76465_Auto_Edited.docx

WORD COUNT	7290	TIME SUBMITTED	22-JUN-2022 06:01PM
		PAPER ID	87560560

Name of Journal: *World Journal of Stem Cells*
Manuscript NO: 76465
Manuscript Type: ORIGINAL ARTICLE

Basic Study

Combination of Mesenchymal Stem Cells and 3D Collagen Scaffold Preserves Ventricular Remodeling in Rat Myocardial Infarction Model

MSC-seeded collagen scaffold for myocardial infarction

Rida-e-Maria Qazi, Irfan Khan, Kanwal Haneef, Tuba Shakil Malick, Nadia Naeem, Waqas Ahmad, Asmat Salim, Sadia Mohsin

Abstract

BACKGROUND

Cardiovascular diseases are the major cause of mortality worldwide. Regeneration of the damaged myocardium remains a challenge due to mechanical constraints and limited healing ability of the adult heart tissue. Cardiac tissue engineering using biomaterial scaffolds combined with stem cells and bioactive molecules could be a highly promising approach for cardiac repair. Use of biomaterials can provide suitable microenvironment to the cells and can solve cell engraftment problem associated with cell transplantation alone. Mesenchymal stem cells (MSCs) are potential candidates in cardiac tissue engineering because of their multilineage differentiation potential and ease of isolation. Use of DNA methyl transferase inhibitor, such as, zebularine and 3D scaffold can promote efficient MSC differentiation into cardiac lineage, as epigenetic modifications play fundamental role in determining cell fate and lineage specific gene expression.

AIM

To investigate the role of collagen scaffold and zebularine in the differentiation of rat bone marrow (BM) MSCs and their subsequent *in vivo* effects.

METHODS

MSCs were isolated from rat BM and characterized morphologically, immunophenotypically and by multilineage differentiation potential. MSCs were seeded in collagen scaffold and treated with 3 μM zebularine in three different ways. Cytotoxicity analysis was done and cardiac differentiation was analyzed at gene and protein levels. Treated and untreated MSC-seeded scaffolds were transplanted in the rat myocardial infarction (MI) model and cardiac function was assessed by echocardiography. Cell tracking was performed by DiI dye labeling, while regeneration and neovascularization were evaluated by histological and immunohistochemical analysis, respectively.

RESULTS

MSCs were successfully isolated and seeded in collagen scaffold. Cytotoxicity analysis revealed that zebularine was not cytotoxic in any of the treatment groups. Cardiac differentiation analysis showed more pronounced results in type 3 treatment group which was subsequently chosen for the transplantation in the *in vivo* MI model. Significant improvement in cardiac function was observed in the zebularine treated MSC-seeded scaffold group as compared to MI control. Histological analysis also showed reduction in fibrotic scar, improvement in left ventricular wall thickness and preservation of ventricular remodeling in the zebularine treated MSC-seeded scaffold group. Immunohistochemical analysis revealed significant expression of cardiac proteins in DiI labeled transplanted cells and significant increase in the number of blood vessels in the zebularine treated MSC-seeded collagen scaffold transplanted group.

CONCLUSION

Combination of 3D collagen scaffold and zebularine treatment enhances cardiac differentiation potential of MSCs, improves cell engraftment at the infarcted region, reduces infarct size, and improves cardiac function.

Key Words: Mesenchymal stem cells; Myocardial infarction; Cardiac tissue engineering; Demethylating agent; Collagen Scaffold; Zebularine.

Qazi REM, Khan I, Haneef K, Malick TS, Naeem N, Ahmad W, Salim A, Mohsin S. Combination of Mesenchymal Stem Cells and 3D Collagen Scaffold Preserves Ventricular Remodeling in Rat Myocardial Infarction Model . *World J Stem Cells* 2022; In press

Core Tip: This study was designed to elucidate the effect of 3D microenvironment provided by the use of natural collagen based scaffold and DNA methyl transferase inhibitor, zebularine, on the cardiac differentiation of mesenchymal stem cells (MSCs) *in vitro* and cardiac regeneration *in vivo*. Collagen scaffold provides a native 3D microenvironment to MSCs and along with zebularine, enhances their cardiac differentiation potential. Transplantation of zebularine treated MSC-seeded collagen scaffold in the rat myocardial infarction (MI) model aids in improving cardiac function and preserves left ventricular remodeling. Translation of this research into clinics can be a better option for the treatment of MI.

INTRODUCTION

Mesenchymal stem cells (MSCs) have been shown to regenerate many damaged tissues including heart, lung, kidney, and liver [1-4]. These cells have several advantages including, immuno-modulatory properties, and high proliferation and migration rates [5]. Several studies have shown that MSCs are ideal cell source for clinical applications [6], including cardiac regeneration. However, transplantation of stem cells to the damaged tissue may lead to undesired differentiation. Therefore, pre-differentiation of stem cells into specialized cell types before transplantation can serve as a better strategy for treatment [2].

Cardiac fate determination is controlled by various agents, such as, cytokines, growth factors, small molecules, and microRNAs [1, 5]. DNA methylation is a key component of epigenetic machinery that regulates gene expression [7]. DNA methyltransferase inhibitors can change the epigenetic landscape, thus promoting differentiation [8, 9]. DNA methyltransferase inhibitor, 5-azacytidine (5-aza) is extensively used in the cardiac differentiation of stem cells [1, 10, 11]. Another demethylating agent, zebularine is a cytidine analog that can inhibit DNA methyltransferase through covalent binding [12]. Unlike 5-aza, zebularine shows minimal toxicity in cell culture and animal models [12, 13]. Several studies have shown the positive effect of zebularine in mesodermal, hepatic, and cardiac differentiation [11, 12, 14].

