Ex-vivo delivered stromal cell-derived factor-1α promotes stem cell homing and induces angiomyogenesis in the infarcted myocardium

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Abstract

We aimed to optimize non-viral transfection of human stromal cell derived factor (SDF-1α) gene into skeletal myoblasts (SkM) and, transplant these cells to establish transient SDF-1α gradient to favor extra-cardiac stem cell translocation into infarcted heart. Optimized conditions for transfection of SDF-1α gene into syngenic SkM were achieved using FuGene™6/phSDF-1α (3:2v/w, 4h transfection) with 125μM $ZnCl_2$ (*p<0.001*). After characterization for transgene overexpression by immunostaining, ELISA, and PCR, the cells were transplanted in female rat model of myocardial infarction. Thirty-six rats were grouped (n=12/group) to receive 70μl DMEM without cells (group-1) or containing 1.5×10^6 non-transfected (group-2) or SDF-1α transfected SkM (group-3). On day-4 post-transplantation (in 4 animals/group), marked expression of SDF-1α/*sry*-gene (*p=0.003*), total Akt, phospho-Akt and Bcl2 was observed in group-3. The number of $CD31^+$, $C-kit^+$ and $CD34^+$ cells was highest in group-3 hearts (*p<0.01*). Blood vessel density in group-3 was higher in both scar and peri-scar regions (*p<0.001*) as compared with other groups. Echocardiography showed improved indices of left ventricle contractile function and remodeling in group-3 (*p<0.05*) as compared with groups-1 and -2. We conclude that ex-vivo SDF-1α transgene delivery promotes stem and progenitor cell migration to the heart, activates cell survival signaling and enhances angiomyogenesis in the infarcted heart.

Keywords

Introduction

SDF-1α protein and gene delivery to the ischemic heart promotes endothelial progenitor cells recruitment into the ischemic myocardium to promote angiogenesis [1,2,3]. SDF-1α specifically interacts with the CXCR4 receptor and orchestrates the mobilization and homing of hematopoietic stem cells from bone marrow (BM) to the ischemic heart [3-6]. Physiologically, BM harbors a heterogenous population of cells positive for CXCR4 receptor.

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BM has the highest hSDF-1α concentration gradient as compared with other tissues, and promotes homing of circulating hematopoietic stem cells into BM stromal adult heart, SDF-1α is expressed constitutively. In the early phase after MI however, elevated SDF-1α levels have been reported in the infarct zone [7-8]. This provides the required stimulus for mobilization of stem cells from BM niches to the damaged site as a part of a natural repair process [9]. The other key players involved in tissue ischemia- induced mobilization of BM progenitors to the circulation include vascular endothelial growth factor, placenta growth factor, stem cell factor, and granulocyte colony stimulating factor [10-13]. The effect of intrinsic SDF-1α up-regulated expression is, however, transient and insufficient for cardiac repair [4,6]. In order to overcome this deficiency, SDF-1α concentration gradient in favor of myocardial scar has been achieved by the delivery of SDF-1α protein or gene encoding for hSDF-1α. The delivery strategies include intramyocardial injection of SDF-1α protein [2], naked plasmid encoding for SDF-1α [3,5,6], transplantation of *ex vivo* genetically manipulated cardiac fibroblasts overexpressing SDF-1α [4] and mesothelial cell transplantation which intrinsically express copious amounts of SDF-1α [1,14]. Moreover, intramuscular injections of GCSF (Filgrastim)[4], the sulfated polysaccharide (Fucoidan) [15-16] or "3 hydroxy-3-methylglutaryl-CoA reductase inhibitor" (Atorvastatin) [17-18] have been reported to increase the plasma concentration of hSDF-1α to a level sufficient enough to induce mobilization of stem cells from BM and their homing into the injured tissue.

The study is aimed to explore the use of synthetic vectors (3 cationic lipid vectors; Lipofectamine™2000, FuGene™6 and Polyethyleneimine-JetPEI™ wih the addition of ZnCl$_2$ for optimal transfection of hSDF-1α into SkM. We anticipate that overexpression of hSDF-1α is cytoprotective and enhances cell survival during the initial phase after transplantation. The donor SkM overexpressing hSDF-1α established a transient and localized hSDF-1α gradient in the heart that favored stem cell translocation into ischemic myocardium, and enhanced angiogenesis.

Materials and methods

SkM purification and culture *in vitro*

Skeletal muscle biopsies from syngenic male Fischer rats were dissected and roughly cut into small fragments in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL). The tissue fragments were enzymatically dissociated using collagenase-IA (2 mg/ml, Gibco BRL) and dispase (2 mg/ml, Gibco BRL) for 1h followed by trypsin-EDTA (0.25%, Gibco BRL) for 20 min at 37°C. The cells were filtered through a 100-μm sieve (Cell Strainer Nylon) and collected by sedimentation at 1500 rpm for 5 min. The enzyme reaction was arrested by adding 10% FBS. The cells were re-suspended in DMEM with 20% FBS and 5μg/ml basic fibroblast growth factor (Sigma) and pre-plated at 1, 2, 12 and 24h to remove cell debris and contaminant populations of cells. The cells obtained from pre-plate 4 were propagated and identified by anti-desmin immunostaining prior to use in all experimental studies.

Preparation of hSDF-1α plasmid

We used an expression vector containing hSDF-1α gene (pORF-hSDF-1α) purchased from InvivoGen, USA. Preparation of the plasmid was performed per suppliers instructions. Briefly, the disk of lyophylized GT100 E. coli bacteria transformed by pORF-hHSDF-1α was dissolved in E. coli FastMedia™ agar medium containing ampicillin. The hSDF-1α gene, was cut out by using SgrA-I and Nhe-I enzymes, followed by Simian virus 40 late polyadenylation (SV40 pAn) and a minimal E. coli origin of replication pMB1-Ori that is necessary for the transcription process. The expression vector had ampicillin resistance gene that allowed the selection of bacteria carrying the pORF plasmid. Plasmid DNA-hSDF-1α was purified with Maxi-prep

protocol (Qiagen) as per supplier's instructions. The purified plasmid was stored at -20°C until used for transfection of cells.

Transfection of SkM

SkM at 80-90% confluence were transfected with phSDF-1α using commercially available cationic vectors; Lipofectamine™2000 (Invitrogen), FuGene™6 (Roche) and Polyethyleneimine-JetPEI™ (Polyplus Transfection). For these vectors, transfection conditions were optimized at various DNA:vector ratios, in the presence of 0-100μM $ZnCl_2$ (Merck, Germany) at 37 °C or room temperature. Cell viability was assessed with a modified MTT reduction test [14]. As maximum transfection efficiency with highest cell viability was achieved using FuGene™6/phSDF-1α (v/w) 3:2 ratio in basal DMEM containing 125μM $ZnCl_2$ (for 4h at 37 °C), these transfection conditions were used throughout the study.

ELISA for hSDF-1α

The amount of hSDF-1α secreted by the transfected SkM *in vitro* and in the rat heart after transplantation was assessed by hSDF-1α ELISA kit (R&D systems) per supplier's instructions. For in vitro hSDF-1α expression, conditioned medium and cell lysate from transfected and non-transfected SkM were collected at regular time intervals after transfection and used for ELISA.

For in vivo hSDF-1α expression, whole left ventricle (LV) tissue samples from groups-2 and -3 were homogenized in 0.1M Tris, 4mM EDTA, 0.1% Triton X-100, pH 7.6 buffer, sonicated and centrifuged (11000g, 15min at 4°C) to extract hSDF-1α for quantification by ELISA. The results were expressed as amount of hSDF-1α/mg protein.

