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Original paper

Adipose Tissue-derived Cardiomyocytes for Enhancing Cardiac Remodeling in a Rat Model of Acute Myocardial Infarction

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Abstract

This investigation was directed towards addressing the pivotal role of adipose tissue-derived cardiomyocytes seeded onto nanofiber (NF) in repairing the deteriorated cardiac tissue in a model of MI. Molecular analysis for *MEF2C* and *Actn* expression was attained to ensure the differentiation of ADMSCs into cardiomyocytes *in vitro*. The *in vivo* study was conducted on forty adult rats assigned into four groups: (1) control; (2) MI; (3) MI treated with adipose tissue-derived cardiomyocytes; (4) MI treated with adipose tissue-derived cardiomyocytes seeded onto NF. Treatment of MI-challenged rats with adipose tissue-derived cardiomyocytes modulates ST height, heart rate, RR, PR, QTc, QRS intervals and P duration as manifested in the ECG. The biochemical parameters corroborated the ECG outcomes as they displayed significant inhibition in serum LDH and CK-MB enzymes activity as well as significant suppression in cardiac cTnT level paralleled with significant elevations in cardiac Cx43 and Actn levels in the treated groups. Molecular analysis of *GATA4* and *NKX2.5* gene expression levels declared significant downregulation in the treated groups. Photomicrographs of cardiac tissue sections of rats in the treated groups showed great renovation in the cardiac microarchitecture. Conclusively, this study delivers a futuristic approach for treatment of MI by applying differentiated cardiomyocytes systematically.

Keywords Adipose tissue-derived cardiomyocytes, Electro-spun nanofibers, *In vitro*, *In vivo*, Myocardial infarction.

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Introduction

Acute myocardial infarction is one of the master causes of human mortality all over the world. It contributes to massive loss of cardiomyocytes, interchanges of unfavorable biological environment and modifying electrical connections by fibrosis scar constitutions. Thus, MI results in shortage of blood supply to the heart, leading to heart spoilage and heart failure (Si et al. 2020). Moreover, perturbation of homeostatic balance produces a series of sequential issues like ventricular stiffness, inflammatory events, necrosis, apoptosis, remodeling that leads to the alteration of heart muscle contractility. Medical and surgical advances such as pharmacotherapies and percutaneous coronary interference can crucially offer their benefaction towards terrible impacts of MI (Yoshizumi et al. 2016). Meanwhile, they display ineffectual towards rejuvenating myocardium and assembling new contractile tissues. As regards to solving this issue, empirically and virtually substantial risk heart transplantation is the unique mandatory medication for MI. Thus, to avoid this difficult therapy, great effort of investigators has led to principles of cell inoculation, a rising therapeutic modality *versus* MI (Liang et al. 2019).

Cell-based regenerative medicine could improve blood supply to the damaged heart, and minimize the area of infarction (Bolli et al. 2013) through secretions of several cytokines such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Zhao et al. 2015). Direct injection of cells into the heart is problematic, because of a significant loss of living cells in addition to the pro-arrhythmic effects (Song et al. 2010).Tissue engineering using cell sheets has been developed to overcome these disadvantages; the cell sheet could improve the viability of cells (Patila et al. 2015) and prolong secretion of cytokines (Memon et al. 2005). Initially, sheets of skeletal myoblast cells have been reported to enhance cardiac functions in patients with severe heart failure. However, collecting skeletal myoblasts from patients requires invasive procedures, and it takes a long time to obtain a sufficient number of myoblasts. In addition, myoblasts secrete low levels of cytokines and lack capability to differentiate into cardiomyocytes (Miyagawa et al. 2010). Thus, an alternative source of stem cells for cell sheets is required.

To date, mesenchymal stem cells (MSCs) have become the most practiced cell types of clinical trials for the intervention of MI (Lee et al. 2015), due to their safety, multi-differentiation potential, nutritional activity, immunomodulatory properties, and abundant donor sources. MSCs have low immunogenicity due to the low expression of major histocompatibility complex MHC II as well as the lack of expression of MHC I on T-lymphocytes, which act as molecules to present processed antigens, leading to immune tolerance and allowing allogeneic transplantation (Miao et al. 2017).

Adipose tissue-derived mesenchymal stem cells (ADMSCs) cultured with the proper stimulating growth factors can differentiate into several lineages, including cardiomyocytes-like cells, suggesting their potential as a cell source for repairing the damaged cardiac tissues. It has been stated that they secrete numerous cytokines and growth factors to counteract cardiac dysfunction and remodeling after MI (Otsuki et al. 2015). However, the poor survival and low engraftment efficiency of the donor cells in the infarcted myocardium challenged its therapeutic efficacy.

Biomaterials can assist cell survival, integration and communication with a proper microenvironment that closely mimics the native tissue architecture (Liu et al. 2012). Therefore, they might promote the appropriate differentiation and maturation of cardiac progenitor cells (CPCs) for cardiac tissue regeneration. Various types of scaffolds have been explored to accommodate heart tissue cells. To guide the organization, growth, and differentiation of cells, biomaterial scaffolds should possess the ability to provide not only mechanical support for the cells, but also the chemical and biological cues needed for stimulating the specific differentiation of cells (Langer and Tirrell 2004). Thus, it should provide a 'microenvironment' for stem cell responsible trans-differentiation effect both at the anatomical and functional dimensions (Scadden 2006). Electrospun nanofibers (NF) are novel materials characterized by an enormous surface to volume ratio, high porosity, and a structure resembling that of the extracellular matrix, thus facilitating their employment in a broad range of biomedical and tissue engineering applications (Agarwal et al. 2008).

The present approach was tailored to establish the possible contribution of adipose tissue-derived cardiomyocytes seeded onto NF in intensifying cardiac relieve in an experimental model of MI.

Materials and methods

Nanofibrous Scaffold Coated Plates

Nanofibrous scaffold coated plates were purchased from Corning® Lab Ware with Ultra-Web™ (Corning Incorporated, USA). Ultra-Web synthetic surfaces are composed of randomly orientated electro-spun polyamide nanofibers with an average fiber diameter of 280 nm. This creates a culturing substrate that mimics the structural components within the basement membrane or extracellular matrix.

Protocol for Isolation of ADMSCs

Adipose tissue was excised from both the omentum (i.e., abdominal) and inguinal regions of male *Wistar* strain rats (Aird et al. 2015). The excised adipose tissues (1 cm^3) were washed extensively with phosphate buffer solution (PBS, Biowest, France) to remove the contaminating debris and red blood cells, then minced with fine tissue scissors. The fragmented tissues were incubated with 0.1% collagenase (NB4 standard grade from Serva Electrophoresis GmbH, Germany) and kept in a slow shaking water bath at 37°C for 60 min. Thereafter, collagenase was removed by diluting the

samples with PBS. The cell suspension was centrifuged twice at 800 xg for 10 min. The supernatant containing mature adipocytes was removed and the precipitate was passed through 100 µm mesh syringe filter generated from 100% borosilicate glass microfiber (Chen et al. 2014).