Human heart development is a complex process that involves various signaling molecules along with other factors. These molecules and three-dimensional (3D) configuration are two important factors that define heart tissue structure and function [15]. The 3D microenvironment is an essential factor that determines cell behavior and fate. Studies have shown that stem cell–extracellular matrix (ECM) interactions influence cardiomyogenic differentiation of stem cells [1]. Collagen is the main structural protein that maintains the biological and structural integrity of ECM. Collagen scaffold has been shown to enhance the expression of cardiomyocyte-specific proteins in bone marrow derived MSCs [16, 17]. Furthermore, collagen scaffold seeded with MSCs have shown structural support to the injured heart [1]. Despite the advancement in tissue engineering technology, development of an efficient, controlled and reproducible protocol for 3D differentiation is always challenging [15].

The present study aims to investigate the combined effect of an epigenetic modifier, zebularine, and a 3D collagen scaffold to enhance the cardiac differentiation of MSCs. The effectiveness of *in vitro* pre-differentiated cells was assessed in the *in vivo* rat myocardial infarction (MI) model. The proposed cardiomyocyte-differentiation protocol represents an efficient model system to investigate the mechanisms that determine cardiac fate and may provide a source of differentiated cardiac cells for drug screening and cell based therapies.

MATERIALS AND METHODS

Flow chart of experimental design is presented in Figure 1.

Animal care and ethical approval

Wistar rats (150-250 gm) were used for bone marrow isolation and MI model development. Ethical approval was obtained from the Institutional Animal Care and Use Committee (IACUC), Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. Experimental procedures were carried out according to the international guidelines for care and use of laboratory animals under protocol number

2020-006. Animals were kept in individual cages in a room maintained at 12h light: 12h dark cycle, 22 °C ± 2 °C temperature and 55 ± 5% relative humidity, and provided with free access to food and filtered water.

Isolation and expansion of MSCs

Rats were euthanized with an over dose of sodium pentobarbital. Bone marrow was isolated according to the protocol described previously [18]. Briefly, rat skin was sterilized with 70% ethanol. Rats were dissected to separate the bones. Bones were cut from the edges using sterile surgical instruments under biosafety cabinet (ESCO, Singapore) and bone marrow was flushed using complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific USA), 100 µg/mL streptomycin, 100 units/mL penicillin, 4 mmol/L L-glutamine and 1 mmol/L sodium pyruvate. Bone marrow was transferred to T75 tissue culture flasks and incubated in CO_2 incubator (NuAire, USA). Non-adherent hematopoietic cells were removed by changing the medium after 3-4 days and adherent MSCs were allowed to proliferate. Cells were sub-cultured when they reached 80-90% confluence. MSCs of passage 1 were used in subsequent experiments.

Characterization of MSCs

MSCs were characterized by morphological examination under phase contrast microscope, by immunostaining using fluorescence microscopy and flow cytometry, and by trilineage differentiation potential, as described previously [18].

Immunostaining: MSCs were cultured in chambered glass slides (IWAKI, Japan) at a cell count of 10,000 cells/mL/chamber. After overnight incubation, medium was aspirated, cells were washed with 1X PBS, fixed with 4% paraformaldehyde (PFA) for 10 min, and permeabilized with 0.1% triton X-100. Cells were washed with PBS and non-specific binding sites were blocked with blocking solution (2% BSA in PBS and 0.1% Tween20) for 1 h at 37°C in the humidified environment. Cells were incubated with primary antibodies against CD29, CD44, CD90, CD117, vimentin, and CD45. After overnight incubation at 4°C, cells were washed with PBS to remove unbound primary antibodies and incubated with Alexa Fluor 546 goat anti-mouse secondary antibody for 1 h at

37°C, followed by washing with PBS 4-5 times. Counterstaining was performed with 0.5 µg/mL DAPI to observe cell nuclei. Finally, cells were washed and mounted using fluorescence mounting medium (Merck, Germany). Mounted slides were observed under fluorescence microscope (90i and NIE, Nikon, Japan).

For immunophenotypic characterization by flow cytometry, cells were detached and washed with FACS buffer (1% BSA, 0.1% Na-azide and 1 mmol/L EDTA). Cells were incubated with CD29, CD44 and CD90 for 2 h at 37°C, and after washing, incubated with Alexa Fluor 488 goat anti-mouse secondary antibody for 1 h at 37°C. Cells were washed and analyzed through FACS Calibur (Becton Dickinson, USA) using CellQuest Pro software. Isotype labeled cells were used as control.

Trilineage Differentiation: MSCs were grown in 6 well plates using complete DMEM. For osteogenic differentiation, MSCs were grown in osteogenic induction medium (complete DMEM containing 0.2 mmol/L L-ascorbic acid-2-phosphate, 0.1 µM dexamethasone, and 10 mmol/L glycerol-2-phosphate) for 28 days. Alizarin Red S staining was performed to detect mineral deposits. For adipogenic differentiation, MSCs were grown in adipogenic induction medium (complete DMEM containing 1 µM dexamethasone, 200 µM indomethacin, and 10 µg/mL insulin) for 21 days. Differentiated cells were stained with Oil Red O stain for the detection of lipid filled vacuoles inside the cytoplasm. For chondrogenic differentiation, MSCs were cultured in chondrogenic induction medium (complete DMEM containing 1 µM dexamethasone, 100 mmol/L ascorbic acid, 20 ng/mL TGFβ, and 10 ng/mL insulin) for 21 days and then stained with Alcian Blue stain for the detection of glycosaminoglycans.

In vitro studies

MSC seeding in collagen scaffold

MSCs were seeded in collagen scaffold as described previously, with slight modifications [17]. Collagen scaffold (Collagen type 1, non-denatured, Pangen, URGO, France) 2.5 x 3.5 cm^2 in size, was cut into 2 pieces; one piece was transferred to a six-well plate. MSCs were detached from the flask and counted using a hemocytometer; 1 million MSCs suspended in medium were seeded in the scaffold. When the cells were

adsorbed, the scaffold was incubated in the CO_2 incubator to allow complete cell penetration inside the scaffold. After 1 h of incubation, 2 mL complete DMEM was carefully added to the scaffold-containing well and cell-seeded scaffold was incubated at 37°C for a specified time duration.