Western immunoblotting

Protein samples were fractionated by 12% SDS-polyacrylamide gel electrophoresis (ISC BioExpress) and electro-transferred onto a Nitrocellulose membrane. The membrane was blocked for 1h with 1×TBS blocking buffer (Cell Signaling Technology) and 5% nonfat dry milk, followed by incubation with gentle shaking at 4°C with anti-hSDF-1α (1:200, R&D System), Akt, and phospho-Akt (1:1000, Cell Signaling Technplogy) diluted in blocking buffer. The primary antibody reaction was detected by incubating for 1h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antidody (1:1000, Cell Signaling Technology). The membrane was washed and developed using LumiGLO® Peroxide reagent and exposed to X-ray for detection of the expressing bands.

Cell Labeling

For identification and tracking the fate of SkM post-transplantation, the cells were labeled with PKH-67 cell tracker dye (Sigma) as per manufacturer's instruction.

Rat heart model of MI and SkM transplantation

All experimental procedures were performed in accordance with the standard human care guidelines of the "Guide for the Care and Use of Laboratory Animals" and Institutional Animal Care and Use of Committee of University of Cincinnati, which conforms to National Institutes of Health guideline.

Acute MI model was developed in young female Fischer-344 rats by permanent occlusion of left anterior descending coronary artery (LAD) as described earlier [19]. The animals were grouped (n=12/group) to receive multiple intramyocardial injections of 70μl basal DMEM without SkM (group-1) or containing 1.5×10^6 non-transfected SkM (group-2) or hSDF-1α transfected SkM (group-3). The chest was closed and the animals were allowed to recover.

Buprenex (0.1mg/kg b.i.d) was adiministered for 24h to alleviate pain. For post-mortem studies the animals were sacrificed using an overdose of sodium pentobarbitol. The heart was excised and sectioned into 5mm blocks. Cryosections of 6-8μm thickness were cut and stained with hematoxylin and eosin for visualization of muscle architecture. Masson trichome staining was carried out to delineate fibrous tissue from the normal tissue.

PCR for gene expression

Isolation of total RNA from the different treatment groups of rSkM, and their subsequent first strand cDNA synthesis was performed using RNeasy mini kit (QIAGEN) and Omniscript Reverse Transcription (QIAGEN) kit respectively as per manufacturer's instruction. Following primer sequences were used for PCR;

hSDF-1α (270bp)	forward-5′CATGAACGCCAAGGTCGTG′3
	reverse-5′TCCAGGTACTCCTGAATCC′3
rat CXCR4 (229bp)	forward-5′GGTCATCAAGCAAGGATGTGA′3
	reverse-5′TGACTCTGTGGAGACGGAAGA′3
rat Akt (381bp)	forward-5′CCACGCACACTCGGGCCG′3
	reverse-5′CAATGCAGAGGGGTGCAGG′3
rat sry (115bp)	forward-5′GAGGCACAAGTTGGCTCAACA′3
	reverse-5′CTCCTGCAAAAAGGGCCTTT′3
ratGAPDH (738bp)	forward-5′TTCTTGTGCAGTGCCAGCCTCGTC3′
	reverse-5′TAGGAACACGGAAGGCCATGCCAG′3

MPCR kit for rat apoptotic genes set-2 (Maxim Biotech Inc.) was used as per manufacturer's instruction.

Immunohistochemical studies

Immunostaining was performed as earlier described [19]. The primary antibodies included mouse anti-hSDF-1α (10μg/ml, R&D Systems), and pre-diluted fast skeletal myosin heavy chain (fast isoform) (Abcam). The detection system was based on immunoperoxidase (Chemicon) followed by nuclear counterstaining by hematoxylin, or using fluorescence-labelled secondary antibodies conjugated with FITC, TRIRC or Alexa Fluor-594, goat anti-mouse IgG (H+L) (2 mg/ml) highly cross absorbed (Molecular Probe, USA).

For detection of the translocated stem cells, histological sections were immunostained with primary antibodies including anti-rat PECAM-1 (1:50, Chemicon), rat anti-mouse c-kit (1:30, Chemicon), CD34 (1:50, Santa Cruz), and rabbit anti-human von Willibrand Factor-VIII (vWF-VIII) (1:50, Chemicon). The total number of stem cells that migrated into the infarct and peri-infarct areas of the heart was determined by counting the cells positive for the respective surface marker in at least 3 slides from each heart.

For blood vessel density analysis, 4 animals per group were assessed after 4 weeks as previously described [19]. Briefly, tissue samples from the respective hearts (6μm thickness) were used for fluorescent immunostaining using vWF-VIII specific antibodies. Counting of blood vessels was carried out in the infarct and peri-infarct areas in the three animal groups. At least 2 microscopic fields each in the infarct and peri-infarct regions were randomly selected and counted in at least 2 tissue sections from each animal. Blood vessel density was expressed as the number of vessels at 200× high power magnification fields (0.74 mm^2).

Heart function studies

LV function and dimension measurements were carried out at 4 weeks after cell transplantation. Transthoracic echocardiography was performed using Compact Linear Array probe CL10-5 on HDI/5000 SONOS CT (HP-Company). Each animal was anesthetized, the chest of the animal was shaven and a layer of acoustic coupling gel was applied to the thorax. The animal was placed in supine position. Echocardiograms were recorded through parasternal long-axis and short-axis views. From the correct image obtained with well-defined continuous interfaces of septum and posterior wall at higher frame rate, numeric acquisitions were obtained. Anterior and posterior end-diastolic and end-systolic wall thickness, LV end-systolic (LVESD) and end-diastolic LVEDD diameters were measured from at least three consecutives cardiac cycles. Indices of LV systolic functions including fractional shortening (LVFS) and ejection fraction (LVEF) were calculated using (LVEDD-LVESD)/LVEDD×100 and LVEF={(LVEDD^3-LVESD^3)/LVEDD^3}×100 relations respectively and the results were expressed as percentage.

Statistical analysis

Statistical analysis was performed with Statview 5.0 software. All values were expressed as means± standard errors using the Student *t*-test, a one-way ANOVA or a two-way factorial ANOVA for repeated measures. *p<0.05* was considered significant.

Results

1. In vitro Studies

a) Transfection of SDF-1α into SkM—SkM during passage 2-4 were >90% pure for desmin expression. The efficiency of phSDF-1α transfection into SkM was markedly influenced by vector/plasmid ratio and optimal efficiency was obtained with FuGene™6/phSDF-1α (v/w 3:2), Lipofectamine™2000/phSDF-1α (v/w 2.5:1) and JetPEI™/phSDF-1α (N/P=5). Short-term $ZnCl_2$ exposure displayed dose-dependent cytotoxicity on SkM. However, cell viability observed with concentrations of <125μM $ZnCl_2$ was significantly higher as compared with concentrations of >250μM (*p<0.001*). Similarly, $ZnCl_2$ concentration of >125μM also caused abrupt reduction in hSDF-1α expression. Consequently, we chose 125μM $ZnCl_2$ to optimize transfection procedures with FuGene™6, Lipofectamine™2000 and JetPEI™. We observed that cell viability insignificantly changed in the presence or absence of 125μM $ZnCl_2$ (*p<0.05*) (data not shown).