The obtained cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biowest) high glucose containing 30% fetal bovine serum (FBS, Biowest) and 1% penicillin-streptomycin (Biowest) for 10 to 14 days in the culture flask. Then, the flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. To exchange the medium, the flasks were washed with PBS in order to remove non-adhered cells and the medium was replaced. This process was performed at least two times/week. Plentiful ADMSCs with confluence of 95 % in the culture plate were harvested using 0.25% trypsin/1 mM EDTA (Biowest) to be re-cultured again as 1stsubculture. This process was repeated till reaching 3rd subculture (Lin et al. 2016).

Identification of ADMSCs

ADMSCs culture flasks were examined under optical inverted microscope in order to determine their morphological feature. The morphology of ADMSCs is one of the hallmarks of MSCs. Flowcytometry technique was accomplished at the 3rd passage of ADMSCs using CD44, CD90 and CD45 cell surface antigens to emphasize whether the isolated ADMSCs preserve their phenotype after expansion in culture (Woodbury et al. 2000). The phycoerythrin (PE)-conjugated CD44 and CD90 antibodies were supplied from Milteny Biotech (Germany) and R&D Systems (UK) respectively. While, the fluorescein isothiocyanate (FITC)-conjugated-CD45 antibody was acquired from Dako Co. (Denmark). The cells were incubated with the antibody against each of the surface antigens for 30 min at 4°C for CD44 as well as CD90 and 10 min at 4°C for CD45 followed by flowcytometry analysis using Beckman Counter Elite XL (USA) equipment. The surface marker antigens represent the second most common trait of MSCs.

Differentiation of ADMSCs into Cardiomyocytes

Differentiation of ADMSCs into cardiomyocytes was induced by adding a cocktail of growth factors for 12 successive days following the method described by Hahn et al. (2008) with slight modification. The differentiation medium consisted of a combination of 2 µl/100 ml bone morphogenetic-2 (BMP-2; R&D Systems, UK), 1 µl/100 ml insulin-like growth factor-1 (IGF-1; Komabiotech, Korea), 5 µl/100 ml fibroblast growth factor-2 (FGF-2; Bio Basic Inc., Canada), 10 µl/100 ml dexamethasone (Sigma, USA) and 146.6 µl/100 ml ascorbic acid (Sigma) in a complete medium composed of antibiotic antimycotic (l ml/100 ml, Biowest), FBS (2 ml /100 ml, Biowest) which was completed to 100 ml DMEM high glucose (Biowest).

Detection of Cardiomyocyte Differentiation by qRT-PCR

After 12 days of ADMSCs culturing in the differentiation media, the cells were harvested using 0.25% trypsin EDTA to be subjected to molecular analysis for both myocyte-specific enhancer factor 2C (*MEF2C*) and alpha sarcomeric actin (*Actn*) to ensure the differentiation of ADMSCs into cardiomyocytes. Total RNA was isolated using an RNA extraction kit (RNeasy; Qiagen, Germany) and 2 µg of total RNA was subjected to reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen) as recommended by the manufacturer manual. Quantitative real-time polymerase chain reaction (qPCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instruction. Specific primer sequence for *MEF2C*, *Actn* and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used for qPCR. The primer sequences for detecting *MEF2C* gene were F: 5′-AGCAGGTGCTGACGGGAACAA-3′ and R: 5′-TCACAGTCGCACAGCACGCTC-3′ (Zhang et al. 2012). While, the primer sequences for detecting *Actn* gene were F: 5′-GACCACAGCTGAAC-GTGAGA-3′ and R:5′-CATAGCACGATGGTCGATTG-3′ (Hahn et al. 2008). Furthermore, the primer sequences for detecting *GAPDH* gene were F:5′-GGCTCTCTGCTCCTCCCTGTT-3′ and R: 5′-GCGGGATCTCGCTCCTGGAAG-3′ (Zhang et al. 2012). qPCR was carried out under the following thermal conditions: an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 94°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s, and a final extension step of 10 min at 72°C. Each reaction was done in a total volume of 20 µl, consisting of 12.5 µl SYBR@ Premix Ex, 0.5 µl each primer (10 µmol/L), 2 µl cDNA and 4.5 µl ddH_2O. The main equations used were ΔCt = Ct (gene of interest) – Ct (housekeeping gene) followed by $\Delta\Delta Ct = \Delta Ct$ (treated sample) – ΔCt (untreated sample) and the overall formula to calculate the relative fold gene expression level was $2^{-\Delta\Delta Ct}$ which was employed to quantify the relative amount of mRNA expression normalized to GAPDH.

Labeling Cells with Iron Oxide Nanoparticles

The Feridex IV (Berlex Laboratories, USA), a sterile aqueous colloid of dextran-coated super paramagnetic iron oxide nanoparticles (at a concentration of 25 µg/ml) and poly-L-lysine (PLL, Sigma-Aldrich, USA) (at a concentration of 375 ng/ml) were used for labeling of the differentiated cardiomyocytes. Feridex was mixed with PLL (1:10) and the mixture was shaked for 30 min at room temperature before being added to the supplemented medium. The differentiated cardiomyocytes were incubated in the labeled medium for 24 hours before infusion in the experimental animals.

Animal Handling

Forty adult male albino rats of *Wistar* strain weighing 150-180 g were provided from the Animal Care Facility of the National Research Centre, Giza, Egypt and housed in polypropylene cages in an animal holding room of Hormones Department under ambient temperature (25±1°C), a relative humidity (60±5 %), 12-hour light and dark periodicity and *ad libitum* access to tap water and a standard rodent chow consisted of 10 % casein, 4 % salts mixture, 1 % vitamin mixture, 10 % corn oil, 5 % cellulose and completed to 100 g with corn starch (Meladco Co., Cairo, Egypt). The animals were kept under observation for about 14 days before the onset of the experiment for accommodation.

Ethical Statement

Housing and management of animals followed the recommendations and guidelines for the care and use of laboratory animals. The study was carried out in compliance with the code of ethics of the World Medical Association (Helsinki Declaration) for animal experiments and the experimental protocol was approved by the institution Ethical Committee for Medical Research of the National Research Centre, Egypt (Code No:15 016).

Creation of Animal Model of MI

Myocardial infarction (MI) was induced in rats by subcutaneous injection of isoprenaline (Sigma-Aldrich, USA) in a daily dose of 100 mg/kg for 2 consecutive days (van Dijk et al. 2011). The calculated dose of isoprenaline/rat was dissolved in 1 ml of saline.

Experimental Groups

Rats (n=40) were randomly grouped into four equal groups: group (1) negative control [control], group (2) myocardial infarction group [MI], group (3) MI-challenged rats infused intravenously once with adipose tissue derived-cardiomyocytes (3×10^6 cells/rat) [Diff. ADMSCs] and group (4) MI-challenged rats infused intravenously once with adipose tissue derived-cardiomyocytes seeded onto nanofiber (3×10^6 cells/rat) [Diff. ADMSCs on NF] (Yip et al. 2008).