Scanning electron microscopy (SEM)

SEM analyses for the soaked collagen scaffold (without cell seeding) and MSC-seeded collagen scaffold, were performed after 21 days of cell seeding. Collagen scaffolds were washed with PBS and fixed in 2.5% glutaraldehyde solution overnight at 4°C. Dehydration of fixed samples was performed using ascending ethanol series (starting from 30% ethanol) and freeze-drying. Samples were mounted, sputter coated with gold palladium, and observed under analytical SEM (JSM-6380A, JEOL) using 10 and 15 kV accelerating voltages.

Zebularine treatment

MSCs were treated with zebularine at a concentration of 3 µM in three different ways: treatment type 1: MSCs were first treated with zebularine for 48 h, and then seeded in the collagen scaffold; treatment type 2: zebularine was added to the MSC suspension (after detachment), and then treated cells were seeded in the collagen scaffold; treatment type 3: MSCs were first seeded in the collagen scaffold, and then zebularine was added with the medium. For control, only untreated MSCs were seeded in the scaffold. All treated and untreated MSC-seeded scaffolds were incubated for 48 h, and cytotoxicity analysis was performed.

Cytotoxicity analysis

MTT cell viability assay was performed to determine the cytotoxicity of 3 µM zebularine on MSC-seeded scaffold. Treated and untreated MSC-seeded scaffolds were washed 2-3 times with 1X PBS after aspirating the culture medium. When the medium completely diffused out from the scaffolds, 10% MTT solution was added, and scaffolds were incubated for 4 h in the CO_2 incubator. The scaffolds were carefully transferred to separate 15 mL falcon tubes, and 2 mL DMSO was added to each tube. Formazan crystals formed inside the scaffold were dissolved in DMSO by vigorous pipetting and

vortexing. Tubes were then centrifuged at 12000 x g for 15 min, and supernatant was transferred to a 96-well plate. The plate was read at 570 nm using spectrophotometer (Thermo Scientific Mutiskan GO, Waltham, US).

Analysis of cardiac differentiation

After 48 h of treatment as described earlier, zebularine was removed from type 2 and type 3 treatment groups. Scaffolds were washed with PBS, and then complete DMEM was added. Treated and untreated MSC-seeded scaffolds were incubated for further 19 days, and medium was changed after every 3 days. After a total of 21-day incubation, both treated and untreated MSC-seeded scaffolds were harvested for gene and protein expression analyses.

Gene expression analysis

MSC-seeded collagen scaffolds were washed three times with PBS to completely remove the medium and crushed in liquid nitrogen using sterile mortar and pestle. The crushed scaffold was collected in microfuge tubes and RNA was isolated using EasyPure RNA kit (TransGen Biotech Co. Ltd, China) according to manufacturer's protocol. RNA was quantified using UV-Vis Spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and 1 µg of isolated RNA was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. cDNA was amplified using cardiac specific gene primers, listed in table 1, by qPCR (CFX96 Touch Real-Time PCR Detection System, Bio-Rad, USA). Gene amplification program comprised of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 58°C for 1 min, then melting curve was run to check the specificity of the amplified products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene to normalize gene expression. ΔΔCt method was used to calculate the fold change. ΔΔCt is the difference between ΔCt of treated and control samples, whereas ΔCt is the difference in Ct values of gene of interest and endogenous control (a house keeping gene).

Protein expression analysis

MSC-seeded collagen scaffolds were washed with PBS and fixed in 4% PFA. Each scaffold was then transferred to a mold containing optimal cutting temperature (OCT) medium (Shandon, Cryomatrix, Thermo, USA), and frozen at -20°C. 15 µm sections were made from the frozen blocks of the scaffolds using cryostat machine (Shandon, Thermo Electron Corporation, USA). The sections were permeabilized with 0.1% triton X-100 and stained with cardiac specific primary antibodies GATA4, cTnT, and Nkx2.5 using the same protocol as described for the characterization of MSCs. Fluorescent images were processed by Adobe Photoshop and ImageJ software was used for the quantitative analysis.

In vivo studies

Experimental groups

Animals were divided into 5 groups (n = 6) to evaluate *in vivo* cardiac regeneration; (i) sham control, (ii) MI model, (iii) MI+collagen scaffold transplantation, (iv) MI+MSC-seeded scaffold transplantation, and (v) MI+zebularine treated MSC-seeded scaffold transplantation.

MI model

MI model was developed by the procedure described in our previous study [19]. Briefly, rats were anesthetized using xylazine and ketamine (7 mg/kg and 60 mg/kg body weight, respectively). Endotracheal intubation was performed using rodent ventilator to initiate artificial ventilation. Left thoracotomy was performed through anterolateral 4th intercostal space, and retractor was used to expose the heart. Left anterior descending coronary artery (LAD) was carefully located and ligated with the help of 6-0 prolene suture (Ethicon, USA) 2-3 mm below the edge of left atrium. Success in artery occlusion was evident with the immediate color change of the left ventricle from red to pale color. Chest cavity was closed and animal was given diclofenac sodium (25 mg/mL) and antibiotics (penicillin and streptomycin 10,000 U/mL) subcutaneously. Animals were monitored till they regained consciousness. For sham control group, suture was passed through LAD without ligation.

Collagen scaffold processing and transplantation

For collagen scaffold only group, collagen scaffold (1.1 x 1.1 cm^2) was soaked in complete DMEM prior to transplantation. For MSC-seeded scaffold group, 1 million MSCs were seeded in the scaffold, and for zebularine treated group, type 3 treatment method was selected. In all cases, scaffolds were incubated for 15 days, with medium change after every three days. After 15 days of incubation, scaffolds were washed with PBS three times to completely remove the medium, and transplanted immediately after LAD ligation in the infarcted region of the heart, as described previously [20]. Scaffold was held at its position by 6-0 prolene suture. Suture was carefully used so that it could not go deep into the myocardium to avoid blockage of other branches of the LAD artery. Rest of the procedure was same as described for the MI model development. To track the engrafted cells in the untreated and treated MSC-seeded scaffold transplanted groups, MSCs were labeled with DiI cell labeling dye (Invitrogen, USA) as per manufacturer's protocol before seeding into the scaffold.