The number of hSDF-1α positive SkM was markedly increased in the presence of 125μM $ZnCl_2$ with FuGene™6, and JetPEI™ (FuGene™6, *p=0.007*; JetPEI™, *p=0.004*) as compared with the absence of $ZnCl_2$ at 4 days after transfection (Figure 1A-C). We observed that transfection efficiency with FuGene™6 was higher than Lipofectamine™2000 and JetPEI™ from day-1 after transfection until 7 days of observation (*p<0.001*) (Figure 1D). Optimal transfection of SkM was obtained with FuGene™6/phSDF-1α at 3:2 (v/w) for 4h in the presence of 125μM $ZnCl_2$ as assessed by ELISA for SDF-1α in conditioned medium and cell lysate. ELISA revealed that peak level hSDF-1α expression was observed between days 3-4 after transfection (30.1±6.5 ng/mg protein in cell lysate and 15.3±0.07ng/mg protein in supernatant) followed by a gradual decline until 12 days of observation *in vitro* (data not shown). The non-transfected SkM secreted negligible amounts of hSDF-1α (1.8±0.1 ng/mg protein in cell lysate vs 0.67±0.1 ng/mg protein in the supernatant) (Figure 2A). RT-PCR showed that hSDF-1α expression in SkM transfected in the presence of $ZnCl_2$ was significantly higher as compared with the ones transfected in the absence of $ZnCl_2$ (*p<0.001*) using non-transfected SkM as control (*p<0.01*) (Figure 2B-C).

b) Cytoprotective effects of SDF-1 against anxoia—LDH release as a marker of cellular injury, was significantly higher in the non-transfected SkM upon exposure of cells to 6h anoxia as compared with hSDF-1α transfected SkM ($p=0.03$) (Figure 2D).

2. In vivo Studies

a) Donor cell survival and in vivo gene and protein expression—PCR for *sry*-gene in the female heart revealed extensive survival of the donor male SkM. Densitometry revealed 5-folds higher *sry*-gene expression in group-3 as compared with group-2 at 4 days after cell transplantation (Figure 3A). Group-1 (control) did not show *sry*-gene. In agreement with improved survival of transfected SkM, expression of mRNA-hSDF-1α was also increased significantly in the LV tissue in group-3 as compared with group-2 on day-4 of observation. When normalized with the *sry*-gene expression, there was 4-fold ($p<0.003$) increase in the expression of SDF-1α in group-3 as compared to group-2 (Figure 3A-B). These observations vividly imply that the transplanted SkM continued to overexpress hSDF-1α after transplantation at the site of the cell graft. ELISA specific for hSDF-1α was perfomed on the LV tissue to show the level of hSDF-1α secretion. The hSDF-1α protein level was 251±3.5 pg/mg protein in group-3 which was significantly higher as compared with group-2 (107±6.5 pg/mg protein) ($p<0.001$) on day-4 after cell transplantation (Figure 3C). These results were confirmed by Western-blotting which showed 1.5-fold elevated level of hSDF-1α protein in group-3 as compared with group-2 (Figure 3D).

Another important observation was the up-regulated expression of total Akt and phospho-Akt in group-3 (Figure 3D). Similar results were obtained for Akt gene expression by RT-PCR (Figure 3E). Multiplex PCR for apoptotic gene cascade showed that Bcl2 expression was higher in group-3 as compared with group-2 thus indicating a possible role for Akt signaling in hSDF-1α mediated survival of the donor SkM after transplantation (Figure 3F).

b) Stem and progenitor cell homing into myocardium—The heart tissue sections were immunostained for the presence of ckit+ (CD117), CD34+, CD31+ and vWF-VIII positive cells in different groups. The number of cells expressing these surface markers was significantly higher in the infarct and peri-infarct areas as compared with the contra-lateral areas in the heart of transfected SkM transplanted animal group-3 as compared with non-transfected SkM transplanted animal group-2 ($p<0.001$) (Figure 4A-B).

c) Effect of SkM transfection on myogenesis and angiogenesis in the heart— The PKH-67 labeled SkM were seen extensively engrafted in the infarct and peri-infarct regions after implantation in both groups-2 and -3 (Figure 5A-B). Immunostaining for skeletal myosin heavy chain (fast-isoform) showed increased DAB-immunoperoxidase staining in infarct as well as peri-infarct regions in group-3 as well as group-2 (Figure 5C-E), indicating their myogenic differentiation.

The angiogenic effect of hSDF-1α overexpression was measured in terms of blood vessel density in the center and peri-infarct regions after immunostaining for vWF-VIII (Figure 5F-L). Blood vessel counts per area (0.74 mm^2) was highest in peri-infarct (130±7.1) and infarct (71.9±3.7) regions in group-3. This was significantly higher ($p<0.001$) as compared with groups-1 and -2. Although the number of blood vessels observed in group-2 was higher as compared with group-1, no significant difference was observed between these groups either in the infarct or peri-infarct regions. Moreover, infarct size was significantly reduced in group-3 as compared with group-1 ($p=0.001$) and group-2 ($p=0.036$) (Figure 6A-D).

d) Heart function studies—Sonography for LV function revealed significant differences amongst all the groups at 4-weeks. LVEF and LVFS in transfected SkM transplanted group-3

were significantly improved (59.2±3.6% and 25.9±2.2) as compared with non-transfected SkM transplanted group-2 (46.7±4.3% and 18.0±2.2%) and DMEM injected group-1 (35.3±5.9% and 13.6±2.7%) respectively (Figure 7A-B). Similarly, significant difference in LVEF and LVFS was observed between group-2 and group-1. Moreover, LVESD, a marker of LV remodeling, was significantly decreased in group-3 (5.5±0.2 *vs* 6.2±0.1mm in group-1, *p<0.01*) (Figure-7C). However, LVEDD changed insignificantly between the groups (*p=0.3*) (Figure 7D). Posterior wall and septum thickness were significantly increased in group-3 versus group-1 and -2 (*p<0.001*).

Discussion

The main findings of our study were that (1) presence of $ZnCl_2$ significantly enhanced the transfection of phSDF-1α into SkM (2) Overexpresion of hSDF-1α rendered the cells resistant to anoxia in vitro and promoted their survival after transplantation (3) higher gradient of hSDF-1α at the site of the cell graft triggered homing of stem and progenitor cells into the infarcted myocardium and (4) transplantation of hSDF-1α overexpressing SkM promoted angiogenesis in the infarcted region and improved indices of LV-function and reverse-remodeling.

Mobilization and homing of BM derived and resident cardiac stem cells into the infarcted myocardium is the first step in the naturally occurring repair process. These mobilized stem cells subsequently differentiate to adopt cardiac phenotype [20,21]. There are reports defining a possible role for SDF-1α in post MI recruitment of extra-cardiac stem cells [6,22]. Nevertheless, the intrinsic up-regulated expression of SDF-1α in response to MI is insufficient and short-lived. Hence, forced overexpression of SDF-1α can result in pronounced stem cell homing to the infarcted heart and to ensure their participation in the repair process. Such findings may have significant clinical implications.

