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Comparative Study between the Attenuation of Cardiac Fibrosis by Mesenchymal Stem Cells versus Colchicine

El Gammal Z. H[1], Rashed L. A[2], Abdel Aziz M. T[2], Ahmed H. M. Elwahy[3], Youakim M. F[4], AlaaEddeen M. Seufi[5]

[1]Graduate student, Faculty of Sciences, Cairo University, Cairo, Egypt, [2]Professor at Biochemistry department, Faculty of Medicine, Cairo University, Cairo, Egypt, [3]Professor at Chemistry department, Faculty of Sciences, Cairo University, Cairo, Egypt, [4]Lecturer at Anatomy department, Faculty of Medicine, Cairo University, Cairo, Egypt, [5]Prof. of Molecular Genetics, Dept of Biology, College of Sciences, Aljouf Univ., Sakaka, KSA and Professor of Molecular Genetics, Dept of Entomology, Fac of Science, Cairo Univ., Giza, Egypt.

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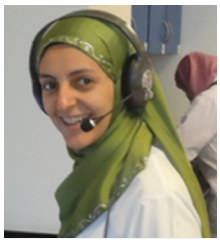

El Gammal Z. H

ABSTRACT

Introduction: Considered as an end-stage for all cardiovascular diseases, cardiac fibrosis leads to the development of heart failure, thus the ultimate goal is to prevent the progression of fibrosis. Indeed, heart can regenerate itself but to a certain limit based upon the number of resident stem cells which is limited. Thus, stem cells transplantation is considered as a promising therapy. This study aims to examine if MSC transplantation can inhibit the progression of myocardial fibrosis in rat model compared to Colchicine treatment; and if the timing of treatment with MSCs or COL affect the progression of fibrosis.

Material & Methods: To induce cardiac fibrosis in 48 female albino rats, Isoproterenol hydrochloride was used. These rats were divided into 2 models: COL-treated group that were treated after 1,2,3 weeks of the last ISO injection by colchicine orally. MSC-treated group that were injected intravenously after 1,2,3 weeks of last ISO injection by MSC. Heart rate and Systolic blood pressure were measured and the levels of Creatine phosphokinase, Lactate dehydrogenase, Matrix Metalloproteinase II and Collagen I were assessed. Moreover, cardiac tissues were examined hitopathologically.

Results & Conclusion: MSC were proved to enhance the effect of anti-remodeling of extracellular matrix significantly by modulating the expression of matrix metalloproteinases, which is superior to COL treatment.

INTRODUCTION

Early in the 21st century, cardiovascular diseases (CVD) contributed to approximately one fourth the deaths of the developing countries and one half the deaths in the developed ones. While in the 20th century, it contributed to less than 10% of all deaths worldwide. Considered as a hallmark of all heart diseases, cardiac fibrosis prevention is a corner stone heart failure treatment.[1,2]

Several approaches were tested for their efficacy to treat cardiac fibrosis. The first approach was to target the remodeling by using antagonists of neuro-humoral factors (such as ACE-inhibitors for angiotensin) that are released by cardiomyocytes.[3,4] Despite the success of this approach in animal studies, the human trials results failed to show similar results. Another approach was using anti-inflammatory drugs such as COL but this approach has serious side effects such as bone marrow depression, peripheral neuritis and myopathy.[5] While the most attractive approach was the heart transplantation, it has serious limitations because of the shortage in organ supply and the possibility of organ rejection. All these therapies do not enable tissue replacement and thus do not turn over the development of remodeling.[6,7]

Indeed, the heart has resident stem cells, but after injury, these cells are not enough to repair the whole damage.[8] To overcome this insufficiency, injection of exogenous stem cells was proposed to reduce pathological processes in the damaged myocardium and promote endogenous repair of cardiac tissue.[9] Thus, several studies focus on the injection of cardiac stem cells into the heart.[10]

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Corresponding Author:
Zaynab Hani Al Gammal, Center of Excellence of Stem Cells and Regenerative Medicine, Zewail City for Science and Technology, Egypt.
Tel.:+201010048565. E-mail: zaynabalgammal@gmail.com