Cardiac function analysis by echocardiography

In vivo cardiac function was assessed by echocardiography at 2-, 4- and 6-weeks post-surgery using Echo machine (Aloka, USA) equipped with 7-MHz transducer. 2D bright mode (B-mode) and motion mode (M-mode) scans were recorded using parasternal long axis view at the level of papillary muscles. Anatomical parameters of left ventricle such as left ventricular internal systolic and diastolic dimensions (LVIDs and LVIDd) were measured using M-mode tracings. Measurements were based on three consecutive cardiac cycles averaged for calculation of the parameters. Percent ejection fraction (%EF) and fractional shortening (%FS), end diastolic and systolic volumes (EDV and ESV), and stroke volume were calculated by in-built software of the echo machine.

Histological analysis

Rats were anesthetized after 6 wk of surgery, perfused with PBS, and fixed with 4% PFA. Hearts were excised and immersed in 4% PFA overnight, dehydrated in ascending alcohol baths, immersed in xylene and xylene-paraffin mixture, and embedded in paraffin. Paraffin blocks were transversely cut into 4 µm thick sections using microtome and Masson's trichrome staining was performed according to the manufacture's

protocol. Stained sections were observed under bright field microscope for histological examination.

Immunohistochemistry

For the analysis of blood vessels in the cardiac tissue, paraffin embedded tissue sections were deparaffinized and antigen retrieval was done using citrate buffer. Sections were permeabilized and stained with anti α-smooth muscle actin (α-SMA) antibody. Alexa Fluor 488 anti-mouse secondary antibody was used for detection and DAPI was used for nuclei staining.

For tracking of cells in the implanted scaffold, DiI labeled MSC-seeded scaffold transplanted rat hearts were fixed in 4% PFA for 4 h, transversely cut from the center, and then snap frozen in molds containing OCT medium. Frozen tissue blocks were sliced into 15 µm thick sections using cryostat machine. DiI labeled tissue sections were carefully examined under fluorescence microscope for the presence of fluorescent cells, permeabilized with triton-X 100, and non-specific binding sites were blocked with blocking solution. Sections were then stained with cardiac specific antibodies, cardiac myosin heavy chain (cMHC), cardiac troponin I (cTnI), and cTnT. Alexa Fluor 488 goat anti-mouse secondary antibody was used for detection and DAPI was used for nuclei staining. Slides were observed under fluorescence microscope and images were analyzed by ImageJ software to measure the fluorescence intensities to determine cardiac differentiation potential of the transplanted MSCs.

Statistical analysis

Statistical analysis was performed using SPSS software (IBM, SPSS statistics, version 26, USA). Independent sample t-test analysis was performed for comparison between two groups, and one-way analysis of variance (ANOVA) with post-hoc Bonferroni corrections for comparison between multiple groups. Data from all experiments were expressed as mean ± SEM and differences between the groups were considered statistically significant at $P<0.05$ (where $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$). Degrees of freedom for *in vitro* and *in vivo* experiments were calculated by SPSS software.

RESULTS

Morphology of MSCs

Rat bone marrow culture showed round morphology and mixed population of floating cells at the time of isolation. MSCs adhered to the surface of culture flask and expanded gradually. After 10 days, passage 0 (P0) cells were fully grown and exhibited fibroblast like morphology. P0 cells were sub-cultured by trypsinization to get the homogenous population of MSCs. MSC culture at respective time points is shown in Figure 2A.

Characterization of MSCs

Immunophenotypic profile: Passage 1 MSCs were characterized by immunostaining and analyzed by fluorescence microscopy and flow cytometry. Cells stained with MSCs specific markers CD29, CD44, CD90, CD117, and vimentin showed positive expression, while CD45, a hematopoietic marker, showed negative expression (Figure 2B). Flow cytometric analysis showed that >90% of the cells expressed CD29, CD44 and CD90, further confirming the presence of MSCs in culture (Figure 2C).

Trilineage differentiation: Trilineage differentiation potential of MSCs was confirmed by the differentiation of MSCs into osteogenic, adipogenic and chondrogenic lineages. Osteogenic differentiation was confirmed by the presence of mineral deposits in MSC culture. Differentiated cells stained orange-red, while large mineral deposits appeared dark red after Alizarin Red S staining. Adipogenic differentiation was evaluated by intracellular accumulation of lipid vacuoles in the treated cells, which turn bright red after staining with Oil Red O stain. Chondrogenic differentiation was analyzed by the accumulation of extracellular matrix that stained intense blue after Alcian Blue staining (Figure 2D).

SEM Analysis

SEM analysis revealed the porous structure of the dry collagen scaffold (Figure 3A). Freeze-dried, medium soaked scaffold also showed the interconnected pores on the surface and deeper inside the scaffold (Figure 3B). When MSC-seeded scaffold was observed after 21 days, cell attachment was observed in between the scaffold fibers (Figure 3C).

Cell viability by MTT assay

Percent viability calculated by the absorbance values obtained through MTT assay revealed that zebularine at 3 µM concentration was non cytotoxic for MSCs in all treatment groups (Figure 3D). Statistical analysis showed significant increase in cell viability of type 2 treatment group, as compared to control. However, there is no significant reduction in the cell viability in any treatment group, hence no cytotoxic effect of zebularine treatment. This is also confirmed by the calculated degree of freedom (Table 3A).

