

Restoration of Heart Function Using Transplantation of Human Umbilical Cord Matrix-Derived Cardiomyocytes and Vascular Endothelial Growth Factor

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Abstract: *Objectives:* In the previous study, although it has been shown that intramyocardial injection of human umbilical cord matrix stem cell (hUCM) improved cardiac function 4 weeks post MI, but angiogenesis has not been observed. Angiogenesis and replacing lost cardiomyocytes with new, live cardiomyocytes are considered as two key agents in cardiac repair. To achieve the above two factors we examined the effects of combination of stem cell and angiogenic therapy approaches by simultaneously injection of hUCM-derived cardiomyocytes with vascular endothelial growth factor (VEGF) in cardiac repair.

Methods: MI-induced animals (by ligation of LAD) received 50 μ l PBS, 5×10^6 differentiated hUCM cells (dhUCM), 5 μ g VEGF in normal saline and 5×10^6 dhUCM cells combined with 5 μ g VEGF in normal saline, intramyocardially. MI group, with no other intervention, served as a control group. We were assessed survival, migration and integration of dhUCM cells, as well as angiogenesis eight weeks post MI induction.

Results: Eight weeks post MI, although dhUCM and VEGF groups have shown that LVEF and LVFS improved significantly, but animals in dhUCM+VEGF group have the highest rise in LVEF and LVFS in comparison to the other MI-induced groups ($p < 0.05$). Histological and morphological analysis have revealed that myocardium of animals in dhUCM+VEGF group have the highest vascular density and the lowest fibrosis tissue in comparison to the other MI-induced groups ($p < 0.05$). Immunohistological assessments revealed that transplanted dhUCM cells have survived, migrated to infarcted area and integrated with recipient cardiac tissue.

Conclusion: we have found that intramyocardial administration of dhUCM cells combined with VEGF improved cardiac function, enhanced angiogenesis and reduced fibrosis tissue formation after MI, eight weeks post MI.

Keywords: Human Umbilical Cord Matrix-derived cardiomyocytes, Myocardial infarction, Angiogenesis, Vascular endothelial growth factor, Cell therapy, Cardiac repair, Rabbit.

INTRODUCTION

Myocardial infarction and the subsequent heart failure are the main causes of morbidity and mortality in western societies [1]. Myocardial infarction is defined as the loss of cardiomyocytes, mostly due to reduced or obstructed blood flow which leads to impaired cardiac function [1, 2]. Despite many recent advances in surgical and medical therapy, infarcted heart therapies need to take more effort and time to

replace lost cardio myocytes and cardiac regeneration. Limited regenerative capacity of the heart has forced scientists to focus on novel therapeutic approaches, such as stem cell and angiogenic therapy, for the treatment of the heart [3-7]. Angiogenesis, defined as the formation of new vessels from existing ones, is a key factor in cardiac repair following myocardial infarction, without which cardiac rupture and fibrosis tissue formation would occur [8]. Nevertheless, angiogenic therapy using VEGF has indicated that the administration of VEGF in infarcted myocardium induces angiogenesis, decreases apoptosis and improves cardiac function. However, cardiac repair requires the replacement of lost cardio myocytes with new, alive cardio myocytes [9-11]. Numerous studies have shown the potential

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of stem cell therapy in replacing lost cardiomyocytes, reducing infarcted size, making less ventricular remodeling and the improvement of cardiac function [12-15]. Each source of stem cells has advantages and disadvantages; currently, hUCM cells are considered for use mostly due to their great expansion capability, non-invasive, comfortable isolation procedure and low risk of immune system reaction and teratoma formation in the recipient [16-20]. Therapeutic potentials of hUCM have been proved for the treatment of some diseases, but their use for myocardial infarction is not well understood yet and therefore its application requires more assessment [21-24]. Latifpour *et al.* have shown that transplantation of hUCM cells in to infarcted myocardium improved left ventricular function 30 days post MI, however, angiogenesis was never observed [25]. On the other hand, precise assessment of hUCM's effects in cardiac regeneration needs more time than 30 days. We assume that the combination of stem cell therapy and angiogenic therapy approaches in the treatment of infarcted heart will cover the limitations related to each one of them and optimize their positive effects.

Here we will describe the results of intramyocardial injection of hUCM- derived cardiomyocytes with and without VEGF into infarcted myocardium on heart function eight weeks post MI. In addition, we will assess survival, migration and integration of dhUCM cells, as well as angiogenesis by histopathological analyses eight weeks after the transplantation in to infarcted rabbit's heart.

MATERIALS AND METHODS

Preparation of hUCM Cells

Umbilical cords were collected after the birth with informed consent of the parents, and were transported to the laboratory in mediums containing Hank's balanced salt solution (HBSS)(Gibco, USA). Within two hours after the collection, under sterile condition, umbilical cord blood vessels were cleared off, and the remained matrices were minced into fragments; these fragments were rinsed with PBS, and then were cultured in culture plates containing a complete medium (DMEM-F12 supplemented with 15% FBS(Gibco, USA), 100 U/ml penicillin, 60 μ g/ml streptomycin). The culture plates were incubated under humidified condition in 5% CO₂ in a 37°C incubator. The medium was changed until fibroblast shaped cells that adhered to the floor of the plates were seen. The fragments were discarded, the cells were trypsinized and suspended in DMEM, and then were cultured in a new complete medium. The mediums were changed every three days.

Flow Cytometric Analysis of hUCM Cells

HUCM cells were lifted by trypsin and were suspended in DMEM, 1×10^6 cells/ml hUCM cells were fixed with 4% paraformaldehyde and were incubated in PBS with 2% normal serum. HUCM cells were incubated with primary antibodies (antibodies against CD73, CD29, CD34, CD44, CD45, CD90, CD105, HLA-ABC (HLA-1) and HLA-DR (all ebiosciences, USA) as described in previous studies [16, 21]) at room temperature followed by fluorescent secondary antibodies. Control hUCM cells were stained with a secondary antibody alone. AFACS can machine (Becton, Dickinson) was used for the flow cytometric analysis.