Stem cells designate the ability of a progenitor cell to divide and renew itself for long periods while remaining undifferentiated and to give rise to specialized cells responding to the internal (controlled by cell genes) and external signals (cytokines, micro-environmental factors and neighboring cells' contact).[11]

Among all the different types of stem cell that can be used in cell therapy, bone marrow mesenchymal stem cells (BM-MSCs) are attractive for clinical therapy since they are multipotent, avoid the rejection by the host immune system after transplantation, and are easily expanded in culture.[12] MSCs transplantation was proved to attenuate the fibrosis in the heart,[13] lung,[14] kidney,[15] and liver[16] significantly. Furthermore, they help recovering cardiac functions by means of secreting angiogenic, antiapoptotic, and anti-inflammatory cytokines.[17]

Some authors suggested that MSCs transplantation is safe and more efficient than traditional therapy using pharmaceuticals.[18,19]

However, it remains a matter of debate if the stem cells contribute to form new cardiac tissue, to trigger endogenous repair mechanisms, or to modify inflammatory processes.[20] Some studies suggest that the anti-fibrotic effect of MSC is related to the production of matrix metalloproteinases (MMPs)[21,22] while others suggested that it's related to the decrease of collagen expression by cardiac fibroblasts.[23]

This study aims to examine if MSC transplantation can inhibit the progression of myocardial fibrosis in rat model compared to Colchicine treatment; and if the timing of treatment with MSCs or COL affect the progression of fibrosis. Our approach is to target the fibrillogenesis that takes place intracardiac not to target the homing of the stem cells to the injured myocardium.[24]

MATERIALS AND METHODS

Animals and Experimental Model

This work was achieved in the Biochemistry and Molecular Biology lab, Medical Biochemistry department, Faculty of Medicine, Cairo University.

This study was carried out on 70 female white albino rats of an average weight 100g. Rats were maintained at the well-controlled animal house under the following conditions: 25°C ± 2°C, 60% relative humidity, 12 Light:12 Dark photocycle and pathogen-free conditions. Chow and water were accessible to animals unlimitedly. Animal treatment protocols were approved by the ethical committee of Cairo University.

Four days before the start of the experiment, rats were acclimatized. Rats were then divided into two models. The negative control model (Con-) (n=8) and the cardiac fibrosis model that was induced in rats by subcutaneous injection of isoproterenol hydrochloride (Sigma Aldrich, USA) in a dose of 170mg/kg for 4consecutive days according to Lili et al.[25]

The Isoproterenol model was further subdivided into two groups: untreated group (Con+) (n=8) and treated group. The treatment of cardiac fibrosis was performed by MSC or COL that were tried at different time intervals by means of intravenous injection or oral intake.[26,27]

The treated group was then divided into the following groups:

COL-treated group: COL1, COL2 and COL3 that were treated after 1,2,3 weeks of the last Isoproterenol injection respectively by single dose of 400µg/kg colchicine orally and on the next day were injected by 1 cm PBS intraperitoneally. (n=8 each group)

MSC-treated group: MSC1, MSC2 and MSC3 that were injected intravenously after 1,2,3 weeks of last Isoproterenol injection respectively by single dose of 150µl of a cell suspension containing 3×10^6 allogeneic MSC from rats at the moment of the boost (when MSC were collected)[25] (n=8).

Heart rate (HR) and Systolic Blood pressure (SBP) were assessed by means of Langendorff apparatus.[28] Blood samples were withdrawn from each rat in order to separate the serum for the estimation of serum levels of Creatine phosphokinase (CPK) (Cusabio CSB-E13327r, China) and Lactate dehydrogenase (LDH) (Cusabio CSB-E11324r, China). At the proper time of sacrifaction, hearts were excised immediately and each of them was divided into 2 portions: one stored in 10% formalin solution to be histologically investigated. The other part was stored in RNA cell lysis to be examined for MMP-II and Collagen I using Real-Time Polymerase Chain Reaction (RT-PCR).