Cardiac differentiation analysis: Gene expression

Cardiac gene expression profile revealed variable results in different treatment types. When compared to control, cTnI, Nkx2.5 ($P<0.01$) and Mef2C ($P<0.05$) genes were significantly upregulated in type 1 treatment group; GATA4, Nkx2.5, cMHC ($P<0.01$) and cTnI ($P<0.001$) genes were significantly increased in type 2 treatment group; and ANF ($P<0.05$), cTnT, Nkx2.5, Tbx20, connexin43 ($P<0.01$) and cMHC ($P<0.001$) genes showed significant upregulation in type 3 treatment group. Comparison among these treatment groups revealed significantly increased expression of cTnI ($P<0.05$), GATA4 ($P<0.01$) and cMHC ($P<0.001$) in type 2 group as compared to type 1 group; ANF ($P<0.05$) and cTnT ($P<0.01$) were significantly upregulated in type 3 treatment group as compared to type 1 group; Tbx20 and connexin43 ($P<0.01$) expressions were significantly increased in type 3 treatment group when compared to both type 1 and 2 treatment groups, while reverse is true for cMHC ($P<0.001$) expression (Figure 4). Calculated degrees of freedom also confirm that there is significant effect of zebularine treatment on the gene expression profile of MSC seeded collagen scaffolds (Table 3A).

Cardiac differentiation analysis: Protein expression

Immunophenotypic profile showed positive expression of GATA4, cTnT and Nkx2.5 in all treatment groups. Quantification of protein expression revealed that the percentages of GATA4, cTnT, and Nkx2.5 were significantly high in all treatment groups as compared to the control group. Fluorescence intensity in case of GATA4 was significantly high (P<0.01) in type 2 treated group as compared to control, while that of cTnT was significantly high in all treatment groups, however it is more pronounced in type 2 and 3 treatment groups (P<0.001). Same is the case with Nkx2.5, but type 3 treatment showed more pronounced results when compared with type 1 treatment group (Figure 5). Calculated degrees of freedom also confirm that there is significant effect of zebularine treatment on the protein expression of MSC seeded collagen scaffolds (Table 3A).

Cell-seeded scaffold: in vivo analysis

Macroscopic evaluation of isolated rat hearts

Collagen scaffold was transplanted immediately after the MI model development (Figure 6A), and hearts were isolated after 6 wk of surgery, for macroscopic and histological evaluation. Macroscopic evaluation showed major difference in the appearance among all the five groups. Hearts from sham control group appeared normal, exhibiting healthy myocardium, whereas left ventricle of the infarcted heart was covered with white fibrous scar. Collagen scaffold patch integrated into the left ventricular wall as shown in other three groups. However, MSC-seeded scaffold transplanted heart exhibited an extensive network of functional blood vessels running through the integrated scaffold patch (Figure 6B).

Cardiac function analysis

Echocardiographic analysis showed significant gradual increase in the LVIDs in the MI group after 2, 4 and 6 wk and significant gradual reduction in the %EF and %FS as compared to sham control (P<0.001). LVIDd was also gradually increased, particularly 6 wk post infarction (P<0.05), whereas ESV was significantly increased after 4 and 6 wk of MI induction as compared to sham control (P<0.01 and P<0.001, respectively). These results demonstrated functional impairment of left ventricle and successful

establishment of MI model. When the treated groups were compared with the MI group, no improvement was observed in LVIDd at any time point, whereas LVIDs was significantly reduced in zebularine treated MSC-seeded scaffold transplanted group after 4 wk (P<0.01) and 6 wk (P<0.001). There was no significant reduction of LVIDs in collagen scaffold transplanted group at any time point. MSC-seeded scaffold group showed significant reduction only after 6 wk of transplantation (P<0.001). %EF and %FS were significantly improved in all treated groups compared to the MI group, but more significant improvement was observed in zebularine treated group, as indicated by comparison with sham control. EDV and SV showed no significant improvement at any time point in all treatment groups compared to the MI group. ESV was significantly reduced in only scaffold and MSC-seeded scaffold groups after 6 wk (P<0.01), but zebularine treated group showed significant reduction after 4 wk (P<0.01) which was further reduced after 6 wk (P<0.001) as compared to MI (Figure 7). Table 2 summarizes mean values of echocardiographic parameters. Calculated degrees of freedom also showed similar pattern (Table 3B)

Histological examination

Cross-sections of paraffin embedded hearts differentially stained with Masson's trichrome stain showed green region of the collagen deposition in the left ventricle of the MI heart with collagen scaffold protruding through the left ventricular wall; and red region of the normal tissue and myocytes. MI hearts showed extremely thin ventricular wall and comparatively large fibrotic area as compared to other groups (Figure 8A). At higher magnification, the sections showed healthy myocytes with interconnected cytoplasmic junctions in the left ventricle of sham control. Infarcted region of the MI heart displayed fibrous tissue containing uniaxially aligned fibroblasts and tightly packed collagen fibrils. There were remnants of myocytes and irregularly large and distorted blood vessels indicating pathological characteristics of the infarcted myocardium. Collagen scaffold transplanted group showed limited fibrosis and neoangiogenesis within the scaffold. MSC-seeded collagen scaffold transplanted group showed more preservation of the left ventricular remodeling and regeneration of the

myocytes. The scaffold seemed filled with the cells and a large number of blood vessels appeared in the scaffold. Zebularine treated MSC-seeded collagen scaffold group showed greater reduction in the infarcted area, more newly formed myocytes in the infracted ventricle and several blood vessels in the scaffold patch (Figure 8B).