SDF-1α binds to CXCR4 receptor and modulates several biological functions through signal transduction pathways. These include increased cell growth, proliferation, cell survival and anti-apoptosis, emigrational and transcriptional activation [23-24]. Furthermore, SDF-1α is co-participant in angiogenesis that is regulated at the receptor level by VEGF and bFGF [25]. Mobilization of angio-competent cells from BM has been achieved by various growth factors and cytokines, either alone or in combination. However, for effective participation in the repair process, their retention at the site of injury is imperative. A recent study by Graunewald et al. (2006) has defined a novel role for SDF-1α as a "retention factor" for BM cells [26]. This ensures that the recruited BM cells stay at the site of recruitment for the duration which is long enough to pledge their participation in the on-going angiogenic cascade. Taken together, SDF-1α participates in the cardiac repair process either directly or via secondary cytokine expression. We observed that forced overexpression of hSDF-1α led to elevated levels of angiogenic factors together with activation of cell survival signaling. There were elevated levels of total Akt and phospho-Akt together with Bcl2 in the hearts transplanted with SDF-1α transfected SkM. Thus cell survival signaling by Akt/Bcl2 led to enhanced cell survival under anoxic conditions in vitro as well as in vivo after transplantation.

Various strategies have been adopted to achieve overexpression of SDF-1α in the heart. Direct gene therapy necessarily encounters a delay between gene delivery and expression which renders it less attractive as an acute-phase therapeutic option. We posit that acute phase delivery of SDF-1α gene will mitigate the ongoing intrinsic molecular cascade involved in the cell death and fibrous scar formation in the ischemic myocardium. In this regard, viral vectors may show some efficiency in gene transfer to the necrotic myocardium which otherwise is resistant to direct gene transfer [27-28]. However, the strategy is not without untoward effects. *Ex vivo* genetic manipulation of donor cells using non-viral vector is a safe alternative and provides a

tissue specific repository of the transgene product [29]. Additionally, the donor cells carrying the transgene will undergo milieu dependent differentiation for *de novo* myocardial regeneration and preservation of LV-function. SkM in this regard are excellent carriers of transgenes and have been extensively used for targeted delivery of therapeutic genes to different tissues and organs including the heart [30-32]. We have previously shown that SkM based delivery of VEGF and angiopoietin-1 transgenes to infarcted heart, either alone or in combination, gave improved neovascularization [33]. Drifting away from the use of viral vectors to alleviate safety and immunological concerns; we have optimized conditions for gene modulation of cells using cationic lipids in the presence of $ZnCl_2$ [14]. Our optimized procedure significantly enhanced the efficiency of hSDF-1α transfection into otherwise transfection resistant cells. The present study showed that hSDF-1α transfection and expression was consistently increased with FuGene™6 in the presence of $ZnCl_2$. However, the mechanism by which $ZnCl_2$ increases cell transfection efficiency is not clearly established. $ZnCl_2$ may act synergistically with cationic polymers to compact the plasmid DNA, or may facilitate DNA entry into cytosol via fusion of the negatively charged DNA with cell membrane. It also inhibits endonuclease activity and prevents pDNA degradation in the cytoplasm thus increasing the probability of pDNA reaching to the nucleus for more stable expression [34].

The elevated levels of hSDF-1α in and around the area of infarct elicited strong signals for homing of the extra-cardiac stem and progenitor cells into the heart including CD31, CD34 and CD117. We have recently shown a significant role of SDF-1α in the accumulation of stem cells in the heart after MI. The population of extra-cardiac cells homing into the injured myocardium was identified by their surface markers [22]. In another recently published work from our lab, we have shown that the mobilized cells exerted paracrine effects via expression of various growth factors and cytokines. This promoted cell growth, proliferation, survival and anti-apoptosis, migration and transcriptional factor activation [35]. Together with the factors from cell survival signaling cascade including Akt and Bcl2, these cellular and biochemical changes in the infarcted heart promoted better cell survival via anti-apoptotic effects, myogenesis and improved neovascularization.

Finally, we have shown significant improvement of cardiac function after hSDF-1α/SKM therapy, with preservation of LVEF%, LVFS% and a decrease in LVESD in the hearts transplanted with transfected cells. An ideal therapeutic intervention for MI requires improved reperfusion of the ischemic myocardium by restoration of regional blood flow together with compensation for cardiomyocytes loss in order to restore myocardial contractility. There is evidence in literature that the cells overexpressing angiogenic growth factors show better survival and neo-vessel formation after transplantation and improve cardiac function by limiting the remodeling process in the scar and/or decreasing apoptosis of hypertrophied myocytes in the peri-infarct region.

In conclusion, our results depict successful and efficient non-viral vector transfection of SkM with hSDF-1α using FuGene™6 in the presence of $ZnCl_2$ and show that transplantation of these transfected SkM in vivo is a useful procedure in the development of techniques of indirect gene therapy after MI. In line with the results of a previous study which showed that combined intramyocardial delivery of SkM and BM cells gave superior prognosis as compared with either of the two [36], our approach indirectly harnesses the beneficial effects of SkM and BM cells, the two potential candidate cell types for heart cell therapy, together with cytokine therapy. Moreover, we demonstrate that the combination of therapeutic gene transfer with cell therapy may represent a promising new avenue of investigation in the treatment of patients with ischemic LV dysfunction.

Acknowledgments

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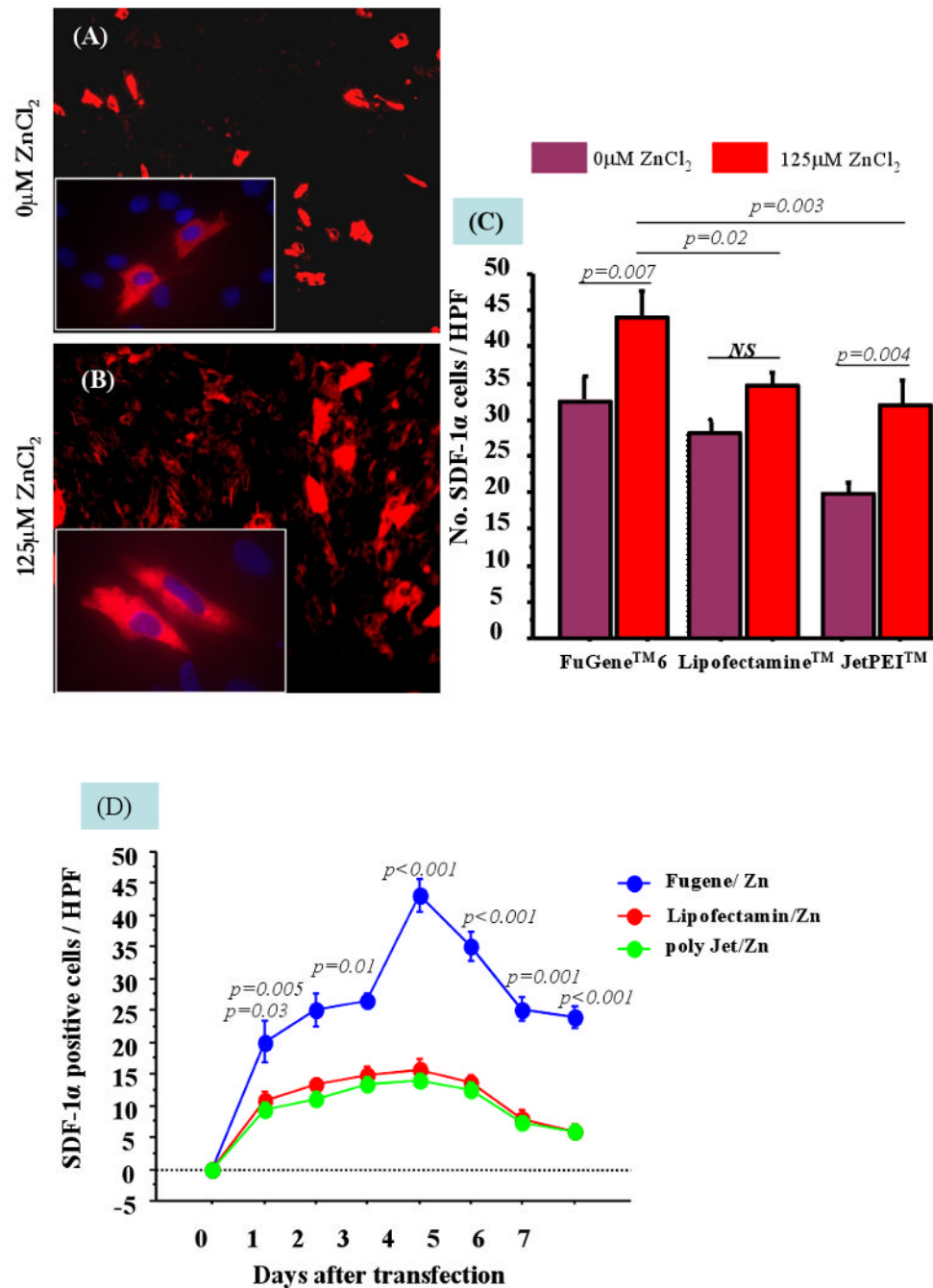
Figure-1.
Immunostaining of SkM for hSDF-1α overexpression in vitro after transfection with
FuGene™6/phSDF-1α (A) in the absence of 125μM $ZnCl_2$ or (B) in the presence of 125μM
$ZnCl_2$. (C) The addition of 125μM $ZnCl_2$ significantly improved the transfection efficiency of
FuGene™6, Lipofectamine™2000 and JetPEI™. (D) The highest transfection and expression
of SDF-1α in the presence of 125μM $ZnCl_2$ was achieved with FuGene™6 until 7 days of
observation.