The Induction of Adipogenic, Chondrogenic, or Osteogenic Differentiation

HUCM cells were induced to differentiate them into adipogenic lineage cells by incubation with serum-free DMEM containing 10% FBS, 50 μ g/ml of ascorbate-1 phosphate, 100 nMdexamethasone and 50 μ g/ml indomethacin [Sigma, St. Louis]), into chondrogenic lineage by incubating them with serum-free DMEM containing insulin-transferrin in-selenium (ITS) + premix [GIBCO, Carlsbad, CA] and 10 ng/ml transforming growth factor [TGF]- β 1 [PeproTech, Rocky Hill, NJ]) and into osteogenic lineage by incubating them with (DMEM-LG containing 10% FBS, 50 μ g/ml ascorbate-2 phosphate, 10 nM dexamethasone, and 10 mM β -glycero-phosphate. Three weeks later, after the fixation of hUCM cells by paraformaldehyde in PBS, these cells underwent Alcian blue, oil red and Alizarin red staining for the detection of chondrogenic, adipogenic and osteogenic differentiation, respectively.

Cardiac Differentiation of hUCM cells

In vitro, the differentiation of hUCM cells into cardiomyocytes was induced by incubating hUCM cells with cardiogenic differentiation medium containing complete medium and 10nM Oxytocin (Wako chemical, Germany) for 72h [26]. Then the differentiation medium was replaced with complete medium, and was regularly refreshed every three days, for three weeks.

Immunocytochemical Analysis

Oxytocin- treated cells (dhUCM) were washed with PBS and fixed with 4% formaldehyde (Sigma, USA) for 10 minutes at room temperature. These cells permeabilized with 0.1% triton X-100 (Sigma, USA) in PBS for 15 minutes, and then were incubated with primary antibodies against cardiac troponin T (cTnT, abcam, UK), sarcomeric actin (sA, abcam, UK), cardiac actin (cA, sigma, USA) and connexin 43 (Cx43, mlllopore, Germany) followed by fluorescent secondary antibodies(FITC and Alexa Fluor). HUCM cells served as a control. Controls for nonspecific binding of secondary antibodies were performed by excluding primary antibodies.

Labeling of the dhUCM Cells with DiI

Cell tracker CM-DiI (Invitrogen, USA) was prepared in dimethylsulfoxide at 2 mg/ml and diluted into HBSS (5 μ l/ml). HUCM and dhUCM cells were labeled by these labeling -solution (2ml added into every tissue culture flask) and incubated in 37 °C incubator for ten minutes. The cells were prepared for transplantation in this way.

Induction of Myocardial Infarction (MI) Model and Cell Transplantation

This investigation was performed in accordance with the principles of the ethical committee at Tehran University of Medical Science, Iran. 60 male New Zealand white rabbits (weight range 2.5-3 kg) were randomly divided into six groups: (a) intact group (non-MI, n=10); (b) MI group (n=10) consisting of MI model; (c) PBS group (n=10); (d) dhUCM group (n=10); (e) VEGF group (n=10); (f) dhUCM + VEGF group (n=10) consisting of MI model with PBS, dhUCM cells, VEGF and dhUCM cells combined with VEGF were injected into the margin of MI region in four

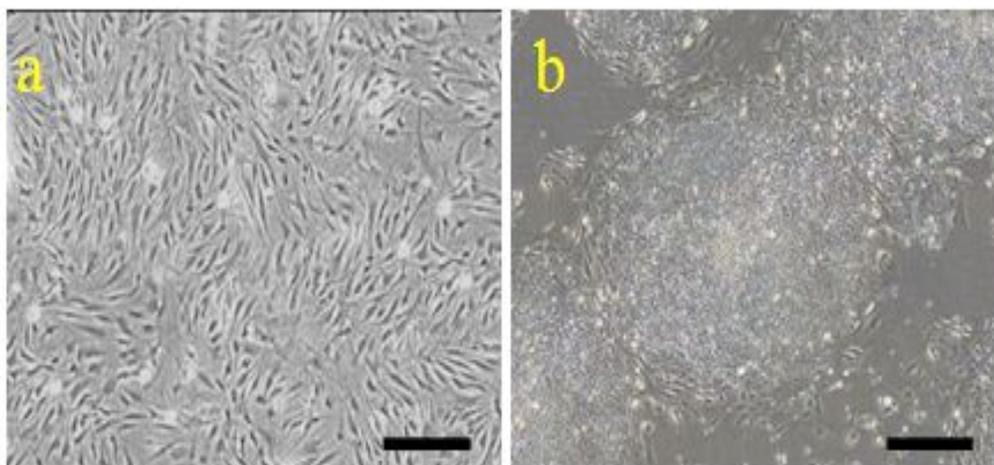


Fig. (1). The hUCM cells appeared in spindle shape cells possess cytoplasmic extensions (a) and high potential of colony formation (b). Scale bar: 100 μ

sites, respectively. Rabbits were anesthetized, intubated and ventilated using a ventilator. After the left thoracotomy, a 0.6 silk ligature was placed and tied closely around the proximal end of the left anterior descending artery (LAD). One hour later, the induction of MI-model was confirmed by a cyanotic appearance of the infarcted area, then 50 μ l PBS, 5×10^6 dhUCM cells, 5 μ g VEGF in normal saline and 5×10^6 dhUCM cells combined with 5 μ g VEGF in normal saline injection was performed in (c, d, e, f) groups, respectively. The injections were applied using an insulin syringe with cooperated 28 gauge needle into the border zone of the infarcted area to maximize graft viability. Animals in group (a) underwent the same procedure without the ligation of the LAD. Cyclosporine A (10 mg/kg, subcutaneously) was given 24 h before the cell therapy and once daily, thereafter.

Functional Assessment by Echocardiography

Echocardiography was performed using a transthoracic echocardiographic device (Vivid3; General Electric) equipped with a 5-MHz linear transducer, five days, four and eight weeks after MI in all of the groups. The echocardiogram provided the following measurements including: ejection fraction (EF) and fractional shortening (FS). The Cardiologist that performed echocardiographic evaluation was not aware of the treatment groups.

Morphometric and Histological Analysis

Eight weeks after the injection, animals from each group was sacrificed, their hearts were excised and sliced into four segments from apex to the site of ligation. Then these segments were fixed in 10% formaldehyde, were prepared as embedded in paraffin and 5 μ m thick sections and stained with hematoxylin and eosin (H&E). Some selected sections were incubated with primary antibody against CD31 (Bioscience, USA), followed by peroxidase conjugated secondary antibody, each one for one hour at room temperature. CD31-positive cells with a vascular structure were counted as a vessel (capillary or artery). The sections underwent trichromemasson staining for the evaluation of the amount of fibrosis tissue using MATLAB software (Mathworks Inc., Natick, MA, USA). Vascular density and fibrosis tissue of myocardium have been quantified as previously described in previous studies [27, 28].