Preparation of BM-Derived MSC from Rats

Isolation of MSC took place following Alhadlaq and Mao protocol.[26] Briefly, 8 weeks old female white albino rats were sacrified, their femurs and tibias were dissected. Using a syringe, the bone marrow was flushed by RPMI 1640 with L-glutamine media (Euroclone, Italy) and the cells were centrifuged for 10 minutes at 3000 rpm. Pellet was then re-suspended in 1ml of the media supplemented by 10% FBS (Euroclone, Italy) and1% Streptomycin-penicillin (Euroclone, Italy) then incubated CO_2 incubator (CO_2 level 5%, Temperature 37°C). Two days later, the cells that didn't adhere to the plastic surface of the flask were discarded and only adherent cells were allowed

for propagation till the fourth passage. Whenever cell colonies reached 80% confluence, cells were trypsinized. Briefly, cells were washed twice with PBS, then trypsin EDTA (0.25%) was added for 5 min. This was followed by centrifugation at 3000 rpm for 10 min, re-suspension with complete culture medium and incubation in 50cm^2 culture flask.

Identification of BM- Derived MSC

MSC in culture were identified morphologically by their fusiform shape, ability to adhere to plastic surfaces, and by their ability of differentiation into osteocytes[29] and chondrocytes[30] and by the expressed surface markers.[31]

Osteocyte Differentiation

100nM dexamethasone, 0.25mM ascorbic acid, and 10mM beta-glycerophosphate were added to the standard medium. Then, cells were stained by Alizarin red staining in order to enable cell visualization.[29]

Chondrocyte Differentiation

500ng/ml bone morphogenetic protein-2 and 10ng/ml transforming growth factor b3 (TGFb3) were added to the standard media and cells were cultured for 3 weeks. Then, cells were stained by means of Alcian blue staining in order to enable cell visualization.[30]

Flow Cytometry

After a brief centrifugation, cells were re-suspended in wash buffer (BD Biosciences, Germany). Three hundred µl of cell suspension was incubated with antibodies against CD29, CD45, CD34 and CD25 conjugated with Allophycocyanin (APC), Cyanine 5 (CY5), Phycoerythrin (PE) and Fluorescein isothiocyanate (FITC) dyes respectively for 45 min at room temperature. Flow cytometry was performed on a FACS Calibur (BD Biosciences, Germany) and Cell Quest software was used for analysis.

Tracking of Stem Cells

CD34$^+$ cells and MSCs cells were harvested during the 4th passage. Then, cells were trypsinized and were put into a single cell suspension. 2X10^7 single cells were placed in a falcon tube, washed once using culture medium free of serum then cells were centrifuged (400xg) for 5 minutes. Finally, cells were labeled with PKH26 fluorescent linker dye (according to the manufacturer's protocol) and examined using fluorescence microscopy (Sigma-Aldrich, Saint Louis, USA).

Detection of Homing of Injected Cells in Rat Heart Tissue

After one month of last ISO injection, heart tissue was examined with a fluorescent microscope to detect the cells stained with PKH26 dye to ensure homing and to trace the injected cells in the heart tissue.

Functional Assessment

According to the manufacturer's instructions, CPK test (Cusabio, China) and LDH (Cusabio, China) were performed to assess the cardiac functions.

Histological Examination

Heart were kept in well-sealed containers in 10% formalin solution prepared in saline till becoming hard enough to be sectioned. Using paraffin blocks, 4µm thick sections were prepared. Next, heart slides were stained with Hematoxylin and Eosin (H and E) staining and Masson's tri-chrome.[32] The image analyzer computer system using the software Leica Quin 500 was used to measure the area percent of connective tissue in a standard measuring frame using a magnification of x200, by light microscopy transferred to the monitor's screen. These areas were masked by a blue color using the computer system. Area percent values for each group were obtained from 5 different fields from different slides. Values were presented as mean and standard deviation values and statistically analyzed.