(A)

ELISA for SDF-1α

(B)

(C)

(D)
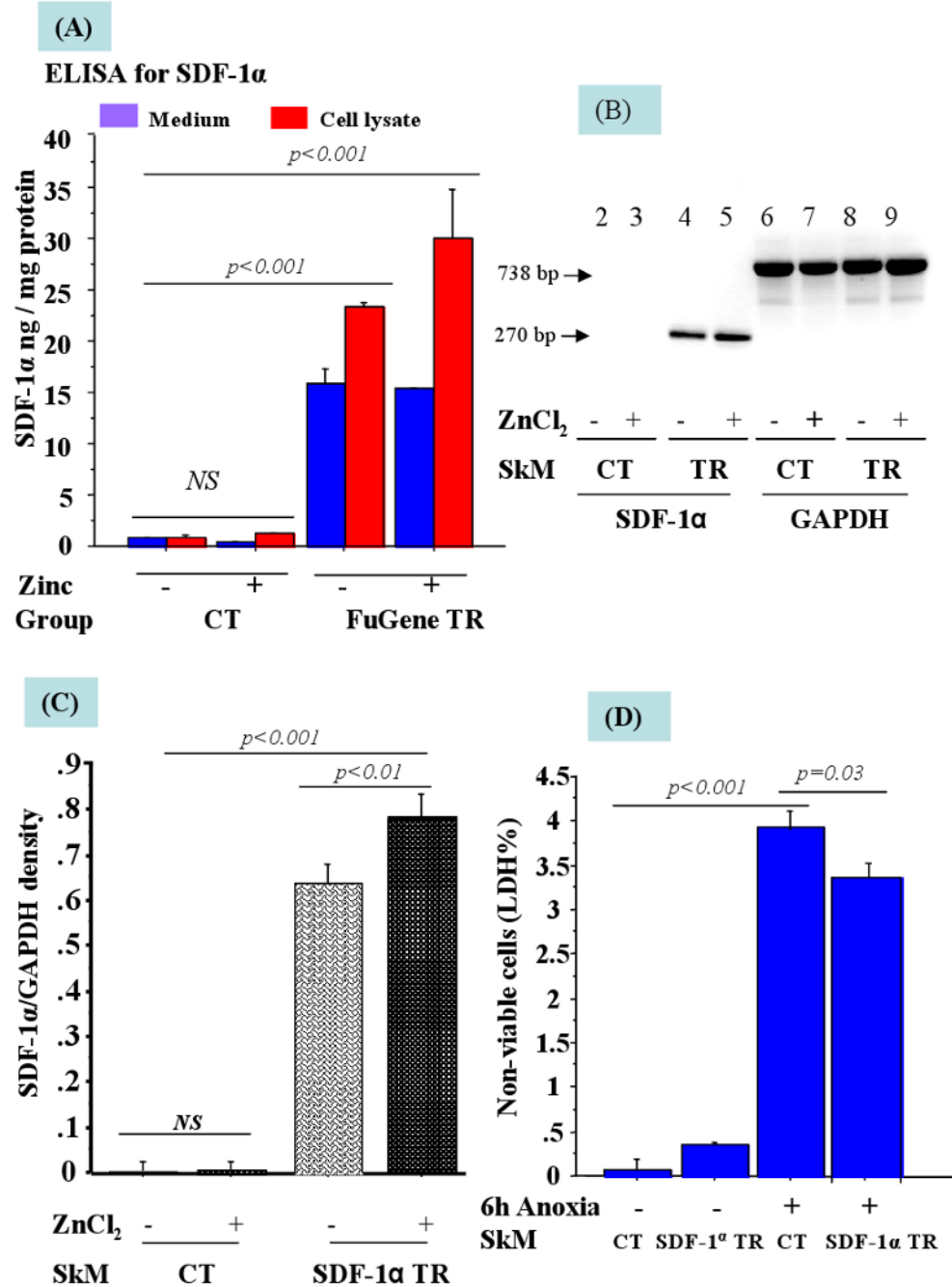
Figure-2.
(A) ELISA for SDF-1α in the supernatant and cell lysate from SkM tranfected with FuGene™6/phSDF-1α in the presence and absence of $ZnCl_2$. The level of hSDF-1α (ng of hSDF-1α /mg of total protein) was significantly higher in the cell lysate as compared with the cell supernatant. (B) Expression of hSDF-1α in non-transfected and transfected SkM without (-) or with (+) 125μM $ZnCl_2$. Lanes 2-5 show PCR product of hSDF-1α and Lanes (6-9) show GAPDH. (C) Densitometry for hSDF-1α to GAPDH ratio from 3 separate experiments is shown as arbitrary units. (D) Overexpression of hSDF-1α in SkM enhanced their resistance to anoxia when SkM were exposed to 6h anoxia as shown by significantly improved cell viability by LDH assay. (CT= Control; TR= Transfected).