Electrocardiography

After two months of differentiated ADMSCs transplantation, the rats in each group were submitted to electrocardiography (ECG) for validation of MI and for verification of the therapeutic influence of the differentiated ADMSCs. Rats were anesthetized with pentobarbital (25 mg/kg, IP), and placed in a prone position on a board and the electrodes were put subcutaneously in the extended limbs of the rat. ECG amplifier was set to channel I and data were acquired from lead II. V+ needle was inserted to the left leg of the rat, the V- needle was inserted into the right arm of the rat and the ground needle was inserted into the right leg of the rat. Then, the ECG parameters were registered using computerized ECG apparatus (Kent Scientific, USA). Heart rate per minute, was achieved from ECG recordings of average one minute, RR, PR, QTc and QRS intervals also P duration and ST height were calculated from ECG recordings by the computer.

Blood and Tissue Sampling and Homogenization Procedure

Next to taking the ECG recordings, the blood samples were drained from the retro-orbital venous plexus after one night of food deprivation. The blood specimens were allowed to coagulate for 45 min at room temperature to obtain sera. The clear serum samples were isolated by centrifugation at 1800 xg for 15 min at 4 °C using cooling centrifuge and maintained at -20 °C until analysis of LDH and CK-MB enzymes' activities. After collection of the blood samples, the animals were euthanized by cervical dislocation and the heart was rapidly and carefully excised, then partitioned into three portions. The 1st portion was weighed and homogenized in ice-cold medium containing Tris-HCl (50 mmol L^{-1}, pH 7.4) (Hartl and Bister 2013) for biochemical determinations. The 2nd portion was immediately frozen in liquid nitrogen and stored at −80°C prior to extraction for molecular analysis. The 3rd portion was fixed in formalin saline (10%) for histopathological procedure.

The acquired homogenates (10% w/v) from the different studied groups were centrifuged (1800 xg/20 min/4 °C) and the supernatants were obtained and preserved at -20°C pending biochemical assessment (Xu et al. 2012).

Determination of Serum LDH and CK-MB Enzyme Activities

Serum LDH and CK-MB enzyme activities were determined by the spectrophotometric kinetic method according to the manufacturer's protocol provided with the LDH and CK-MB assay kits (Chronolab Systems, Spain).

Assessment of Cardiac Biochemical Variables

Troponin T, Cx43 and Actn levels in cardiac tissue were quantified using ELISA kits, (Glory Science Co., USA) following the manufacturer's manuals.

Molecular Genetic Analysis

Total RNA was isolated from heart tissue using the RNeasy Mini Kit (Qiagen). Approximately 1 µg of total RNA was subjected to reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instruction. qPCR was carried out using the QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's protocol. Specific primers for *GATA4*, *NKX2.5* and housekeeping gene; *GAPDH* were used for qPCR. The primer sequences for detecting *GATA4* gene were F: 5′-GCCAACCCTGCGAGACACCC-3′ and R: 5′-TCAGCGTTACGGCGCCACAG-3′ while, for detecting *NKX2.5* gene were F: 5′-CCACCTGGCGCTGTGAGACC-3′ and R: 5′-GAAGCGCCGCTCCAGCTCAT-3′. Furthermore, the primer sequences for detecting *GAPDH* gene were F: 5′-GGCTCTCTG-CTCCTCCCTGTT-3′ and R: 5′-GCGGGATCTCGCT-CCTGGAAG-3′ (Hahn et al. 2008). qPCR was performed under the following thermal conditions: an initial denaturation step at 94°C for 4 min, followed by 40 cycles of 94°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s, and a final extension step of 10 min at 72°C. Each reaction was done in a total volume of 20 µl, consisting of 12.5 µl SYBR@ Premix Ex, 0.5 µl each primer (10 µmol/L), 2 µlcDNA and 4.5 µl ddH_2O. The main equations used were ΔCt = Ct (gene of interest) – Ct (housekeeping gene) followed by ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample) and

the overall formula was $2^{-\Delta\Delta C}$ to calculate the relative fold gene expression level.

Hematoxylin and Eosin Staining

After fixation of heart tissue in 10% neutral formalin saline for 24 hours, washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were utilized for dehydration. Specimens were cleared in xylene and embedded in paraffin bee wax at 56 °C in hot air oven for 24 hours. Paraffin bee wax tissue blocks were submitted for sectioning at 4 microns thickness by rotary microtome. After routine de-waxing and hydration, hematoxylin and eosin staining was used and the histopathological alterations of heart tissue were noticed using optical microscope (Bancroft et al. 1996).

Statistical Processing

The results of this study were expressed as mean ± SD. Graph Pad Prism 5 software was used to estimate the statistical analysis. A p value of<0.05 was considered statistically significant using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

Results

Identification of ADMSCs

Morphological Feature

ADMSCs exhibited a spindly, fibroblast-like morphology and formed clusters under optical inverted microscope at the 5th day of culturing which represents the traditional phenotypic attitude of MSCs. On the7th day, the cells appeared with evident expansion and proliferation, generating small colonies with several fusocellular, triangular, and polygonal cells. In fact, few cells were triangular or polygonal in shape; however, most of the cells were basically with short or long spindle-shaped appearance. Thereafter, the cells manifested typical morphology with multilayered flat cell bodies having short cell processes connected to adjacent cells on the 14th day with 95% confluence **(Fig. 1)**.

Surface Antigen Profile Analysis

The ADMSCs were also characterized *via* their surface antigen profile using flowcytometry technique. The cells were positive for CD44 by 92.9 % and CD90 by 95.2 % and negative for CD45 by 12.6 % **(Fig. 2)**.

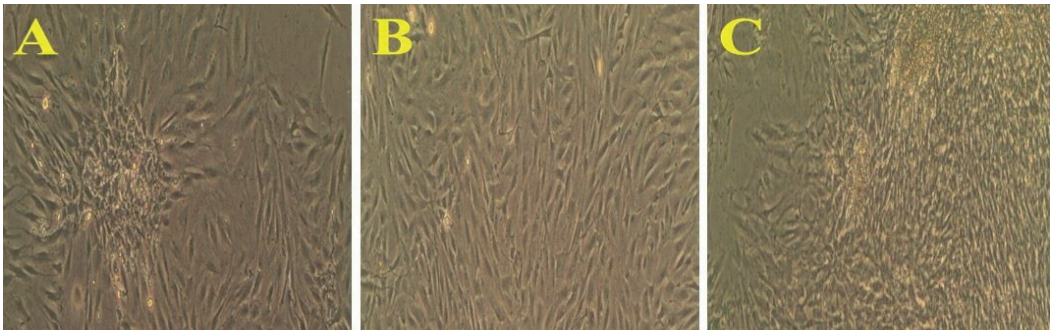

Figure 1. Morphological characteristics of ADMSCs (A) ADMSCs cultured for 5days. (B) ADSCs cultured for 7 days. (C) ADMSCs cultured for 14 days with 95% confluence before 1st subculture.