Immuno Histochemical Analysis

Survival, migration and integration with recipient cardiac tissue of transplanted dhUCM cells were assessed by tracking DiI-labeled cells using fluorescence microscope as well as by immunohistological evaluation as the following: The sections were incubated in 3% H₂O₂, blocked in 5% normal goat serum, then incubated with primary antibody, overnight at 4 °C with primary antibodies against cardiac α -actinin (cAn, Sigma, USA), cardiac troponin T (cTnT, Sigma, USA) and connexin 43 (Cx43, Millopre, Germany) followed by FITC and Alexa Fluor for one hour.

Statistical Analysis

Statistical analysis was conducted with ANOVA using SPSS 19.0 software. A P value of <0.05 was considered significant. Data is expressed as means \pm SD.

RESULTS

Morphology and Flowcytometry Analysis of hUCM Cells

10-15 days after the initiation of hUCM segment cultures, hUCM cells were expanded from matrix segments, then separated from segments and cultured in new mediums. These cells demonstrated short doubling time(16-24h), strong proliferative ability, and high potential of colony formation(colonies formed within 3-4 days);they adhered to the floor of tissue culture flask, and appeared in spindle/fibroblast shape cells with different size cytoplasmic processes which remained in similar morphology after several passages (Fig. 1a,b). The isolated hUCM cells from four different umbilical cords at passage fourwere selected for flow cytometry. The results of the flowcytometric analysis suggested that hUCM cells were positive for mesenchymal specific markers(CD73(98%), CD29(94%), CD44(89%), CD90(91%)) and were negative for CD34 and CD45, which indicated that hUCM cells did not belong to endothelial or hematopoietic linages. Moreover, hUCM cells showed HLA-ABC(95%)but didn't show HLA-DR;this fact proves that these cells have a low risk of recipient inflammatory reaction (Fig. 2). The mesenchymal entity of hUCM cells was approved following their differentiation into chondrogenic, osteogenic and adipogenic linages (Fig. 3a-c).

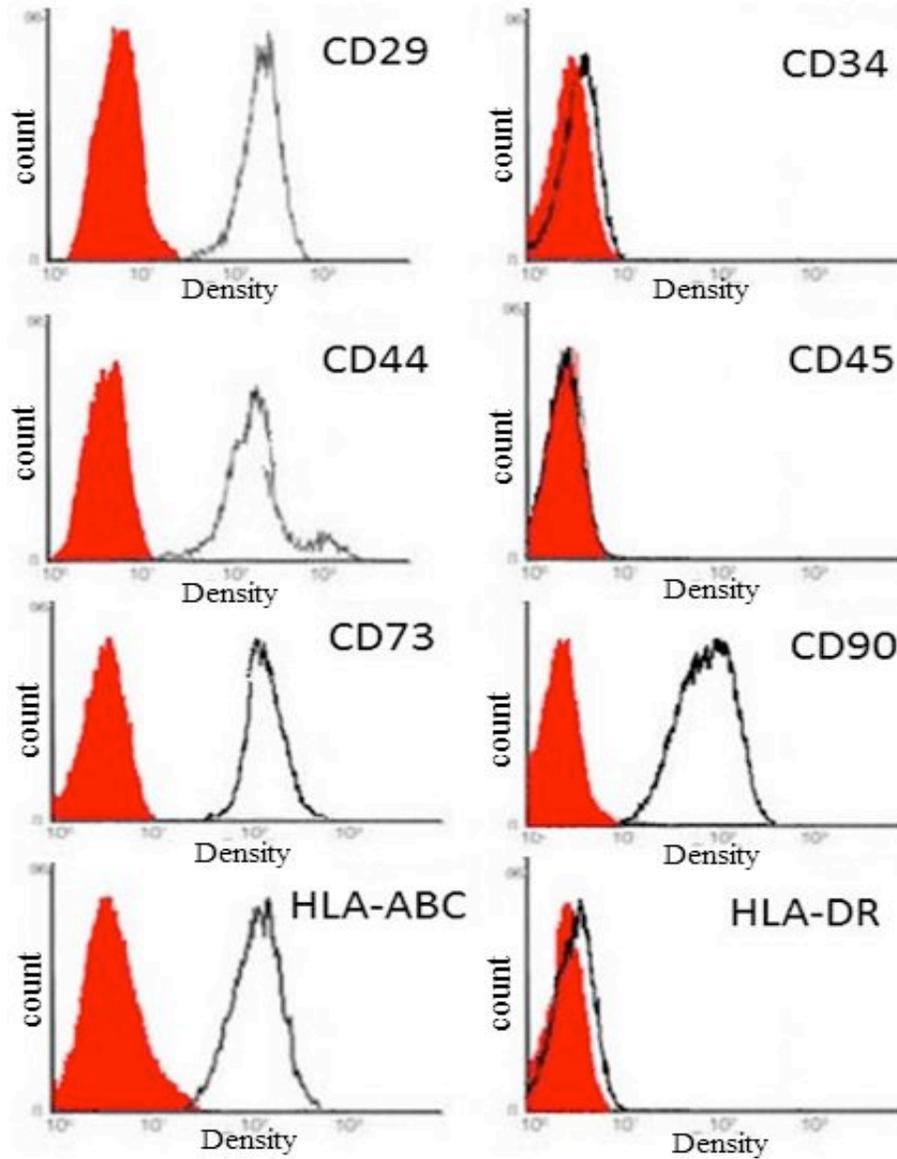


Fig. (2). Flow cytometric analysis of hUCM cells. Red histogram indicates background signal; open histogram represents positive reactivity with the indicated antibody. HUCM cells have expressed the mesenchymal specific markers (CD29, CD44, CD73 and CD90), but didn't express hematopoietic specific markers (CD34 and CD45). As you see, hUCM cells were positive for HLA-1 but negative for HLA-DR which indicated the low risk of recipient inflammatory reaction. The represented data obtained after 3 experiments.

