysis and interpretation. M.R.; Data analysis and interpretation, and manuscript writing. All authors read, reviewed, and approved the manuscript.

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