Immunohistochemistry

Further confirmation of neoangiogenesis was done by immunostaining of the paraffin embedded sections by α-smooth muscle actin staining. Fluorescence microscopic analysis showed pathological characteristics of MI in the microvasculature of the myocardium. All three scaffold transplanted hearts showed new vessel formation at the border zone of the scaffold and myocardium. MSC-seeded collagen scaffold transplanted hearts showed arterioles in the scaffold and myocardium along with numerous small blood vessels, whereas zebularine treated transplanted hearts showed venules and mature blood vessels in the myocardium near the border zone (Figure 9A). Quantification of the data revealed significant increase in the number of blood vessels in the scaffold only transplanted group ($P<0.01$), and untreated and zebularine treated groups ($P<0.001$) as compared to MI. Zebularine treated MSC- seeded group showed significant increase in the number of blood vessels as compared to sham control ($P<0.01$), MI ($P<0.001$), scaffold only group ($P<0.01$), and MSC-seeded scaffold transplanted group ($P<0.05$) (Figure 9B).

Cell tracking

Microscopic analysis of DiI labeled MSC-seeded collagen scaffold showed cell migration at the border of the scaffold and myocardium in both MSC-seeded and zebularine treated MSC-seeded scaffold transplanted heart sections. Immunohistochemical analysis of these sections with cMHC, cTnT, and cTnI revealed that the transplanted cells were differentiated towards cardiac lineage. However, more cardiac differentiation and cell density was observed in the zebularine treated collagen scaffold transplanted group ($P<0.05$) (Figure 10).

DISCUSSION

In this study, we investigated the role of 3D collagen scaffold in combination with zebularine treated MSCs on cardiac differentiation *in vitro* and functional regeneration of cardiac tissue *in vivo* following myocardial infarction (MI) in rats. MSCs were isolated from rat bone marrow and identified on the basis minimal criteria established by the International Society for Cellular Therapy [21]. MSCs were characterized by their plastic adherence capability, and presence of MSC markers CD29, CD44, CD90, CD117 and vimentin, and absence of CD45, a hematopoietic marker, thus confirming the presence of a pure population of MSCs in culture. MSCs were further characterized by their multilineage (osteogenic, adipogenic and chondrogenic) differentiation ability under *in vitro* culture conditions. MSCs are good candidates for cell transplantation due to ease of isolation and having low immunogenic and immunomodulatory effects. Their rapid proliferation and high genetic stability make their *in vitro* proliferation simple and easy. Therapeutic advantages of MSCs have been extensively demonstrated in various MI models [22]. However, poor survival and engraftment of transplanted cells decrease their advantage as therapeutic agent [23]. Various approaches have been explored to overcome these hurdles, including the use of natural or synthetic scaffolds [24]. The strategy of transplanting stem cells along with biomaterials, mimicking the *in vivo* microenvironment, provides substantial support for cellular retention and engraftment [25]. The use of mimicking extracellular matrix (ECM) is one of the tissue engineering strategies that can regenerate the myocardium and improve contractility. Various natural and synthetic polymers offer the construction of ECM, however, natural biomaterials provide better construction ability and biocompatibility to mimic the endogenous tissue, compared to synthetic materials [26]. Natural ECM-derived biomaterials create a dynamic environment that can be remodeled, processed and replaced during cell therapy [27]. Among natural scaffolds, collagen represents the most appropriate candidate due to its excellent biocompatibility and mechanical stability [28, 29].

Collagen type 1 is the most abundant structural protein, and most common constituent of cardiac extracellular matrix (ECM) [27, 30]. It accounts for 85-90% of collagens and

forms fibrillary network along with type III collagen in the heart tissue. It provides structural support and participates in cellular signaling [30]. It is one of the earliest identified and isolated ECM component, which provides 3D environment that affects cell morphology, function and cell growth [27]. Collagen based patches were well studied for cardiac regeneration and can be transplanted directly on the epicardial surface at the infarcted region. They can provide better mechanical support to the weakened ventricular wall after ischemic injury, as compared to the hydrogel having low mechanical properties. Collagen patches can provide higher cell engraftment at the infarcted region, compared to the injectable hydrogels and can be used to cover large infarct area, whereas, injectable hydrogels can target smaller focal regions [29]. Therefore, we used a non-denatured type 1 collagen scaffold to provide 3D microenvironment to the cells. MSCs were seeded on the collagen scaffold using a similar strategy as described previously [17]. Scanning electron microscopic analysis was performed to analyze the porous network of collagen fibers and cell attachment.

MSCs have the potential of differentiating into cardiomyocytes both *in vitro* and *in vivo* [31]. *In vitro* cardiac differentiation was first described by Makino *et al*., in 1999, in which a demthylating agent, 5-azacytidine, was used to treat bone marrow stromal cells. They observed cardiomyocyte-like characteristics and beating of cells in culture [32]. Toma *et al*., demonstrated the *in vivo* cardiac differentiation of BM-MSCs upon transplantation into adult murine heart. Transplanted cells showed migration, proliferation, and differentiation in the infarcted region [33]. These two principle discoveries led scientists to make advances in the MSC transplantation strategy for the treatment of ischemic cardiovascular diseases [34].

Epigenetic changes are the landmarks of early cardiac differentiation as they cause activation of cardiac specific genes and repression of non-cardiac and cell cycle progression genes. Epigenetic modifications of DNA or nucleosomes are tightly regulated by enzymes. DNA methyltransferases block access to the promoter and halt subsequent transcription of downstream genes. DNA demethylation of CpG island of the promoter region clears this blockage and allows access to the promoter for active

transcription [35]. Previous studies reported the use of DNA methyltransferase inhibitors such as zebularine, 5-azacytidine and 2-deoxycytidine for cardiac differentiation of MSCs [11, 36]. Previously, we have reported that zebularine is non-cytotoxic to cells at 3 µM concentration and has shown better cardiac differentiation of MSCs [11]. Here, we also used same concentration of zebularine to induce cardiac differentiation in three different ways. In the type 1 treatment, MSCs were first treated with zebularine for 48 h, and then seeded in collagen scaffold; in type 2 treatment, zebularine was added in the cell suspension which was then seeded in the scaffold, while in the third type, cells were first seeded in the scaffold and then zebularine was added in the medium. All three treatment types were non-cytotoxic to the cells, as indicated by the MTT assay.