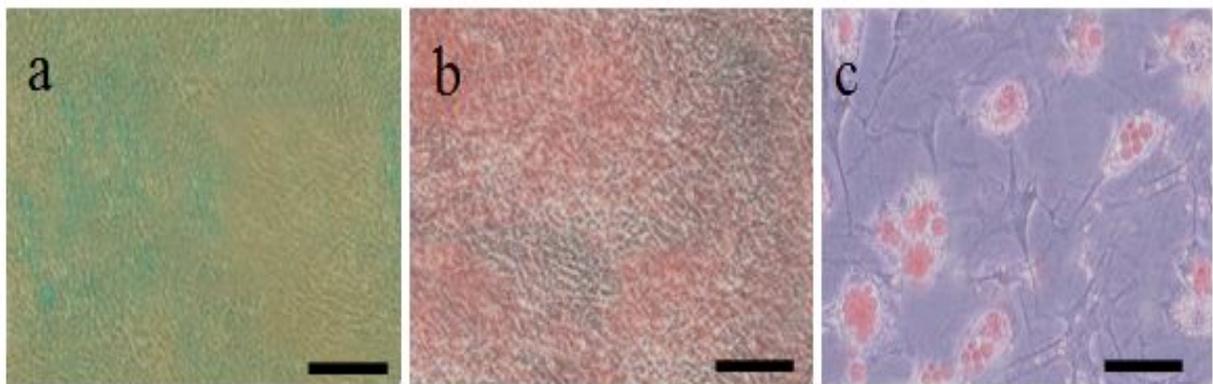


Fig. (3). Mesenchymal entity of hUCM stem cells. Chondrogenic differentiation verified by Alcian blue Staining (a) Scale bar: 100 μ . Osteogenic differentiation verified by Alizarin Red Staining (b). Scale bar: 100 μ . Adipogenic differentiation approved by Oil-Red Staining (c) Scale bar: 50 μ .

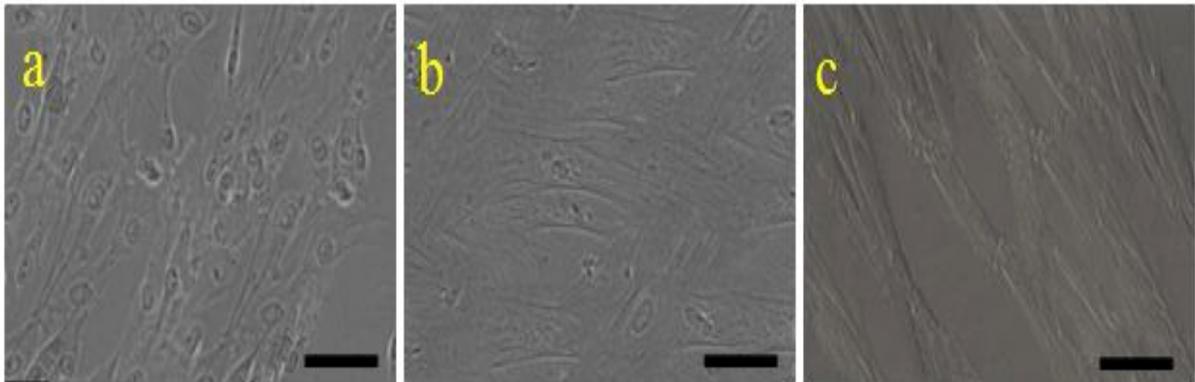


Fig. (4). Cardiac Differentiation of hUCM cells. Fibroblast shape hUCM cells (a) were turned into flat shape cells with short cytoplasmic extensions as cardiomyocytes. Fibrillar structure and spontaneous beating of dhUCM cells were seen under light (b) and phase contrast microscope (c). Scale bar: 50 μ

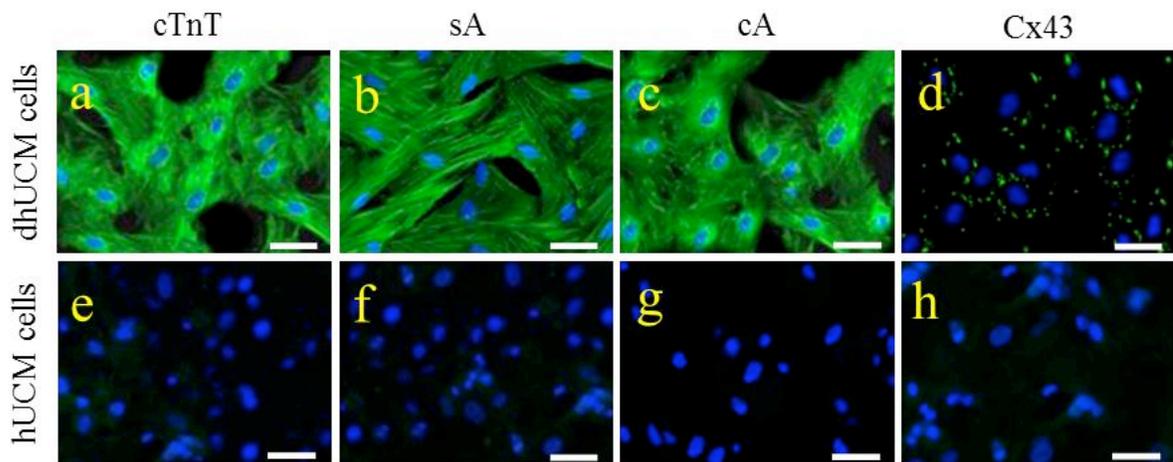
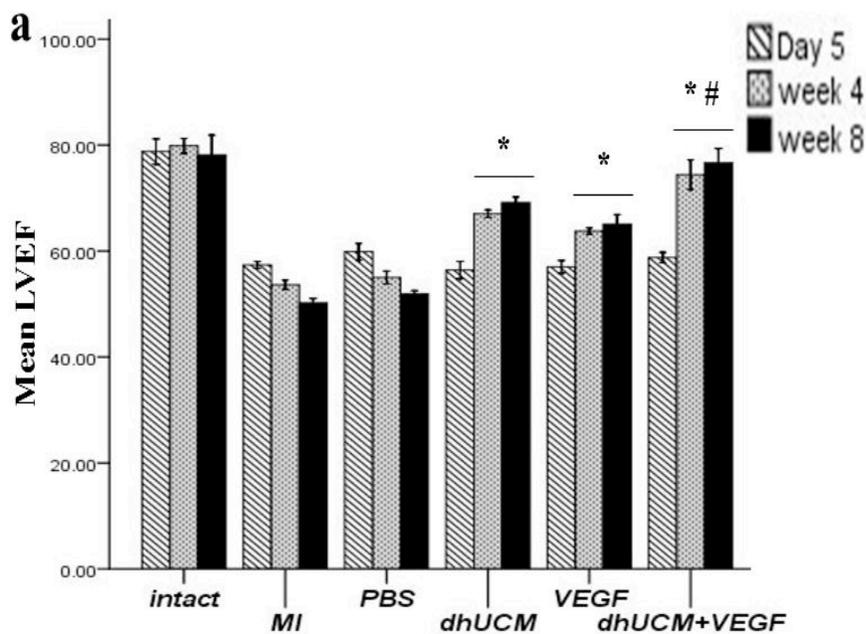