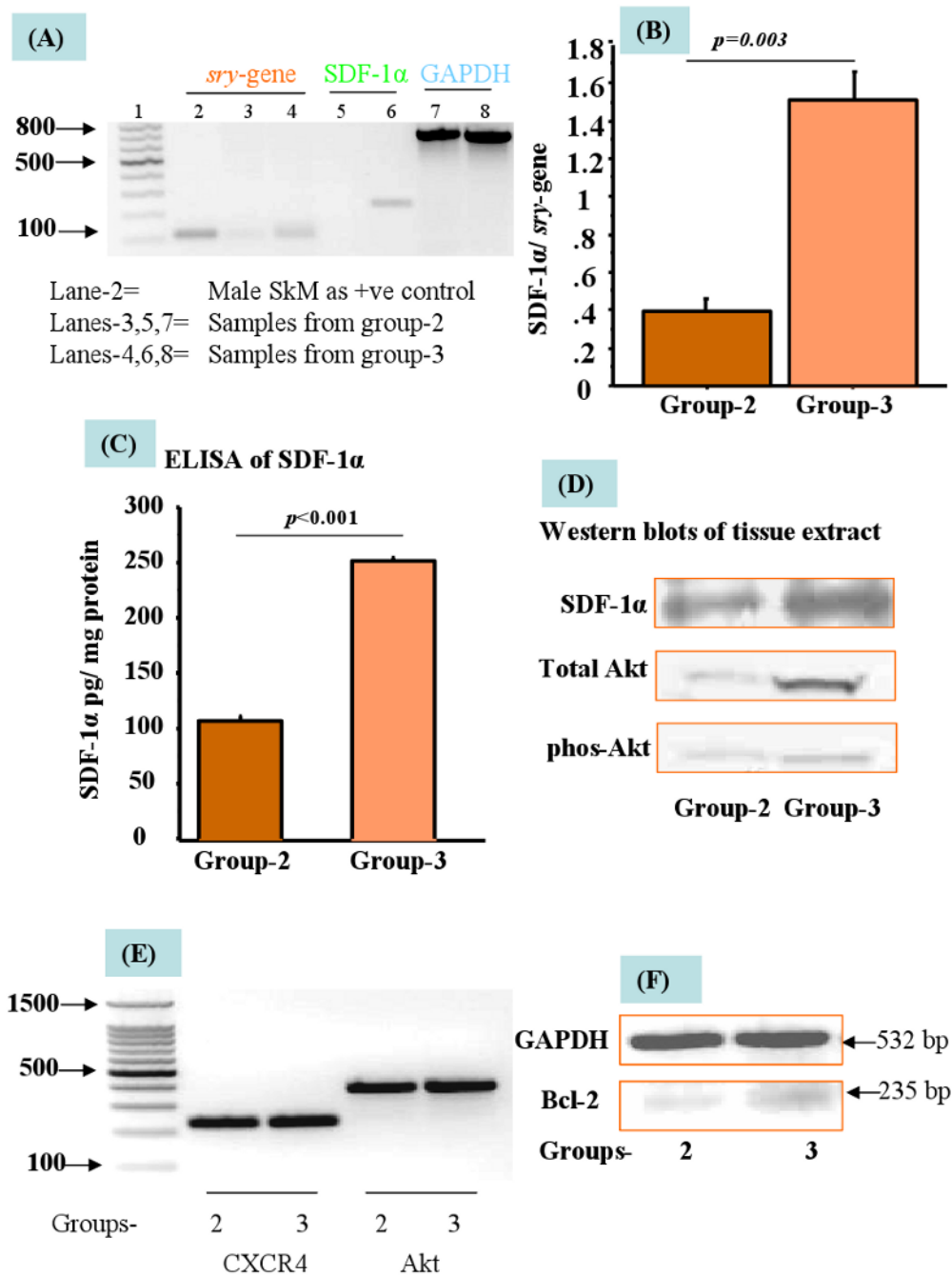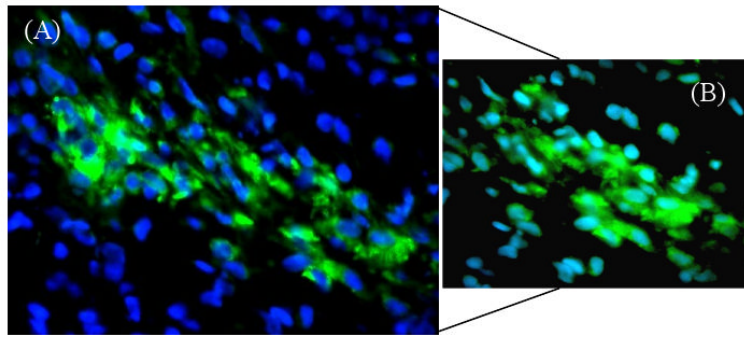
Figure-3.
The effect of SDF-1α overexpression in the rat heart. (A) Significantly higher *sry*-gene in group-3 was observed as compared with group-2. (B) The ratio of hSDF-1α to *sry*-gene was significantly higher in group-3 as compared with group-2 thus indicating a relationship between hSDF-1α expression with the survival of SkM in the rat heart. (C) ELISA showed significantly higher hSDF-1α (pg/mg of total protein) in group-3 as compared with group-2 at day 4 after SkM transplantation. (D) Western-blotting of rat heart ptrotein extract