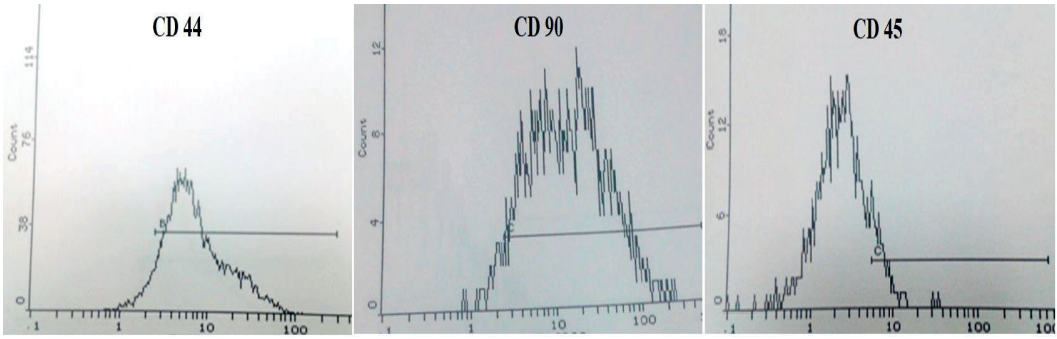

Figure 2. Flowcytometric identification of ADMSCs showing that the cells are positive for CD44 by 92.9 % and CD90 by 95.2 % and negative for CD45 by 12.6 %.

Differentiation of ADMSCs into Cardiomyocytes

Phenotypic characteristics of ADMSCs were examined on six and twelve days from culturing with a cocktail of growth factors to show the stages of differentiation of ADMSCs into cardiomyocytes using inverted light microscope with Euromex camera (Netherlands) **(Fig. 3)**.

Differentiation of ADMSCs into Cardiomyocytes Seeded onto NF

Morphological aspects of the generated cardiomyocytes seeded onto NF under the inverted light microscope were captured by Euromex camera attached to the inverted microscope. Cardiomyocytes cultured on NF in the presence of a cocktail of growth factors on the 3rd day are shown in Fig.(4A). Cardiomyocytes after 7 days of culturing on NF with a cocktail of growth factors are represented in Fig. (4B). Cardiomyocytes after propagation on NF with the involvement of a cocktail of growth factors on the 12th day showing complete confluence are illustrated in Fig. (4C).

Genetic Portrait of the Differentiated Cardiomyocytes

The differentiation of ADMSCs into cardiomyocytes was verified by molecular analysis of *MEF2C* and *Actn* genes expression levels (Fig. 5). Significant ($P < 0.05$) upregulation in the gene expression level of *MEF2C* and *Actn* has been demonstrated in the differentiated ADMSCs *versus* the undifferentiated ADMSCs. Further significant ($P < 0.05$) upregulation in the expression levels of the two genes was noticed in the differentiated ADMSCs seeded onto NF compared to the undifferentiated ADMSCs. *MEF2C* and *Actn* gene expression levels exhibited significant ($P < 0.05$) upregulation when comparing the differentiated ADMSCs seeded onto NF with those lacking NF.

Homing of the Infused Cardiomyocytes

To ensure the accommodation of the transplanted cardiomyocytes into heart tissue of MI-challenged rats, the adipose tissue-derived cardiomyocytes were labeled with iron nano-oxide prior injection into the MI-challenged rats and the rats were left for 2 months. Then, the rats were euthanized and the heart was carefully excised. Cross sections of heart tissue were examined after Prussian blue staining to detect the homing of adipose tissue-derived cardiomyocytes into the heart. The results showed that the stain is absent in the heart tissue section of the MI group. While, it was detected as blue spots upon treatment of MI-challenged rats with the adipose tissue-derived cardiomyocytes without NF or upon treatment with the adipose tissue-derived cardiomyocytes on NF **(Fig. 6)**.

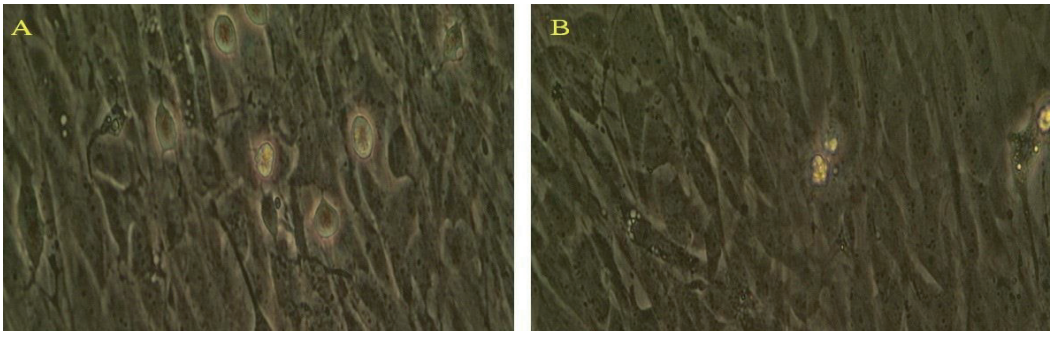

Figure 3. Morphological characterization of cardiomyocytes showing at (A) primary differentiation to cardiomyocytes after 6 days from culturing with growth factors cocktail and (B) colony forming sheets of cardiomyocytes after 12 days.

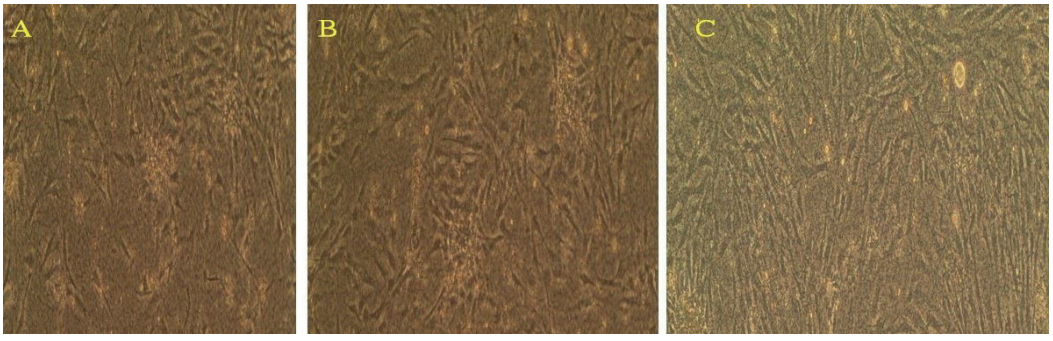

Figure 4. Morphology of cardiomyocytes (A) at the 3rd day of culturing on NF in the presence of growth factors cocktail, (B) at the 7th day of culturing on NF and (C) at the 12th day of culturing on NF displaying complete confluence.

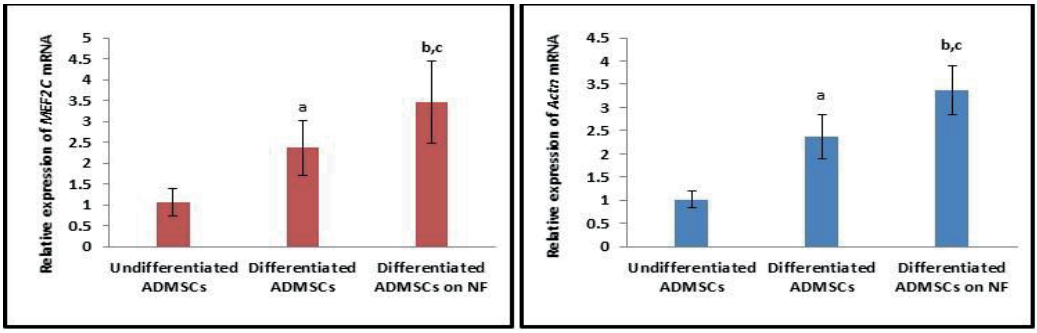

Figure 5. The mRNA expression levels of *MEF2C* and *Actn*.
a: Represent statistically significant value for differentiated ADMSCS VS undifferentiated ADMSCs.
b: Represent statistically significant value for differentiated ADMSCs on NF VS undifferentiated ADMSCs.
c: Represent statistically significant value for differentiated ADMSCs on NF VS differentiated ADMSCs without NF.
All values are expressed as mean ± SD with a statistically significant difference at $P<0.05$.