Fig. (5). Immunohistological analysis have shown that dUCM cells were positive for cardiac troponin T (cTnT), sarcomeric actin (sA), cardiac actin (cA) and connexin 43 (Cx43). The hUCM cells as a control group were negative for these cardiac specific proteins and didn't express any one of them. Cell's nuclei stained by DAPI. Scale bar = 50 μ m.



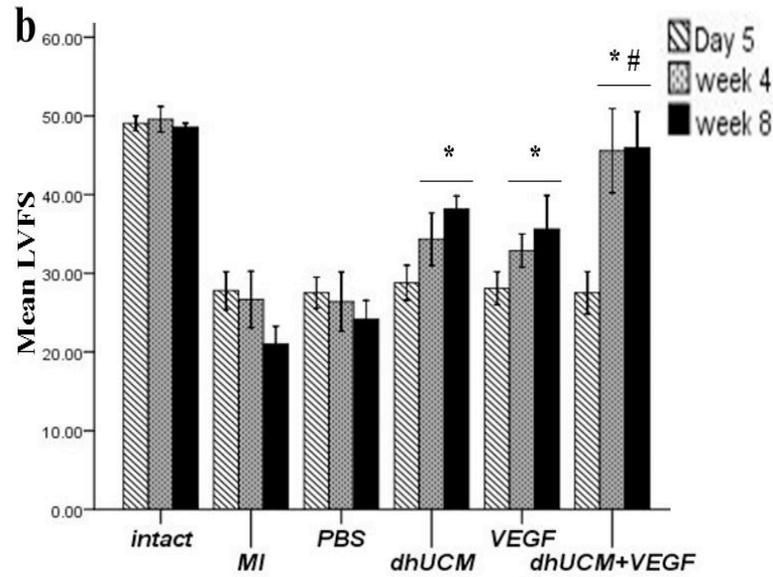


Fig. (6). Echocardiography assessments were applied in different time points. After 8 weeks, the treated animals by dhUCM cells combined with VEGF have shown the highest significant improvement in left ventricular function as well as animals in dhUCM and VEGF groups have demonstrated the increasing of EF (a) and FS (b) compare to MI and PBS groups with a significant difference.

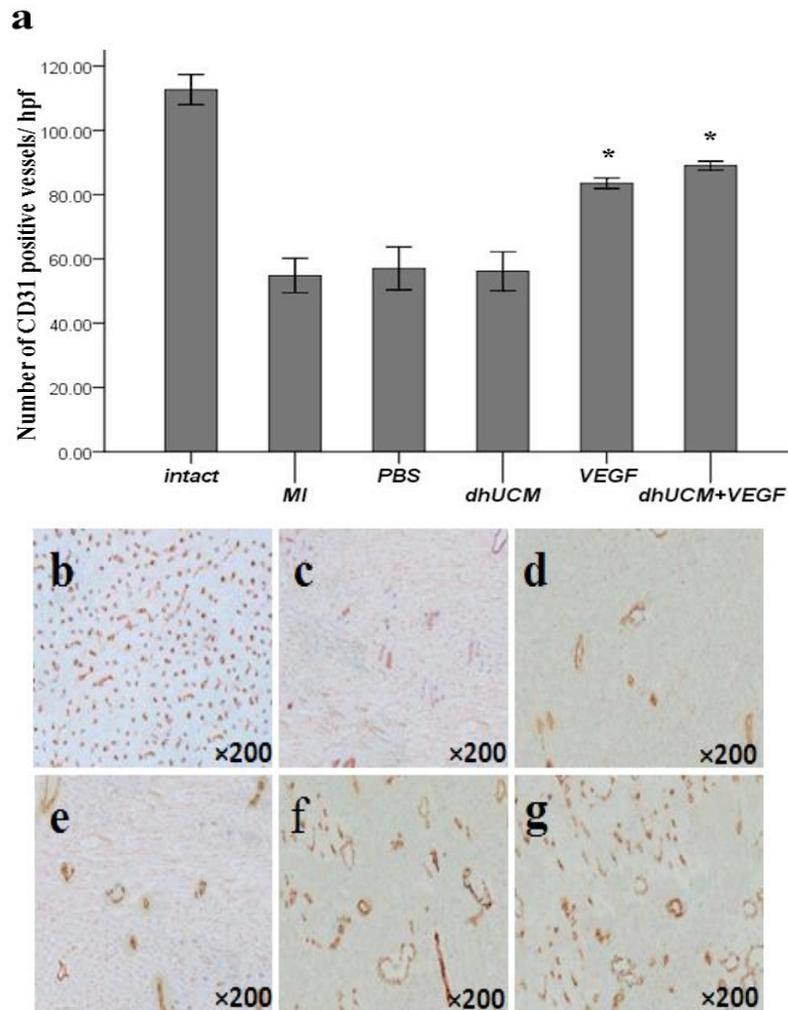


Fig. (7). Vascular density. bar graphs showing the quantification of CD31 positive vessels per high power field of microscope (a). Representative micrographs ($\times 200$) have shown the blood vessels after CD31 staining 8 weeks post MI in intact (b), MI (c), PBS (d), dhUCM (e), VEGF (f) and dhUCM + VEGF (g) groups, respectively. * $p < 0.05$ vs. MI, PBS and dhUCM.

* $p < 0.05$ vs. MI and PBS. # $p < 0.05$ vs. dhUCM and VEGF.