Real-Time Quantitative Analysis for MMPII and Collagen I Gene Expression

Heart tissue was homogenized to extract total RNA by using RNeasy Mini Kit total RNA extraction kit (Quiagen, Germany). Then, 10µl of total RNA, 1µl antisense primer (20pmol), 1µl reverse transcriptase enzyme were used for the preparation of cDNA(15 min at 42°C).

5ul of these cDNA along with 2X SYBR Green PCR Master Mix (Applied Biosystems) and 5pmol of each primer were used to perform quantitative RT-PCR. Amplification conditions consist of the initial denaturation step (15 min at 95°C), followed by 40 cycles of denaturation (15 s at 94°C), annealing (60°C for 30 sec) and extension (30s at 72°C).

Gene Runner Software (Hasting Software, Inc., Hasting, NY) was used to design the specific primers used to amplify the required genes (Table 1).[25]

Using the comparative Ct method (Step one applied biosystem software), data from real-time were used to calculate the relative expression of MMPII and Collagen I mRNA. β-actin gene was used as a housekeeping gene to enable the normalization of all values. Finally, values are reported as fold change over background levels.

Statistical Analysis

Data were reported as mean ± SD. ANOVA with multiple comparisons post hoc test were used for normally distributed quantitative variables while non-parametrical kruscal-wallis test and mann-whitney test were used for non-normally distributed variables. Only at at $p<0.05$, results

were considered significant. Pearson correlation was done to test for linear relations between quantitative variables.

RESULTS

Morphology and Characterization of MSC

Five days after initial plating, MSC (fibroblast-like cells) were about 110 cell/cm^2 of total heterogeneous cells. Two weeks later, fibroblast-like cells were predominant in culture (Figure 1a). MSC in vitro osteogenic (Figure 1b) and chondrogenic (Figure 1c) differentiation were confirmed by morphological changes and special stains: Alizarin red and Alcian blue respectively.

Cell Surface Marker Expression Analysis Using Flow Cytometry

Cells were uniformly negative for CD25 (Figure 2a), CD34 (Figure 2b) and CD45 (Figure 2c) and positive for CD29 (Figure 2d).

Tracking of Stem Cells

MSCs were labeled by PKH26 to track its engraftment in the heart tissue (Figure 3)

Histopathological Results

Con- shows normal appearance of cardiac myocytes arranged in longitudinal bundles with central nuclei (Figure 4a, b). Con+ shows marked cardiac myocyte loss, decreased amount of viable cells, increased fibrosis and focal necrosis (Figure 4c, d). COL1 shows increased viable nucleated cardiac myocytes with less marked fibrosis and slightly congested vessel (Figure 4e, f).

MSC1 shows less myocardial damage with decreased amount of fibrosis and slightly congested vessels (Figure 4g, h). COL2 shows minimal fibrosis with increased viable cardiac myocytes and normally appearing vasculature (Figure 4i, j). MSC2 shows markedly decreased fibrosis with increased viable cardiac myocytes and minimal congested vessels (Figure 4k, l).

COL3 shows unnoticed myocardial fibrosis with viable cardiac myocytes arranged in bundles preserving normal cardiac muscle architecture (Figure 4m, n). MSC3 shows very minimal fibrosis with normally appearing bundles of cardiac myocytes respecting normal histological pattern (Figure 4o, p).

The image analysis results confirm that the area of the connective tissue increased significantly in Con+ group (p=0.008). Moreover, COL treatment 1,2 and 3 weeks after last ISO injection had attenuated this increase significantly but still there is a significant difference than Con- group (p=0.008). Also, MSC treatment had attenuated the area of the connective tissue especially after 3 weeks of last ISO injection (No significant difference than Con- group) (Figure 5).