showed that besides elevated hSDF-1α expression, higher total Akt and phospho-Akt was observed in group-3 as compared with group-2 on day 4 posttransplantation. (E-F) Akt and Bcl2 gene expression normalized with GAPDH was also higher in group-3.
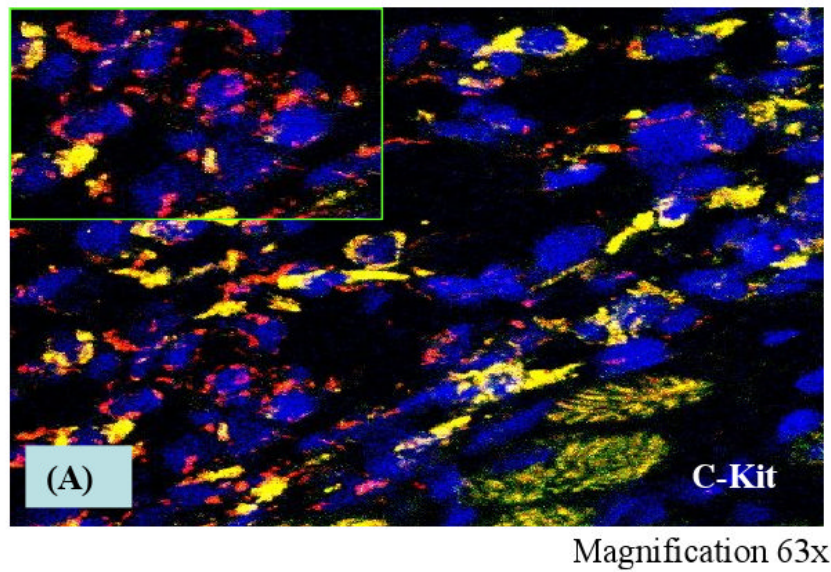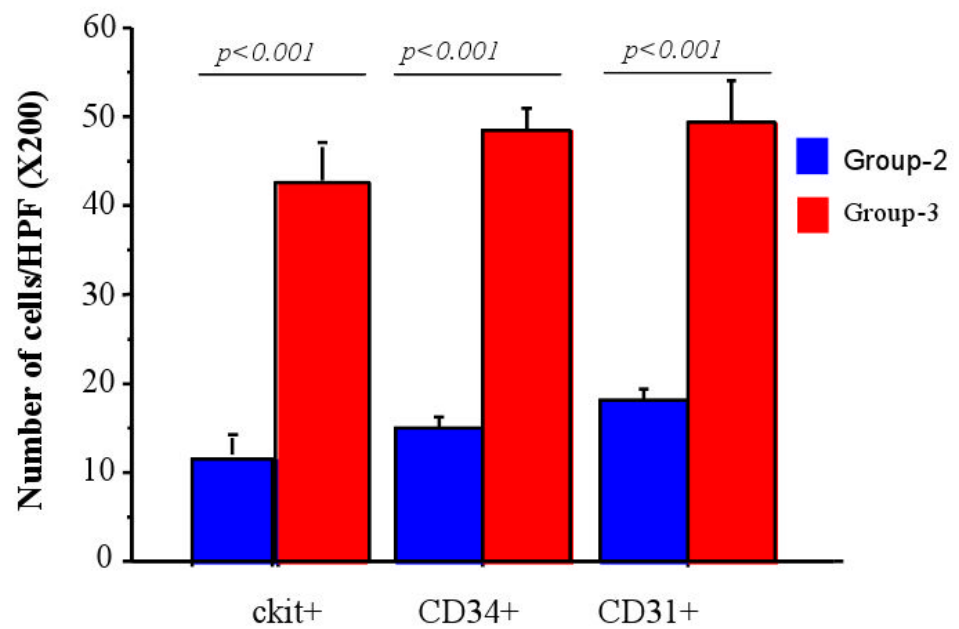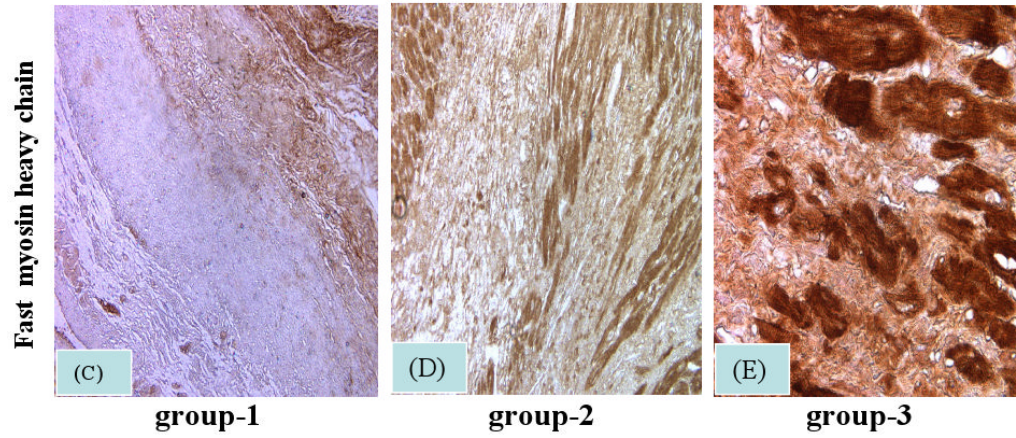
Figure-4.
Confocal image after immunostaining of rat heart tissue grafted with SkM (PKH-67 labeled, green) and enriched with ckit+ cells (red fluorescence 63× oil immersion) with their nucleus stained with DAPI (blue) in group-3. The number of ckit+, CD34+ and CD31+ were counted per high power field.
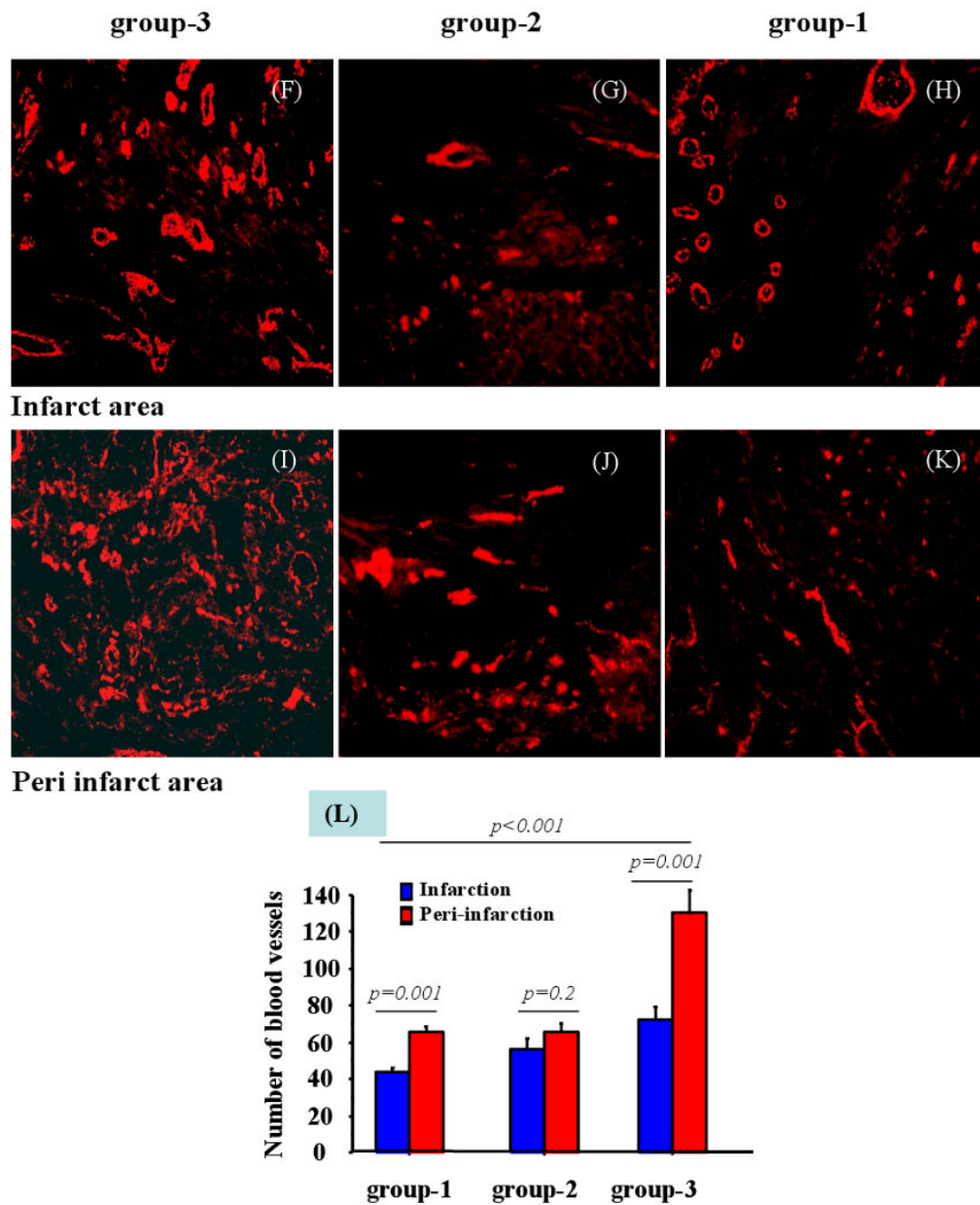
Myogenic differentiation