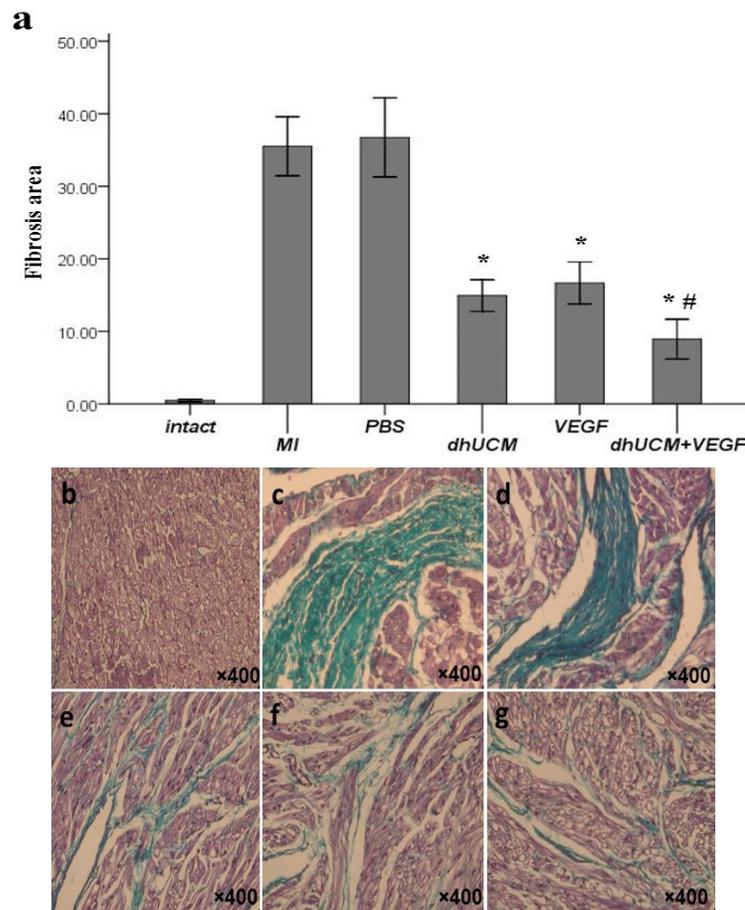


Fig. (8). Fibrosis tissue formation post MI. bar graphs showing the quantification of fibrosis formation in myocardium (a). Representative micrographs have demonstrated the Masson's Trichrome stained sections taken from experimental animal's hearts 8 weeks post MI in intact (b), MI (c), PBS (d), dhUCM (e), VEGF (f) and dhUCM+VEGF (g) groups, respectively. Scale bar: 100 μ .

* $p < 0.05$ vs. MI and PBS. # $p < 0.05$ vs. dhUCM and VEGF.

Cardiac Differentiation of hUCM Cells

Three weeks after treating hUCM cells with Oxytocin, as given in material and methods, the fibroblast-like shape hUCM cells were turned into flat shape cells with short cytoplasmic extensions as cardiomyocytes. DhUCM cells appeared in striped cytoplasm. The indications of contractile filaments and spontaneous beating of these cells have been observed under microscope (Fig. 4a-c). DhUCM cells were stained positively with some cardiac specific markers including cTnT, sA, cA, which indicated fibrillar, contractile structure of dhUCM cells (Fig. 5), as well as with Cx43, which is a gap junction protein that is required for cell-to-cell interaction (Fig. 5), in green fluorescence. HUUCM cells, as a control group, didn't show any cardiac specific proteins (Fig. 5). About 73% of hUCM cells have been differentiated into cardiomyocytes.

Echocardiographic Measurements

Echocardiographic assessment of heart function was done at different time points. Five days after the induction of MI (acute phase), echocardiographic measurements have shown that both of the LVEF and LVFS significantly decreased ($p < 0.05$) in all MI groups (MI, PBS, dhUCM, VEGF, dhUCM + VEGF) compared with the intact group, which indicated that cardiac function was damaged (Fig.

6a,b). There wasn't any significant difference between MI groups at this time point. Four weeks post MI (midterm), LVEF and LVFS increased in dhUCM, VEGF, dhUCM + VEGF groups which indicated that the cardiac function had improved (Fig. 6a,b). The animals in dhUCM + VEGF group have shown the highest rise in LVEF and LVFS in comparison to the other groups. Moreover, dhUCM and VEGF groups have suggested that cardiac function improved, which is a significant difference ($p < 0.05$) when we compare them with non-treatment groups. Eight weeks post MI (long term), echocardiograms suggested that LVEF and LVFS had continued rising in dhUCM, VEGF and dhUCM + VEGF groups but in comparison with the obtained results from the same groups four weeks post MI, no significant difference ($p > 0.05$) was observed (Fig. 6a,b). A significant difference ($p < 0.05$) between treated animal groups (dhUCM, VEGF, dhUCM + VEGF) and non-treated animal groups (MI, PBS) was continued.

Morphometric and Histological Analysis

Endothelial cells were stained positively with CD31 (Fig. 7). This assessment has shown that vascular density decreases after MI, a significant difference ($p < 0.05$) between the intact group (112.6 ± 3.78) and the other ones, but it didn't show any significant difference between MI (54.8 ± 4.32), PBS (57.04 ± 5.33) and dhUCM (56.16 ± 4.84) groups