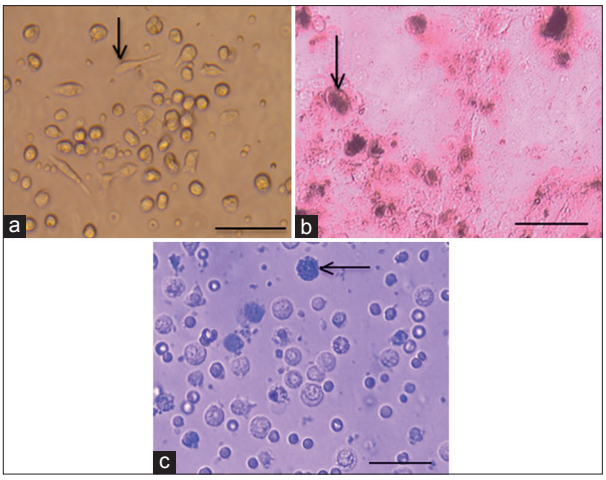

Figure 1: Morphological identification of BM-MSCs, (a) Adherent, elongated cells Bone Marrow MSC in conventional culture after 5days, (b) Alizarin red staining showing the formation of calcium deposits, (c) Alcian blue staining showing the differentiation into chondrocytes. The bar represents 100 um

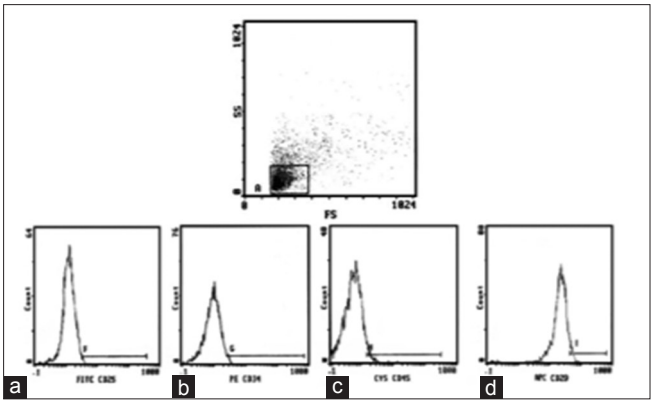

Figure 2: Flow cytometric characterization analyses of bone marrow-derived MSC. Cells were uniformly negative for CD25 (a), CD34 (b) and CD45 (c) and positive for CD29 (d)

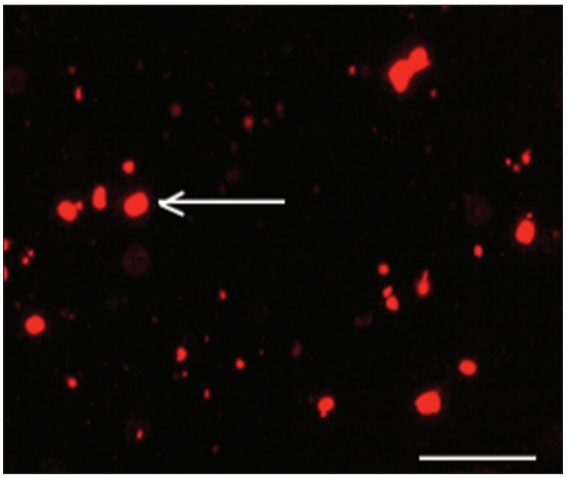

Figure 3: PKH26-labelled injected stem cells showing the engraftment in heart tissue. The bar represents 100 um