Gene expression analysis by qRT-PCR showed significantly enhanced expression of cardiac specific genes Nkx2.5, Mef2C, and cTnI in type 1 treatment group. Nkx2.5 is the crucial protein that leads the mesoderm towards the development of heart by commencing the transcription of other cardiac specific transcription factors, like GATA4 and Mef2C, which in turn, enhances the expression of cardiac myosin heavy chain (cMHC), actin and atrial natriuretic factor (ANF) [35]. Type 2 treatment group, showed significant upregulation of Nkx2.5, GATA4, cMHC and cTnI as compared to the control group. The later three genes also showed significantly enhanced expression when compared to type 1 treatment group. Nkx2.5, Tbx20, ANF, cTnT, connexin43, and cMHC were significantly upregulated in type 3 treatment group. cMHC expression was more significant in this treatment group when compared to control. cTnT and cTnI are involved in the regulation of muscle contraction in response to intracellular calcium flux. cMHC is the main thick filament protein that participates in muscle contraction. Connexin43 is a gap junction protein, providing connections to the neighboring cardiac cells. This protein plays a pivotal role in electrical signal transduction for the coordinated contraction of cardiac cells [37].

When protein expression was analyzed, all treatment groups showed enhanced expression of GATA4, cTnT and Nkx2.5 as compared to control, however, type 2 and 3 treatment groups showed significantly higher expression of these proteins. Previous

studies also reported the expression of these cardiac genes and proteins in the differentiated cells [2, 11, 16, 36]. Type 3 treatment showed more pronounced expression of cardiac markers, including both functional and gap junction proteins, hence, this treatment was selected for *in vivo* analysis.

Previous studies demonstrated the use of adipose derived stem cell-seeded collagen scaffold in rat and porcine models of MI with improvement in ventricular remodeling and cardiac function, mainly associated with enhanced cell engraftment [25, 38]. In relevance to these reported results, we used a combination approach of pretreated MSC-seeded collagen scaffold for the regeneration of infarcted myocardium. We transplanted the collagen only scaffold, and untreated and zebularine treated MSC-seeded collagen scaffolds in acute MI model of rats. Functional parameters assessed by echocardiography showed significant increase in LVID and ESV in case of the MI group, while EF and FS were significantly reduced as compared to sham control. This functional impairment of left ventricle indicates successful establishment of the MI model. When compared with the MI group, LVIDs was significantly reduced after 6 wk in both untreated and zebularine treated MSC-seeded collagen scaffold transplanted groups. However, zebularine group showed significant reduction in LVIDs just after 4 wk. Also, significant reduction in ESV was observed after 4 wk of transplantation in the zebularine group which was more pronounced after 6 wk. Significant reduction in ESV was also observed in the scaffold only group and untreated MSC-seeded scaffold group, but only after 6 wk. Moreover, this reduction is less significant than the zebularine treated group. EF and FS showed significant improvement in the untreated and treated MSC-seeded scaffold transplanted groups after 2 wk, better than the scaffold only transplanted group as compared to MI. However, when compared to sham control, EF and FS showed significant reduction in all treatment groups. After 4 wk, EF and FS were as significant in zebularine treated MSC-seeded scaffold transplanted group as in sham control, when compared with MI. No significant reduction in EF was observed when compared with the sham control, but FS was slightly reduced. After 6 wk, EF and FS showed significant improvement in both

untreated and zebularine treated groups, compared to MI. In summary, zebularine treated MSC-seeded collagen scaffold transplanted group showed more pronounced improvement in the cardiac functional parameters than the untreated scaffold group, comparable to the sham control. Our results are in accordance with the previous studies, which showed better functional improvement in cell-seeded scaffold or only scaffold transplanted groups as compared to the MI group [20, 38, 39].

When hearts were harvested after 6 wk of surgery, scaffold transplanted hearts showed whitish residual scaffold integrated into the host myocardium. MSC-seeded scaffold transplanted group showed blood vessels running along the entire transplanted region of the myocardium. Similar result was also reported in a study where MSC-seeded collagen-GAG scaffold was transplanted in the rat heart [40]. Histological analysis showed that the scaffold only transplanted heart sections have reduced fibrotic area than that in case of the MI group, with few patches of regenerated myocytes in the infarcted region and some blood vessels in the scaffold region, while MSC-seeded scaffold group showed better accumulation of regenerated myocytes. Zebularine treated MSC-seeded scaffold group showed much reduced fibrosis, compared to the other groups. Immunohistochemical analysis showed enormous blood vessels in the scaffold region. Arterioles were obvious in the MSC-seeded scaffold and in the peri-infarct region below the transplanted patch. Zebularine treated MSC-seeded scaffold group showed significant number of blood vessels including venules.

Immunohistochemical analysis of the frozen heart sections of DiI labeled cell-seeded scaffold group with cardiac functional proteins cMHC, cTnT and cTnI, showed co-localized expression of DiI and cardiac proteins in the scaffold near the border of the host myocardium. Quantitative analysis showed significantly enhanced grafted cell density and cardiac protein expression in the zebularine treated MSC-seeded collagen scaffold group, compared to the untreated group. This data supports our histological findings which showed significantly reduced fibrosis and enhanced regeneration of the infarcted myocardium. Previous reports have confirmed restricted migration of the engrafted cells to organs other than the heart, while robust cell retention was observed

when scaffold was used, with the cells being detectable even after 1 mo of implantation [41]. Our data is in agreement with these findings, as we also observed cells in the scaffold after 6 wk of surgical transplantation. Taken together, our findings suggest that zebularine treated MSC-seeded collagen scaffold better preserved cardiac function and ventricular remodeling, and promoted regeneration.

Study Limitations: The current study used small animals; large animals should be used along with other sources of MSCs, such as human MSCs, in future studies. In addition, the *in vivo* effects of cell-seeded scaffolds were evaluated after a duration of 6 wk, while long term effects should to be explored to monitor the complete biodegradability of the transplanted scaffold and complete healing of the injury. Also, large sample size may offer more credibility to the obtained results.