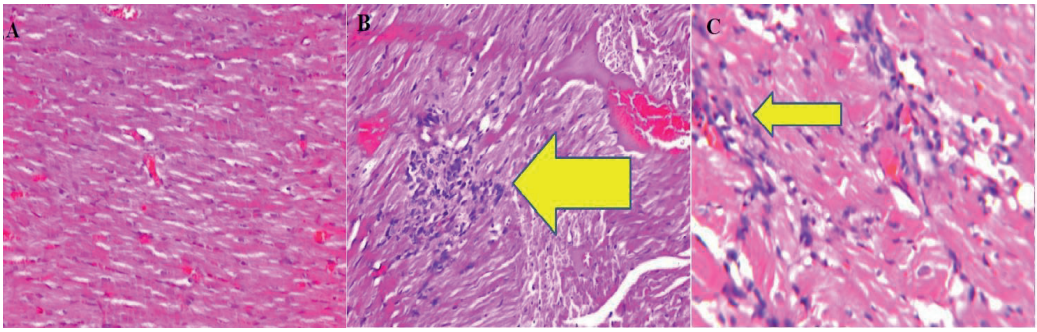

Figure 6. Photomicrophotographs of Prussian blue-stained heart tissue cross sections. (A) MI group, (B) Adipose tissue-derived cardiomyocytes-treated group and (C) Adipose-tissue-derived-cardiomyocytes seeded onto NF-treated group. The yellow arrows showing iron nanooxide blue dots in the treated groups.

Electrocardiogram (ECG) Recordings

Different diagnostic ECG parameters were used to ensure the onset of MI at the beginning of the experiment as well as the efficacy of the treatments after two months. Considered parameters were ST height, heart rate, RR, PR, QTc, QRS intervals and P duration, the ECG measurements of MI-challenged rats showed depressed ST height, heart rate and PR interval with elevated RR, QTc and QRS intervals along with elevation in P duration (Fig. 7). These clear variables are signs for the development of MI. The same elements measured by ECG revealed an improvement after treatment with adipose tissue-derived cardiomyocytes where moderate elevation in ST height, heart rate and PR interval with a moderate decrease in RR, QTc and QRS intervals along with a moderate drop in P duration. The ECG recordings of MI-challenged rats treated with the adipose tissue-derived cardiomyocytes seeded onto NF demonstrated great improvement as indicated by the marked elevation in ST height, heart rate and PR interval with obvious reductions in RR, QTc and QRS intervals along with pronounced drooping in P duration (Fig. 7). Both the decrease and the increase in ECG variables were statistically significant ($P < 0.05$).

Biochemical Findings

The data obtained from the biochemical analyses demonstrated the outcome of treatment with adipose tissue-derived cardiomyocytes and adipose tissue-derived cardiomyocytes seeded onto NF in MI rat model **(Fig. 8)**. The obtained findings clarified that the MI-challenged rats exhibit significant ($p<0.05$) elevation in serum LDH and CK-MB enzyme activities as well as cardiac cTnT level paralleled with significant ($p<0.05$) decline in cardiac Cx43 and Actn levels *versus* their control counterparts. On the opposite side, the treatment of MI-challenged rats with adipose tissue-derived cardiomyocytes or adipose tissue-derived cardiomyocytes seeded onto NF elicited significant ($p<0.05$) inhibition in serum LDH and CK-MB enzyme activities as well as cardiac cTnT level in association with significant ($p<0.05$) enhancement in cardiac Cx43 and Actn levels in comparison with MI-challenged rats. Noteworthy, the MI-challenged rats treated with adipose tissue-derived cardiomyocytes seeded onto NF evoked significant ($p<0.05$) inhibition in serum LDH and CK-MB enzyme activities and cardiac cTnT level along with significant ($p<0.05$) elevation in cardiac Cx43 and AcTn levels *versus* the MI-challenged rats treated with adipose tissue-derived cardiomyocytes.