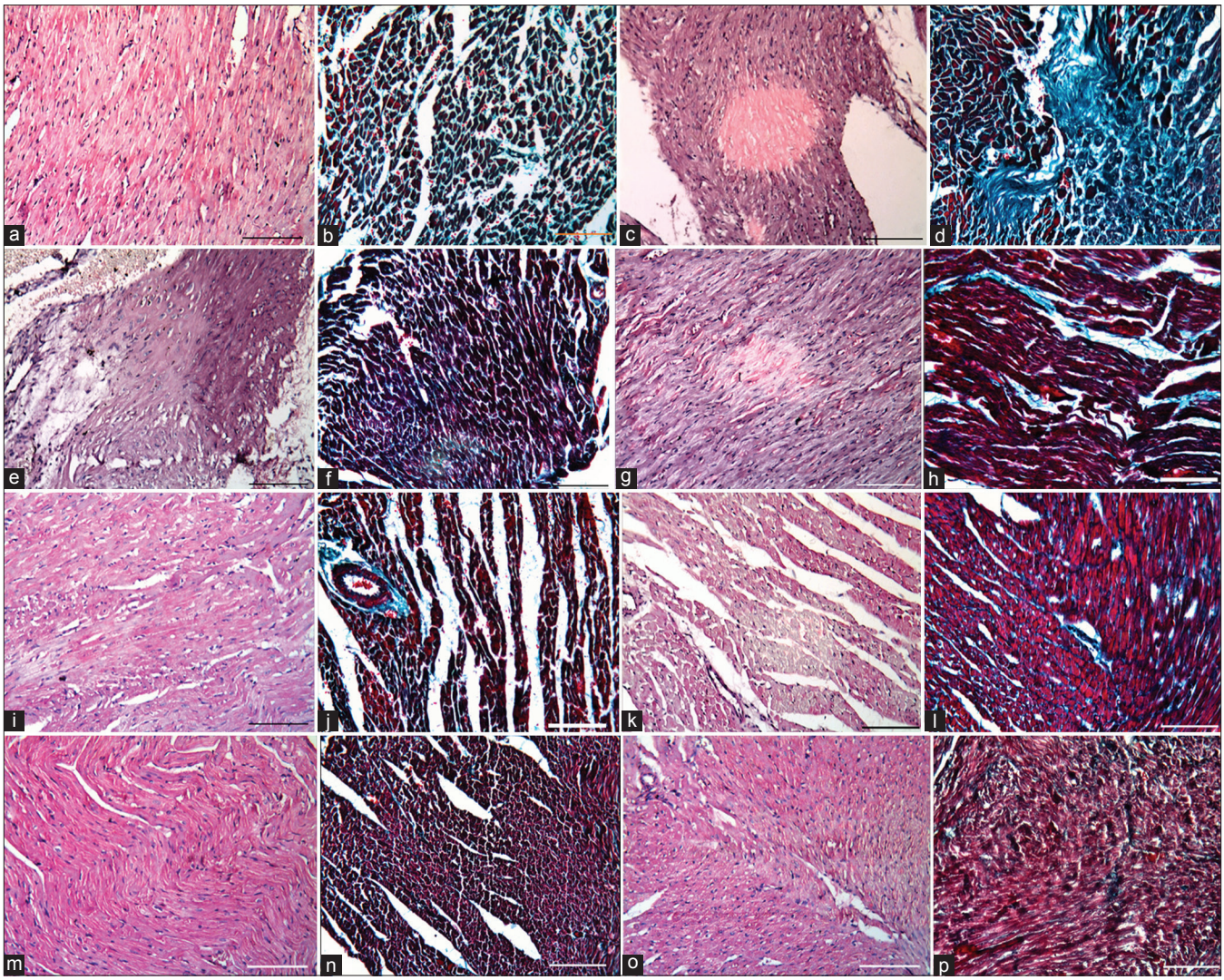

Figure 4: Histological analysis of the cardiac tissue extracted from each group by H&E and Masson's trichrome respectively at 200X (a,b) Control negative, (c,d) Control positive, (e,f) Colchicine treated group after 1 week, (g,h) Mesenchymal stem cells treated group after 1 week, (i,j) Colchicine treated group after 2 weeks, (k,l) Mesenchymal stem cells treated group after 2 weeks, (m,n) Colchicine treated group after 3 weeks, (o,p) Mesenchymal stem cells treated group after 3 weeks. The bar represents 100 um

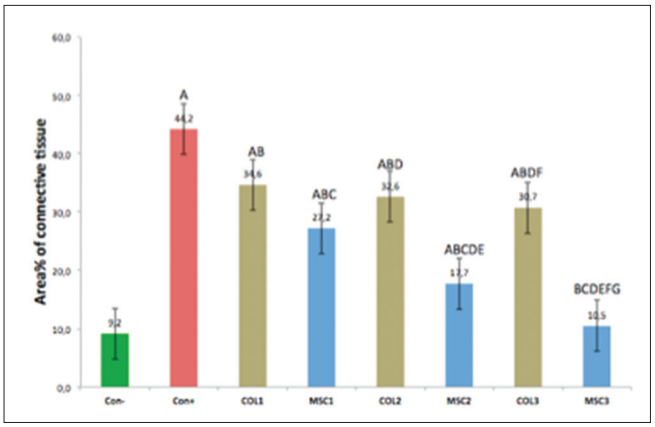

Figure 5: Image analysis of the connective positive group, COL treated group 1,2 and 3 weeks and MSC treated group after 1,2,3 weeks of tissue area in control negative group, controllast ISO injection.