CONCLUSION

This study focused on the combined effect of zebularine, a demethylating agent, and 3D collagen scaffold on the cardiac differentiation of rat bone marrow MSCs and their subsequent role in the preservation of ventricular remodeling in the rat MI model. Zebularine treatment in the 3D microenvironment enhanced the expression of cardiac markers in MSCs, both at the gene and protein levels as compared to the control. Transplantation of zebularine treated 3D collagen scaffold in the rat MI model provided beneficial effects in terms of improvement in cardiac function, cell engraftment, neovascularization and preservation of ventricular remodeling, better than collagen scaffold only group and untreated MSC-seeded scaffold group. The results obtained from the conducted preclinical study suggest the exploration of this tissue engineering based approach for future clinical trials involving MI patients.

ARTICLE HIGHLIGHTS

Research background

Cardiovascular diseases are the leading cause of death globally. Adult heart tissue possesses impaired self-renewal capability and thus shows inadequate capability of

restoring its structure and function after injury. Stem cell based therapy to treat cardiac injuries has achieved moderate success due to some limitations. Cardiac tissue engineering constructs cardiac patch or scaffold to restore cardiac function following injury. Mesenchymal stem cells (MSCs) have great potential to be used for myocardial regeneration due to their multilineage differentiation potential. Controlled fate of grafted cells can be achieved by inducing *in vitro* cardiac differentiation by demethylating agent such as zebularine.

Research motivation

MSCs are potential candidates for the regeneration of damaged cardiac tissue but their insufficient survival and engraftment at the injured tissue is a major hurdle. This can be overcome by pre-differentiation of MSCs using a demethylating agent and providing 3D microenvironment through biological scaffold. *In vivo* transplantation of pre-differentiated cell seeded scaffold can provide mechanical support and enhance cell survival, engraftment and regeneration of cardiac tissue and pave the way to develop an improved cardiovascular therapeutic strategy.

Research objectives

The study was aimed to enhance the differentiation of MSCs by treating them with demethylating agent, zebularine, in 3D microenvironment provided by collagen scaffold, and subsequent enhancement of cell engraftment, survival and myocardial regeneration upon *in vivo* transplantation in the rat myocardial infarction model.

Research methods

MSCs were isolated from rat bone marrow and characterized on the basis of specific cell surface markers and trilineage differentiation potential. MSCs were seeded in collagen scaffold and treated with zebularine to induce cardiac differentiation. MSC-seeded scaffolds were transplanted in the rat myocardial infarction model. Cardiac function assessment was done by echocardiographic analysis and ventricular regeneration by

histological analysis. Neovascularization was analyzed by immunohistochemistry with α-SMA staining. DiI labeled cell seeded scaffolds were transplanted to track the cells and their *in vivo* cardiac differentiation was analyzed by immunohistochemistry.

Research results

In vitro results showed significantly enhanced cardiac differentiation of MSCs after zebularine treatment in 3D culture. Transplantation of pre-differentiated MSC-seeded collagen scaffold in rat myocardial infarction model improved cardiac function more efficiently than untreated MSC-seeded collagen scaffold group. Histological analysis also showed improvement in myocardial regeneration, ventricular wall thickness and reduction in fibrotic tissue. Immunohistochemical analysis showed significantly enhanced vasculature and *in vivo* cardiac differentiation of transplanted MSCs in zebularine treated MSC-seeded collagen scaffold group.

Research conclusions

Pre-differentiation of MSC-seeded collagen scaffold transplantation improves cardiac function, preserves ventricular remodeling and enhances myocardial regeneration after acute myocardial infarction. This strategy provides 3D microenvironment to the transplanted cells, enhanced their survival and engraftment at the injured tissue, as well as increased blood supply by forming new vascular system.

Research perspectives

The combination approach using pre-differentiated MSCs and 3D collagen scaffold can open a new insight to repair the damage caused by ischemic cardiovascular injuries.

76465_Auto_Edited.docx

ORIGINALITY REPORT

1 %
SIMILARITY INDEX

PRIMARY SOURCES

1	Santosh Gupta, Akriti Sharma, Archana S, Rama Shanker Verma. "Mesenchymal Stem Cells for Cardiac Regeneration: from Differentiation to Cell Delivery", Stem Cell Reviews and Reports, 2021 Crossref	15 words — < 1%
2	Akon Higuchi, Qing-Dong Ling, Shih-Tien Hsu, Akihiro Umezawa. "Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation", Chemical Reviews, 2012 Crossref	13 words — < 1%
3	Oyama, Kyohei, Danny El-Nachef, Yiqiang Zhang, Patima Sdek, and W. Robb MacLellan. "Epigenetic regulation of cardiac myocyte differentiationâ€ ", Frontiers in Genetics, 2014. Crossref	13 words — < 1%
4	G. Bhuvanalakshmi, Frank Arfuso, Alan Prem Kumar, Arun Dharmarajan, Sudha Warrier. "Epigenetic reprogramming converts human Wharton's jelly mesenchymal stem cells into functional cardiomyocytes by differential regulation of Wnt mediators", Stem Cell Research & Therapy, 2017 Crossref	12 words — < 1%
5	Syeda Roohina Ali, Waqas Ahmad, Nadia Naeem, Asmat Salim, Irfan Khan. "Small molecule 2'-	12 words — < 1%

deoxycytidine differentiates human umbilical cord-derived MSCs into cardiac progenitors in vitro and their in vivo xeno-transplantation improves cardiac function", Molecular and Cellular Biochemistry, 2020
Crossref

EXCLUDE QUOTES	ON	EXCLUDE SOURCES	< 12 WORDS
EXCLUDE BIBLIOGRAPHY	ON	EXCLUDE MATCHES	< 12 WORDS