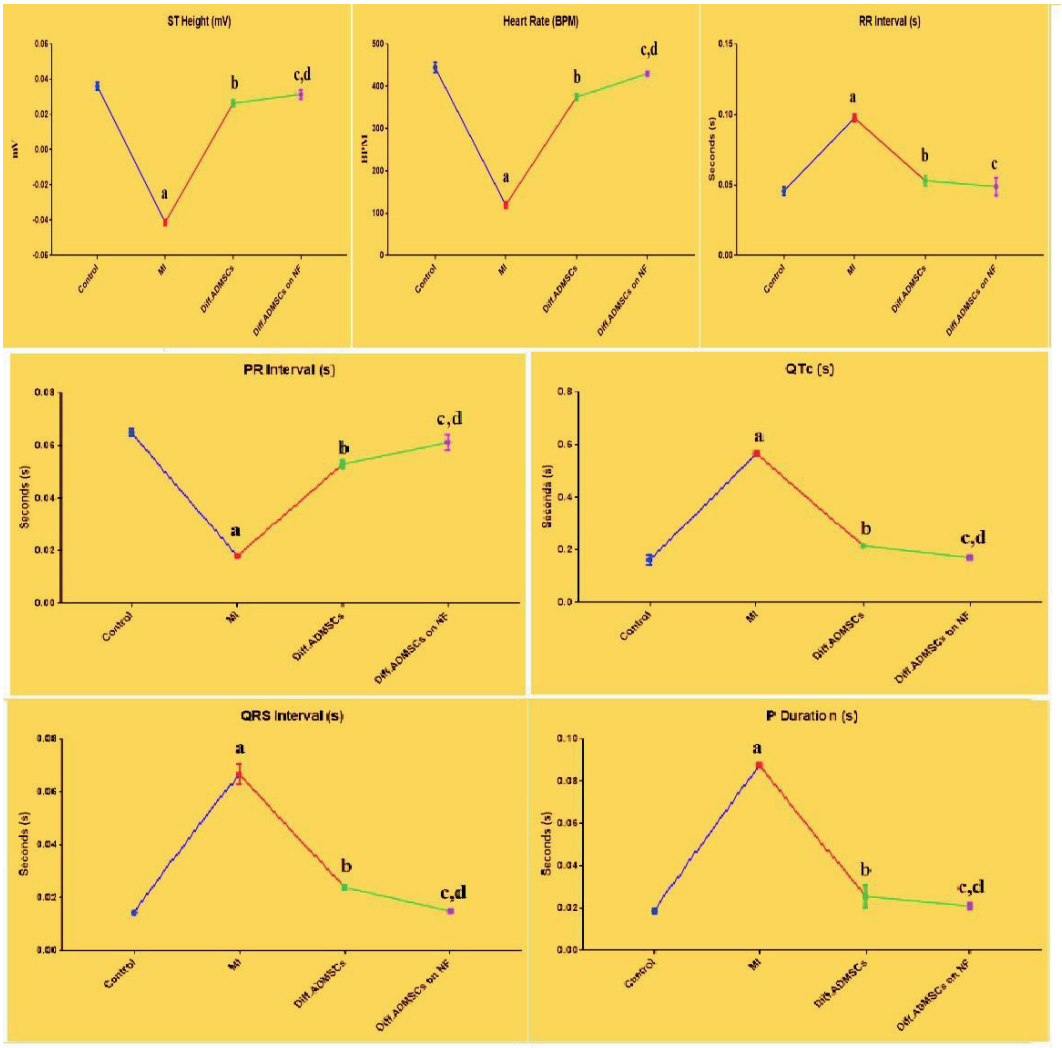

Figure 7. Schematic diagrams for ECG recordings in ST Height, heart Rate, RR, PR, QTc, QRS intervals and P Duration. In each diagram the four groups are represented as control, MI, Diff. ADMSCs and Diff. ADMSCs on NF.
a: Represent statistically significant value for MI VS control.
b: Represent statistically significant value for Diff. ADMSCs VS MI.
c: Represent statistically significant value for Diff. ADMSCs on NF VS MI.
d: Represent statistically significant value for Diff. ADMSCs on NF VS Diff. ADMSCs.

All values are expressed as mean ±SD with statistically significant difference at P<0.05.

Molecular Genetic Outcomes

Figure (9) represented the effect of the infusion with adipose tissue-derived cardiomyocytes and adipose tissue-derived cardiomyocytes seeded onto NF on the gene expression levels of cardiac *GATA4* and *NKX2.5*. The attained data elucidated that the MI-challenged rats display significant ($p<0.05$) upregulation in *GATA4* and *NKX2.5* gene expression levels *versus* the control ones. Significant ($p<0.05$) downregulation in the gene expression levels of *GATA4* and *NKX2.5* has been detected in MI-challenged rats treated with adipose tissue-derived cardiomyocytes or adipose tissue-derived cardiomyocytes seeded onto NF when compared with MI-challenged rats.

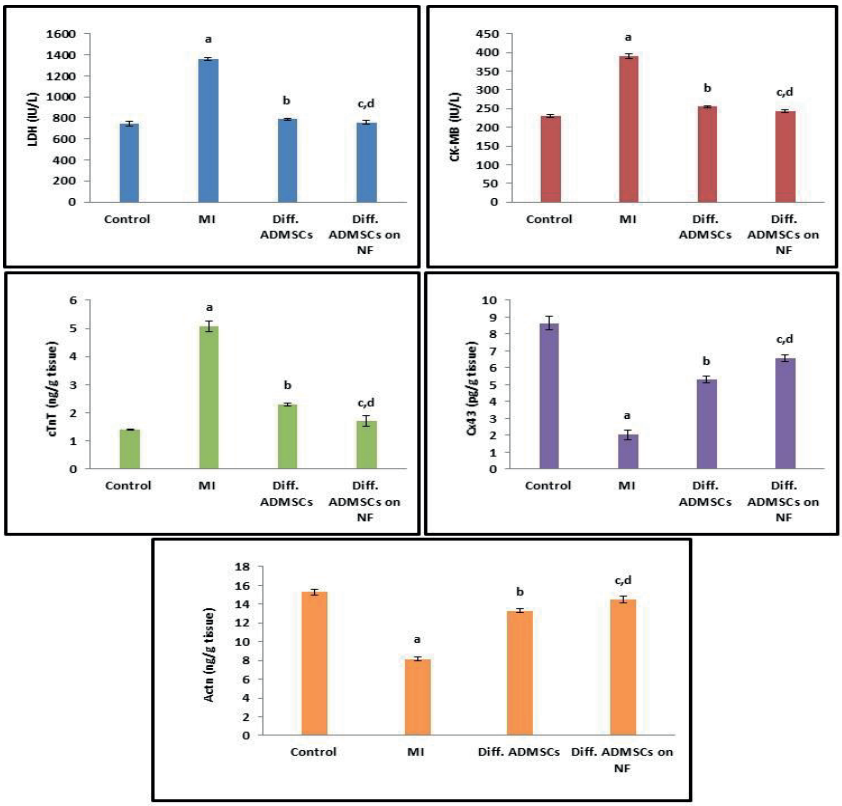

Figure 8. The outcome of treatment with adipose tissue-derived cardiomyocytes and adipose tissue-derived cardiomyocytes seeded onto NF on serum LDH and CK-MB enzyme activities as well as cardiac cTnT, Cx43 and Actn levels in the MI rat model.
a: Represent statistically significant value for MI VS control.
b: Represent statistically significant value for Diff. ADMSCs VS MI.
c: Represent statistically significant value for Diff. ADMSCs on NF VS MI.
d: Represent statistically significant value for Diff. ADMSCs on NF VS Diff. ADMSCs.

All values are expressed as mean ± SD with statistically significant difference at $P < 0.05$.

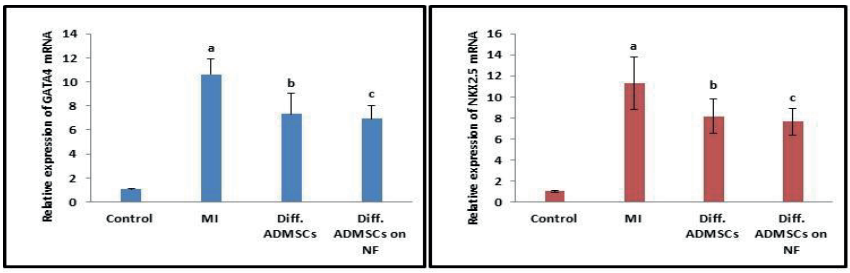

Figure 9. The mRNA expression levels of cardiac *GATA4* and *NKX2.5* in the different studied groups.
a: Represent statistically significant value for MI VS control.
b: Represent statistically significant value for Diff. ADMSCs VS MI.
c: Represent statistically significant value for Diff. ADMSCs on NF VS MI.

All values are expressed as mean ± SD with statistically significant difference at $P < 0.05$.

Histopathological Findings

Transverse cross section of heart tissue obtained from the control rat showed no histopathological alteration and the normal histological structure of the myocardium is noticed **(Fig. 10A)**. While, photomicrograph of heart tissue section of rat in the MI group revealed focal degenerated myocardium with leucocytes inflammatory cell infiltration and myofibroblast proliferation **(Fig. 10B)**. Microscopic investigation of heart tissue section of rat in the group treated with adipose tissue-derived cardiomyocytes disclosed focal area in the myocardium and myofibroblast proliferation with coagulative necrosis in the adjacent area, as well as severe congestion in the myocardial blood vessels **(Fig. 10C)**. Optical micrograph of heart tissue section of rat in the group treated with adipose tissue-derived cardiomyocytes seeded onto NF demonstrated focal area of inflammatory cell infiltration, whilst the intact histomorphological appearance of the heart is seen **(Fig. 10D)**.