Effects of COL and MSC Transplantation on Heart Function

Consistent with ISO induction of heart failure, rats had increased SBP and HR (compared to negative control rats) at the different time intervals after ISO injection. Then, rats were treated MSC or COL injection.

ISO injection had significantly increased HR in Con+ve group. Col and MSC treatment had insignificantly decreased HR after 1 week. However, Col & MSC treatment after 2 & 3 weeks had significantly decreased HR (Table 2).

Regarding SBP, ISO injection significantly increased SBP. Col treatment significantly decreased SBP after 3 weeks (Table 2).

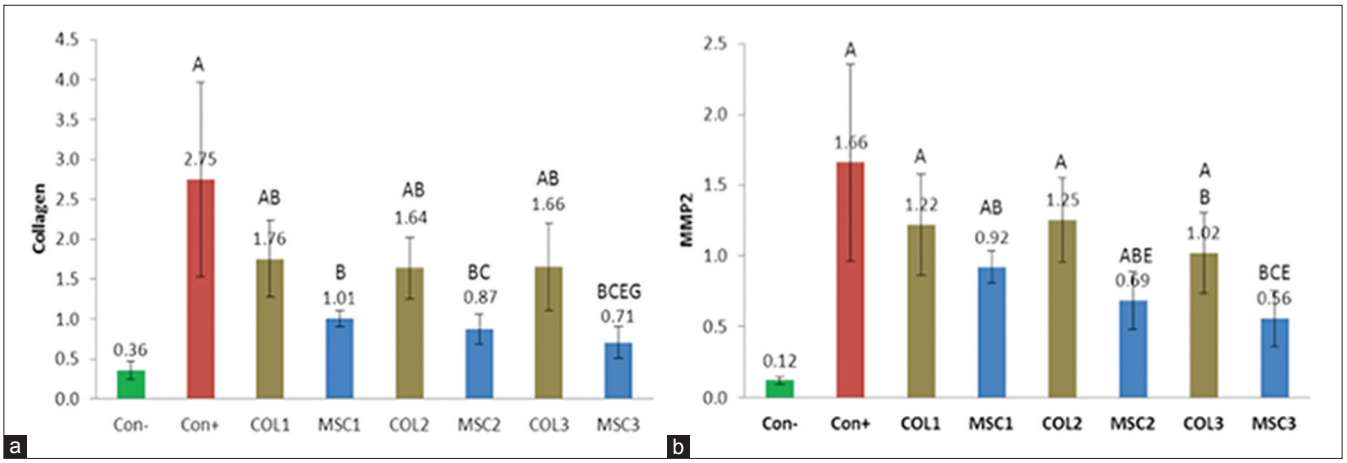

Figure 6: Effect of colchicine and MSC treatment on: Collagen expression level (collagen/ beta actin ratio) (A) and MMPII expression level (MMPII/ beta actin ratio) (B) level in normal and isoproterenol treated female albino rats. Statistically significant (P<0.05) compared to the corresponding value in A: Control Negative, B: Control Positive, C,E,G: COL treated after 1,2,3, weeks after last ISO injection respectively, D,F: MSC treated after 1,2 weeksrespectively.