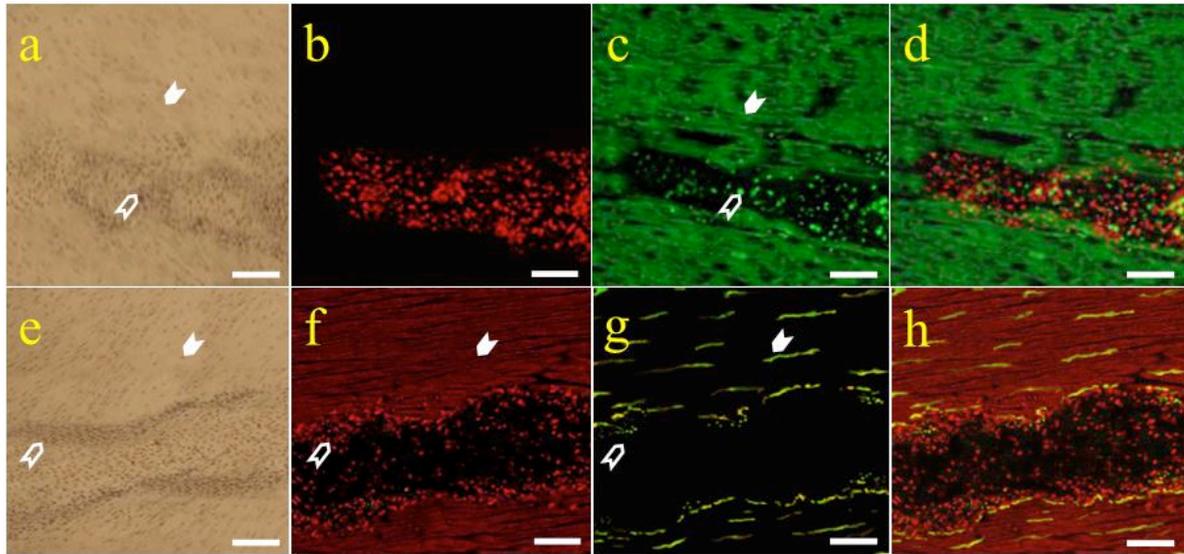


Fig. (9). Transplanted cells (non-filled chevron) and recipient cardiac tissue (filled chevron) after H&E staining (**a** & **e**). Transplanted DiI-labeled cells appeared in spots within dark zone (**b**). The α -actinin stained sections that shown integrated area (filled chevron) as a non-infarcted myocardium (**c**), spots (non-filled chevron) as a dhUCM cells in infarcted area (**e**) and a merged image of them that indicating survival and migration of transplanted cells in to infarcted myocardium (**d**). Connexin 43 and cardiac troponin T positive cells, respectively (**f** & **g**), appeared in both of infarcted area (non-filled chevron) and non-infarcted area (filled chevron), a merged image of them have shown that connexin 43 appeared in border zones between transplanted cells and recipient cardiac tissue (**h**). Scale bar = 100 μ m.

(Fig. 7a-g). DhUCM + VEGF group has the highest vascular density compared to the other MI groups, which indicates a significant difference ($p < 0.05$) with MI, PBS and dhUCM groups but not with the VEGF group (83.54 ± 1.31). Trichromemasson staining was applied to determine the amount of fibrosis tissue formation in myocardium. The fibrosis tissue appeared in green color (Fig. 8). The fields were selected in a similar way like the vascular density evaluation and the obtained images were analysed using MATLAB software. Animals that received dhUCM+VEGF have shown smaller fibrosis (8.9 ± 2.21) compared to other MI-animal groups, ($p < 0.05$). Also, animals that received dhUCM cells only (14.93 ± 1.75) and VEGF only (16.67 ± 2.33) have represented attenuation of fibrosis tissue formation with a significant difference ($p < 0.05$) with PBS (36.73 ± 4.39) and MI (35.52 ± 3.27) groups (Fig. 8a-g).

Immunohisto Chemical Analysis

The survival of dhUCM cells (DiI-labeled cells) is examined by tracking the red cells in cardiac tissue sections under light (Fig. 9a,e) and fluorescence microscope (Fig. 9b). The red spots in the dark zone are determined as transplanted dhUCM cells (Fig. 9b). Having been stained with monoclonal anti- α -actin in antibody (α -actinin), the dark zone turns into green. Integrated green areas are non-infarcted areas, and non-integrated areas represent infarcted areas that contain transplanted cells which appeared in the green spots (Fig. 9c). Merged pictures have shown that transplanted cells survive and migrate to the infarcted; they also show heart specific marker (α -actinin) (Fig. 9d). The sections were stained with cTnT. The red zones in the infarcted area represent cardiogenic entity of the transplanted cells, and integrated red zones are the recipient cardiac tissue (Fig. 9f). Moreover, sections that underwent staining with Cx43 and green fluorescence demonstrated Cx43 in both of the infarcted and non-infarcted areas (Fig. 9g). The pictures

have been merged; green spots between cardiac tissue and transplanted cells indicate that transplanted cells integrate with the recipient cardiac tissue and Cx43, which is a requirement for electrical cell-to-cell coupling that exists between them (Fig. 9h). This integration proves the existence of functional solidarity of dhUCM cells and the recipient cardiac tissue.

DISCUSSION

In the present study, we have investigated the effects of direct intramyocardial injection of dhUCM cells combined with VEGF on cardiac repair. The achieved data indicates that intramyocardial injection of dhUCM cells combined with VEGF improves left ventricular function, enhances angiogenesis and attenuates fibrosis tissue in infarcted myocardium eight weeks after MI. Recent studies in the field of cell therapy have shown new therapeutic potentials in the treatment of cardiac disease [27]. Previous studies had shown that various stem cell resources such as; bone marrow (BM-MSCs) [28], Embryonic (ESCs) [29], adipose tissue (ADCs) [12, 30], cardiac (CSCs) [31] and umbilical cord blood stem cells (UCBSCs) [32] have beneficial effects on the restoration of cardiac function after MI, but practical application of these sources is limited. Low cell yields, invasive collection procedure, long culture periods, ethical issues and the risk of recipient immune reaction are but a few of the associated problems that prove to be serious and troublesome barriers in choosing a sufficient stem cell source. Trapped MSCs in hUCM (Wharton's jelly) provide sufficient source of stem cells as a hUCM-MSCs. They are obtained from the umbilical cord that routinely discards them at parturition, thus eliminating the concerns related to other stem cell resources [16-20]. The hUCM cells don't face some of the problems associated with other stem cell resources, therefore, we tried to partake of them in this study. The hUCM cells were differentiated into adipogenic,