Figure-5.
Assessment of survival and angiomyogenesis in the infarcted myocardium. (A-B) Extensive survival of PKH-67 labeled SkM was observed in group-3 in the infarcted myocardium. (C-E) Immunostaining of rat heart tissue for fast skeletal myosin heavy chain expression showed extensive myogenesis in the infarct and peri-infarct areas in both (D) group-2 and (E) group-3 using (C) group-1 as control. Blood vessel density was assessed in the infarct (F-H) and peri-infarct areas (I-K) in all the groups which showed that (L) blood vessel density was significantly higher in group-3 as compared with groups-1 and -2.
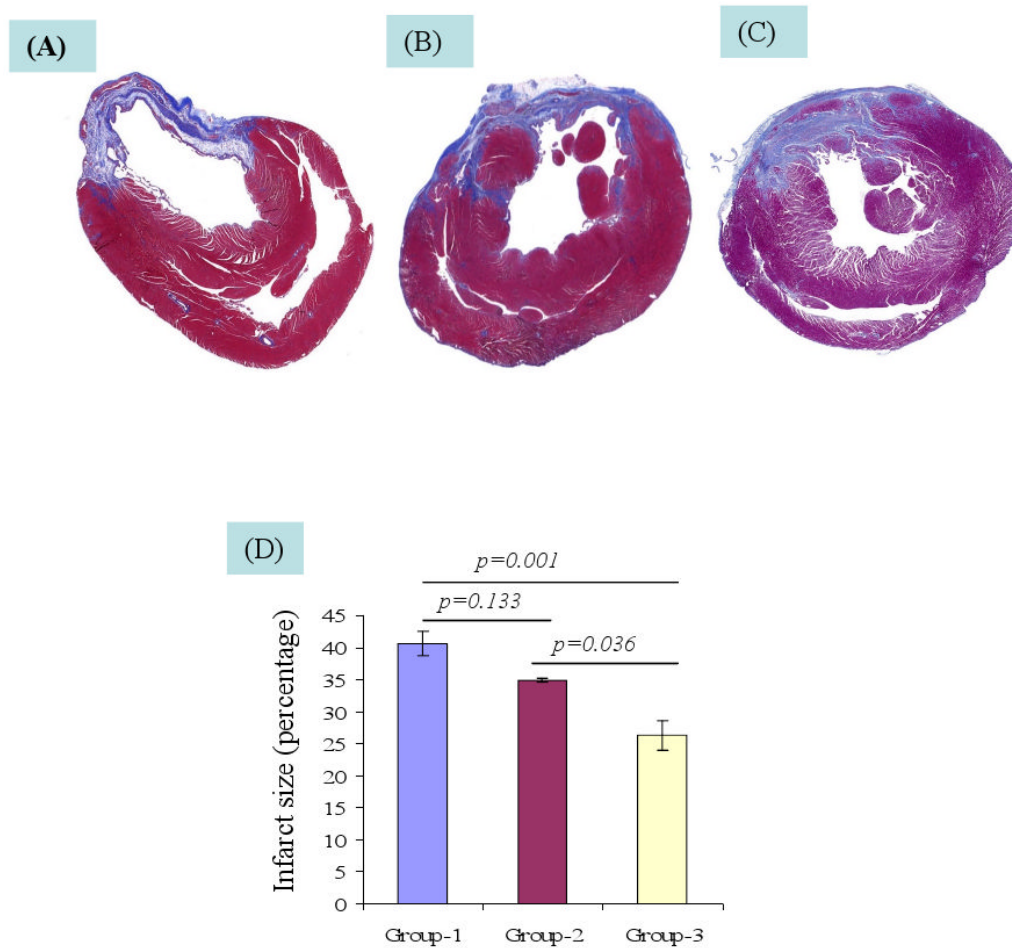
Figure 6.
Infarction size was measured after Masson trichome staining of the rat heart tissue sections (A) DMEM injected group-1 (B) non-transfected rSkM transplanted group-2 (C) SDF-1α transfected group-3. (D)Whereas the infarct size changed insignificantly in group-2 as compared with group-1 (*p=0.133*), significantly reduced infarct size was observed in group-3 as compared with group-1 (*p=0.001*) and group-2 (*p=0.036*).
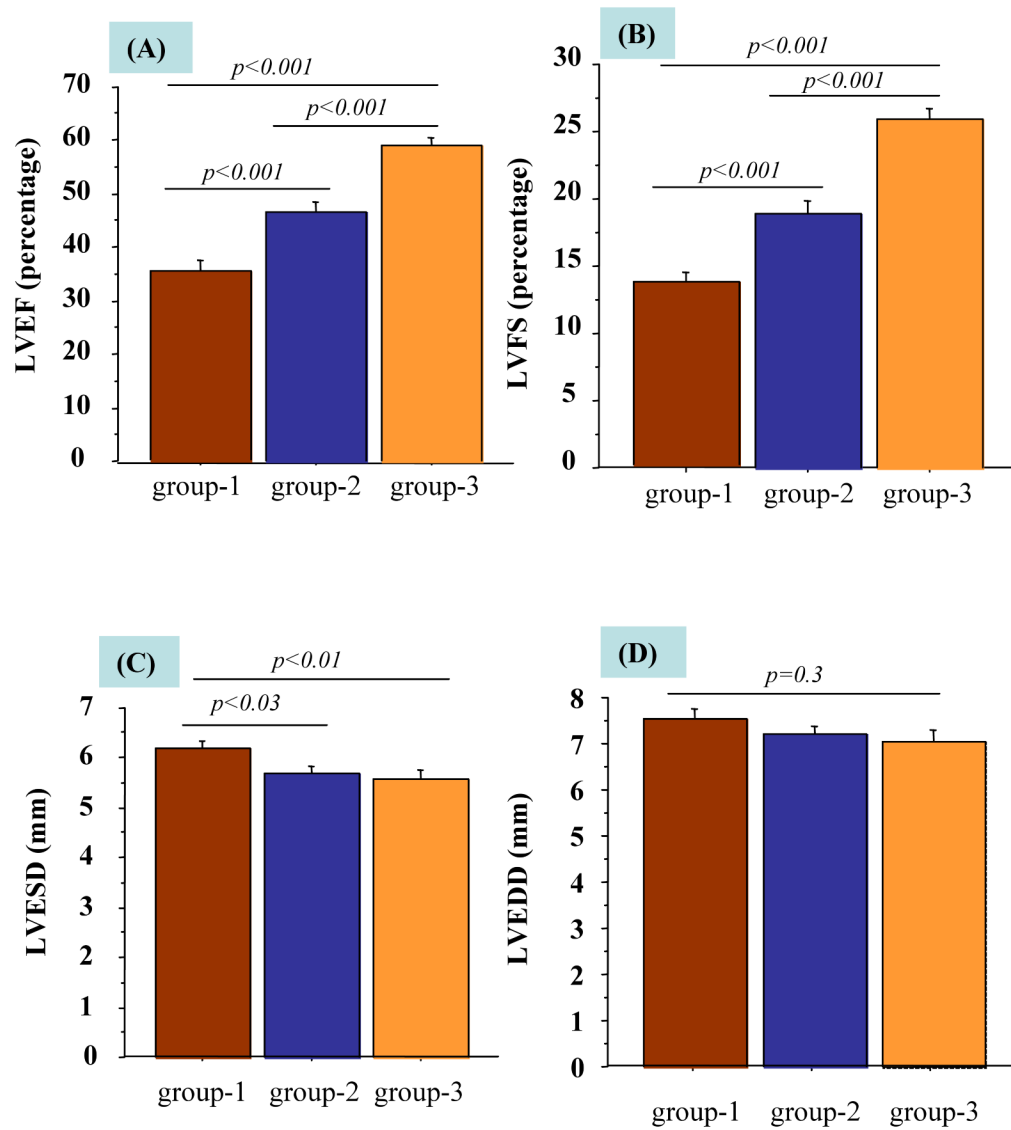
Figure 7.
Echocardiographic assessment of heart function. (A) LVEF (B) LVFS (C) LVESD and (D) LVEDD were compared in all the groups after 4 weeks of respective treatment.