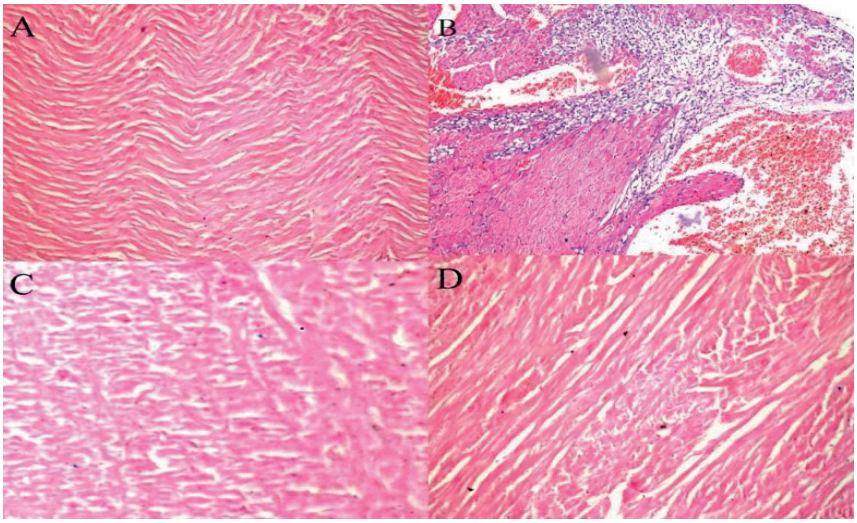

Figure 10. Transverse cross section of the heart tissues of rats in the groups under investigation stained by H&E stain. (A) Control group, (B) MI group, (C) MI treated with adipose tissue-derived cardiomyocytes and (D) MI treated with adipose tissue-derived cardiomyocytes seeded onto NF.

Discussion

The idea of employing stem cells to address cardiac diseases has emerged in the last decade as a leading approach for the regenerative medicine (Karantalis et al. 2012). In 2006, the International Society for Cellular Therapy established the requirements for MSC definition: 1) adherence to plastic in standard culture conditions with a spindly, fibroblast-like morphology; 2) expression of the surface molecules CD73, CD90, and CD44 in the absence of CD34, CD45, CD14, CD11b, CD79a, or CD19; 3) a capacity for differentiation into osteoblasts, adipocytes, and chondroblasts *in vitro*. In the present investigation, the morphological appearance of ADMSCs and the expression of the surface markers (CD44, CD90, CD45) emphasized that the isolated cells are MSCs according to the criteria of the International Society for Cellular therapy (Dominici et al. 2006).

Colony forming units (CFU), could be forced to particular cell lineages through the application of bioactive factors *in vitro* or *in vivo*. The factors that have been shown to induce CFU differentiation into cardiac cells, include dexamethasone and ascorbic acid (Shim et al. 2004), BMP-2 and FGF-2 (Yoon et al. 2005). Additionally, IGF-1 was also used, it is an anabolic growth hormone, that regulates cellular proliferation, differentiation, and senescence, in various tissues (D'Amario et al. 2011). FGF-2 has long been known to stimulate proliferation of cultured mesenchymal cells and it is also involved in regulation of cell survival, migration, and matrix production (Detillieux et al. 2003). BMPs play important roles during various stages of cardiac development, including early cardiogenic differentiation of mesoderm, cardiac tube assembly, looping and jogging, cardiac chamber identity, cardiomyocyte differentiation, and cardiac cushion formation (Van Wijk et al. 2007). It has been cited that, these growth factors exerted anti-apoptotic effects on MSCs which can be implemented by at least 2 mechanisms: 1) FGF-2 prolonged telomerase length and life span of MSCs and it is useful in obtaining a large number of cells with preserved differentiation potential (Bianchi et al. 2003) and 2) FGF and IGF-1 activate PI3-kinase/Akt pathway, which is known to mediate antiapoptotic signaling (Debiais et al. 2004). The differentiation of ADMSCs to

cardiomyocytes was achieved in the current study by using a cocktail of the abovementioned growth factors according the study of Hahn et al. (2008) and the derived cardiomyocytes started to form colonies then connected sheets similar to those demonstrated by Li et al. (2007).

For tissue engineering, using biodegradable scaffolds in combination with stem cell is considered as an alternative strategy that provides a repository for cell delivery leading to enhancement of cell survival (Bokhari et al. 2005; Zhang et al. 2005). In this study, we used nanofiber tissue culture plates as a scaffold material which replicates the extracellular matrix of the heart for the purpose of providing anisotropic support for cardiomyocytes (Davis et al. 2006). Culturing of CFU on NF plate stimulates cells to form a mineralized extracellular matrix (Khan et al. 2015). This explains what happened in our study after culturing ADMSCs-derived cardiomyocytes on NF.

To confirm the molecular changes during the differentiation of ADMSCs into cardiomyocytes by the cocktail of growth factors, we have studied the expression of a panel of specific cardiac genes. Data from qPCR analysis showed that the cardiogenic gene expression levels (*MEF2C* and *Actn*) are upregulated after differentiation of ADMSCs into cardiomyocytes. In cardiogenesis, *MEF2C* and *Actn* are known as key regulators in cardiac development and the recorded upregulation of these genes in the current study agrees with Planat-Bernard et al. (2004). The prominent upregulation of *MEF2C* and *Actn* gene expression levels upon the differentiation of ADMSCs into cardiomyocytes on NF comes in line with the study of Li et al. (2017). These investigators stated that the growing of stem cells on nanofiber intensifies their potentiality to differentiate.

Several paramagnetic contrast agents have been successfully used for *in vivo* cell tracking and labeling of different mammalian cell types (Weissleder et al. 1989). In our study, iron nanooxide was employed to track the differentiated cardiomyocytes to ensure their accommodation in heart tissue. Transverse sections across the heart tissues were examined after Prussian blue staining for the treated groups. It has been shown that the blue spots appear in the group treated with adipose tissue-derived cardiomyocytes and they are more pronounced in the group treated with adipose tissue-derived cardiomyocytes seeded onto NF. These findings emphasize the success of the transplanted cardiomyocytes in sticking to heart tissue.

Concerning the biological experiment of this investigation, the induction of MI in rats was done by isoprenaline, which is considered as a reliable and simple model mimic MI in human (Garson et al. 2015). Large doses of isoprenaline lead to overexcitation of heart beta receptors, increased heart rate, enhancement of cardiac muscle contractility and elevated myocardial oxygen consumption. The excitation of beta receptors, the expansion of peripheral blood vessels and the reduction of their resistance lead to falling of blood pressure, especially diastolic blood pressure resulting in myocardial ischemia (Zhang et al. 2008). Also, the overexcitement of heart beta receptors contributes to the increased local cardiovascular angiotensin, which promotes the overloading of Ca^{2+} inside cardiomyocytes. The overloading of Ca^{2+} in the cardiomyocytes can activate phospholipase causing decomposition of membrane phospholipids; overproduction of free radicals derived from xanthine oxidase leading to lipid peroxidation of the cellular membrane which ultimately leading to cell membrane destruction or even cell death (Hu et al. 2013).