osteogenic and chondrogenic lineages so as to prove their multi-lineage differentiation potential (Fig. 3a-c). Many scientists have administered various stem cells directly to infarcted heart before a differentiation of those cells into cardiogenic *in vitro* [11, 28, 31]. Latipour *et al.* have demonstrated that hUCM and dhUCM transplantation in to infarcted myocardium improved cardiac function thirty days after MI [25]. Although cardiac microenvironment potentially introduced stem cells to cardiomyocyte differentiation, some surveys have revealed that less than 5% of cardiomyocytes are derived from stem cells in the recipient heart tissue [33-35]. In this regard, approximately one billion cardiomyocytes are lost in post MI, therefore, the number of cardiomyocytes, derived from stem cells *in vivo*, seems not to be enough to influence the contractility of the injured myocardium [36]. With this in mind, and to prevent mal-differentiation of hUCM cells in cardiac tissue, we induced them to cardiomyocyte differentiation by oxytocin. Although 5-azacytidine is known as a routine agent for the induction of cardiogenic differentiation, but conflicting data about its efficacy has been reported [37]. On the other hand, oxytocin, which is another agent, has shown greater potential than that of 5-azacytidine, therefore, we selected oxytocin to increase and improve the qualification of cardiomyocyte differentiation [26, 38, 39]. The dhUCM cells have shown some contractile proteins such as cardiac actin, cardiac troponin T and sarcomeric actin as well as connexin 43, which indicates the electrical coupling of them together remarkably (Fig. 5). In this study, EF and FS, in MI groups decreased significantly compared to the intact group, five days post MI, which suggests that the treatment of the injured heart's function (dhUCM, VEGF and dhUCM+VEGF) didn't have any advantage at this point. Four weeks post MI, EF significantly increased in three treated animal groups, compared with the non-treated animal groups, in accordance with Latipour's results [25]. Although increased EF was significantly higher in dhUCM+VEGF group compared with dhUCM only and VEGF only groups, the difference between the last two groups was trivial and negligible. Eight weeks post MI, EF enhanced in treated animal groups compared with the same measurements from the same groups four weeks after MI, but this difference was not significant. In addition, animals treated by dhUCM cells combined with VEGF have shown the highest rise in EF, with significant difference ($p < 0.05$), compared with other MI groups eight weeks post MI. Surveys that investigated synergic effects of stem cell therapy and angiogenesis therapy are rare. Yang *et al.* have suggested that BM-MSCs combined with HGF (proangiogenic factor) improve cardiac function and angiogenesis, but no significant difference was observed between BM-MCs combined with HGF and non-combined with HGF groups [40]. Also, they couldn't see the gap junctions between engrafted stem cells and cardiac tissue. Our study indicates that the combination of dhUCM cells and VEGF have more positive effects than the sole application of cell therapy or angiogenic therapy in infarcted myocardium. Synergetic effects between dhUCM cells and VEGF may be explained as the following: hUCM cells were differentiated into cardiomyocytes with high quality prior to the *vivo* administration, therefore delivering the contractile/functional cardiomyocytes to infarcted hearts that

prevent the loss and mal-differentiation of hUCM cells in cardiac microenvironment. The dhUCM cells, before and after transplantation, demonstrated Cx43 expression as a gap junction protein; in addition, Cx43 was observed between engrafted cells and host cardiac tissue (Fig. 9g). Optimal delivery route for cell therapy was unclear, but it seems that direct intramyocardial injection results in a large number of transplanted cells that accumulated in the infarcted border zone, and then migrated to the infarcted area [41]. Also, we delivered a considerable number of cardiomyocytes to infarcted myocardium that fused with the host cardiomyocytes, and integrated functionally with the recipient cardiac tissue. On the other hand, VEGF diminished cardiomyocyte apoptosis, stimulated cell proliferation, and mediated the migration of cardiac stem cells to infarcted myocardium post MI [30, 42, 43]. According to the above statements, we delivered and protected enough cardiomyocytes in ischemic hearts that hypothetically participated in cardiac regeneration, but the exact mechanism of how they function remains unclear yet. Histopathological analysis has revealed that vascular density enhanced significantly in VEGF and dhUCM + VEGF groups compared to the other groups. No significant difference in vascular density was observed between VEGF and dhUCM+VEGF groups. Despite the beneficial effect of dhUCM cells on cardiac function improvement, no angiogenesis was reported. Latipour *et al.* have found, by using hematoxylin and eosin staining, there wasn't any significant difference between non-treated animals and treated animals (with hUCM and dhUCM cells) in number of myocardium's vessels [25]. Lack of the ability to induce angiogenesis is considered as a defect for dhUCM cells, therefore, we eliminated this defect by using VEGF. Many studies have proved that VEGF increases angiogenesis, improves left ventricular function and reduces scar tissue after MI, however, conflicting data about its efficacy was reported that was primarily associated with an exposed dose of it [44-49]. Here we administered 5 μ g VEGF in normal saline as a sufficient dose [50]. VEGF injection has shown greater number of vessels in infarcted border zone in VEGF and dhUCM+VEGF groups. VEGF stimulated neovascularization through the induction of growth, proliferation and migration of endothelial cells to infarcted myocardium [51, 52] as we observed greater number of CD31 positive vessels in VEGF and dhUCM+VEGF groups (Fig. 7). After MI, injured myocardium and blood vessels were replaced by fibrosis tissue. It has been proved that stem cell engraftment by activating signaling pathways decreases fibrosis tissue [53]. Fibrosis tissue in animal treated with dhUCM cells was significantly lower than that of MI and PBS groups. We thought that after dhUCM migration and integration with the recipient cardiac tissue, fibroblasts have less chance of finding more space for proliferation and collagen production. VEGF treated animals have shown low fibrosis tissue compared with MI and PBS groups, but no significant difference was observed with the dhUCM group. Previous studies had indicated that VEGF declined scar tissue in infarcted heart [54, 55]. A possible explanation for the way VEGF decreases fibrosis formation is: VEGF induces the migration of endothelial, CSC and aortic smooth muscle cells to the infarcted area, triggers angiogenesis and facilitates the survival of vessels, promotes the proliferation

and migration of cardiomyocytes and prevents their deaths by apoptosis. All of the above suggests that the chance of fibroblasts presence in the infarcted area is limited by the characterized subsequent of VEGF administration. Effects of VEGF only and dhUCM cells only on declining the fibrosis tissue is duplicated in animals treated with both of them together. As a result, the dhUCM+VEGF group has shown the lowest fibrosis tissue formation compared to the other groups (Fig. 8).

In conclusion, we have found that intramyocardial administration of dhUCM cells combined with VEGF can optimize the beneficial effects of dhUCM cells, or the sole administration of VEGF; so simultaneous transplantation of dhUCM cells and VEGF improved cardiac function, enhanced angiogenesis and reduced fibrosis tissue formation after MI, eight weeks post MI.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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