ECG measurements in this study pointed to depressed ST height, heart rate and PR interval with elevated RR, QTc and QRS intervals along with abnormal elevation in P duration. These indices are clear signs of MI as described previously by Hopenfeld et al. (2004). All the parameters recorded by the ECG demonstrated improvement after treatment of MI-challenged rats with adipose tissue-derived cardiomyocytes as the ST height is moderately recovered. In addition, the moderate elevation in heart rate and PR interval paralleled with a moderate decrease in RR, QTc and QRS intervals along with P duration decrement are recorded after treatment with adipose tissue-derived cardiomyocytes. These observations are quite similar to those manifested by kakkar et al. (2006). More success was observed upon treatment of MI-challenged rats with adipose tissue-derived cardiomyocytes seeded onto NF as ECG parameters showed that normal ST height is demonstrated. Also, considerable elevation in heart rate and PR interval with marked decrease in RR, QTc and QRS intervals along with a P duration reduction are registered. The observed signs of recovery obtained from ECG measurements after treatment with adipose tissue-derived cardiomyocytes seeded onto NF concur with the findings of the study of Kijtawornrat et al. (2006).

Various relevant biochemical variables were analyzed in this study to detect the biochemical responses after the treatment of the rat model of MI with the generated cardiomyocytes. The elevated serum activity of lactate dehydrogenase (LDH) and MB fraction of creatine kinase (CK-MB) showed a statistically significant positive correlation with MI in clinical practice (Sivaranjani et al. 2014). In our MI model, these two enzymes demonstrated a significant increase in MI-challenged rats. This observation is in harmony with that demonstrated by Zeng et al. (2018). The overload of calcium caused by isoprenaline to induce MI causes oxidative stress which in turn decreases ATP production that directly affects LDH and CK-MB by a mechanism including the decrease in oxygen supply and the increase in anaerobic glycolysis that lead to the accumulation of lactate and a decrement of pH in the cardiomyocyte resulting in the activation of lysosomal enzymes (Chen and Frangogiannis, 2016). On the other side, these enzymes exhibited a significant decrease in the MI-challenged group treated with adipose tissue-derived cardiomyocytes. Further inhibition in the activity of these enzymes has been detected in the group treated with adipose tissue-derived cardiomyocytes seeded onto NF. The study of Lv et al. (2016) proved that the implantation of MSCs differentiated into cardiomyocytes to infarcted rat improves cardiac functions; this finding is validated in our study by the significant inhibition of serum LDH and CK-MB enzymes activity upon treatment of MI-challenged rats with the generated cardiomyocytes.

Upon onset of MI by isoprenaline in the current study, cardiac cTnT showed significant elevation while cardiac Cx43 and Actn displayed significant reduction as compared

to controls. These results match those observed in infarcted cardiac tissue as reported in the previous studies (Fan 2019). It has been concluded that the formation of highly reactive hydroxyl radicals as a result of oxidation stress yielded from isoprenaline administration induces an alteration and disintegration of proteins such as cTnT, Cx43 and Actn (Huang et al. 1999). Huang et al. (1999) stated that acute ischemic changes in Cx43 phosphorylation are followed by Cx43 loss due to degradation. On the opposite hand, cardiac cTnT displayed significant reduction and cardiac Cx43 as well as Actn showed significant elevation after treatment of MI-challenged rats with the adipose tissue-derived cardiomyocytes or adipose tissue-derived cardiomyocytes seeded onto NF, indicating an obvious sign of cardiac functions improvement as illustrated by the study of Khan et al. (2015). Noteworthy, the influence of treatment with adipose tissue-derived cardiomyocytes seeded onto NF on these cardiac specific biomarkers was more prominent than adipose tissue-derived cardiomyocytes, indicating more advancement in cardiac functions (Wu et al. 2017). It has been demonstrated that the treatment of MSCs with the specific growth factors yielded a great expression of cardiac specific genes, including Cx43 and a preferable gap junction formation (Hahn et al. 2008). The cytoprotective effects of MSCs are suggested to be mediated not only by paracrine actions, but also by direct cell-to-cell communication *via* gap junctions and the treatment with growth factors could potentiate their gap junction-mediated cytotherapeutic action (Krysko et al. 2005).

GATA4 and *NKX2.5* are the early differentiation indices in cardiac cells; they ensure electrical and metabolic coupling between cardiomyocytes and coordinate their contractility. In this study, the gene expression levels of *GATA4* and *NKX2.5* were first upregulated in MI-challenged rats while they showed down regulation after treatment with adipose tissue-derived cardiomyocytes or adipose tissue-derived cardiomyocytes seeded into NF. The noticed upregulation in the gene expression levels of *GATA4* and *NKX2.5* in MI-challenged rats could be allied to the cardiomyocyte hypertrophy resulted from isoprenaline (Sachdeva et al. 2012). While, the observed downregulation in the gene expression levels of *GATA4* and *NKX2.5* in the treated groups with the generated cardiomyocytes could be attributed to the reduction in cardiomyocyte hypertrophy through their paracrine, regenerative and cytoprotective actions (Hahn et al. 2008).

The histomorphological investigation of heart tissue sections in the present approach indicated that isoprenaline injection evokes focal degeneration of the myocardium with leucocytes inflammatory cell infiltration as well as myofibrobalsts proliferation. These histopathological alterations match those observed in the infarcted heart tissue (Talman and Ruskoaho, 2016). The group of rats treated with adipose tissue-derived cardiomyocytes manifested myofibrobalsts proliferation with coagulative necrosis in the adjacent area, as well as sever congestion in the myocardial blood vessels, suggesting that the transplanted cardiomyocytes successfully survived among the myocardial cells and contributed to the healing of cardiac tissue (Wang et al. 2006). The group of rats treated with adipose tissue-derived cardiomyocytes seeded onto NF demonstrated focal area of inflammatory cells infiltration and the connections between the myocardial cells have been clearly defined, whereas certain cells were able to self-assemble to form vessel-like structures, resulting in increased blood circulation within the myocardium. Of note, the recovered myocardial tissues were promoted in the transplanted cardiomyocytes. These observations are in parallel with those noticed in other studies (Li et al. 2017; Gómez-Heras et al. 2019) as the differentiated cardiomyocytes were attracted to the inflammatory-reparative area and they infiltrated the infarcted tissue of the heart (Mazo et al. 2012).

In conclusion, the present investigation provides documents for the successful generation of cardiomyocytes from ADMSCs. Also, this study offers clear pre-clinical evidences for the favorable employment of implantation of the differentiated cardiomyocytes in the recovery from MI. The differentiated cardiomyocytes succeeded in the accommodation within the myocardial infarcted site and proved eminent efficacy in cardiac renovation with heightened cardiac functions, particularly in case of the differentiated cardiomyocytes seeded onto NF. These outcomes were profoundly validated by ECG recordings, cardiogenic biomarkers ratings and histomorphological examination of cardiac tissue. The current approach establishes a new avenue for therapeutic strategy of cardiac remodeling after acute MI.

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