Table 1: Primer sequencesrequired for real-time PCR

	Forward primer sequence	Reverse primer sequence
Beta actin	5'- ATCATGTTTGAGAC CTTCAACACC-3'	5'- TAGCTCTTCTCCAG GGAGG-3'
Collagen I	5'-TGCCG TGAC CTCAAGATGTG-3'	5'-CACAA GCGTGCTGTAGGTGA-3'
MMPII	5'-GGCCATGCCATGGG GCTGGA-3'	5'-CCAGTCTGATTGATG CTTC-3'

Primer sequences of collagenI and matrix metalloproteinase enzyme (MMPII) used to assesstheirlevels by means of real time PCR. Beta actinwasused as housekeepinggene

The level of cardiac enzymes (CPK and LDH) had been increased by the injection of ISO. Col treatment insignificantly reduced CPK and significantly reduced LDH after 2 and 3 weeks. However, MSCs treatment significantly reduced CPK only after 3 weeks and significantly reduced LDH after 1,2 and 3 weeks (Table 2).

The expression of collagen and MMPII had been increased by the injection of ISO. Col treatment had significantly reduced Collagen expression (at the 3 time intervals) and MMPII only after 3 weeks. MSCs treatment had also significantly reduced Collagen and MMPII expression at the 3 time intervals (Figure 6a and b).

DISCUSSION

The ultimate goal of new therapeutic approaches is to reverse myocardial remodeling, reduce cardiomyocytes loss caused by the apoptotic processes, and prevent myocardial wall rigidity caused by fibrotic processes. Thus, research had been focused on bone marrow–derived stem cells in different types of tissues, including the heart.[33]

This study aims to examine if MSC transplantation can inhibit the progression of myocardial fibrosis in rat model compared to Colchicine treatment; and if the timing of treatment with MSCs or COL affect the progression of fibrosis.

Therefore, BM-MSCs were isolated from female rats and engrafted into the myocardium of 48 rat model of Isoproterenol-induced myocardial fibrosis subdivided into 6 groups according to the type of injection (COL or MSC) and the time of injection (1,2 or 3 weeks).

It was hypothesized that BM-MSCs transplantation in cardiac fibrosis model may have beneficial effects through altering the extracellular matrix. Injection of human MSC into rat model of ischemic heart attenuates fibrosis, apoptosis, and left ventricular (LV) dilatation. Furthermore, it preserves systolic and diastolic cardiac function by increasing the myocardial thicknesss.[34] Through paracrine actions, MSCs were proved to inhibit fibrosis by expressing molecules involved in the synthesis of ECM such as collagens, metalloproteinases (MMPs), serine proteases and their inhibitors.[23]

We had compared the effect of MSC to the effect of a well-known anti-fibrotic agent which is colchicine.

Our results show that compared with Con-ve group, Isoproterenol-treated group (Con+ve) has significantly increased HR and SBP since it greatly decreases the mean arterial pressure and the diastolic blood pressure.[35]

Isoprenaline increases the strength of muscular contraction (Positive inotropic effect) and increases HR (Positive chronotropic effects) by means of acting on cardiac β1 receptors and β2 receptors on smooth muscle within the tunica media of arterioles.[36] Also, it has vasodilatory effect that decreases the total peripheral resistance.

COL showed decrease in HR and SBP compared to the con+ve group. It inhibits the release of fibroblast growth factors while increasing the activity of collagenase retarding the formation of collagen: COL inhibits the polymerization

Table 2: Assessment of cardiacfunctions

	Con-	Con+	Col 1	MSC 1	Col 2	MSC 2	Col 3	MSC 3
HR	112.75±4.8	156.6±10.4 A	156.7±8.3 A	152.4±5.79 A	140.8±4.8 ABC	139.4±8.05 ABC	132.8±7.31 ABCD	131.45±7.31 ABCD
SBP	104.10±1.95	137.48±1.96 A	128.60±0.94 A	120.81±2.27 AB	130.61±1.69 A	114.65±3.18 ABCE	124.71±1.81 AB	104.26±2.85 BCDEFG
LDH	135.14±5.99	219.45±6.47 A	204.3±3.42 A	195.41±3.18 AB	192.34±3.01 AB	185.4±3.03 AB	191.8±3.53 AB	181.99±4.63 ABC
CPK	91.61±3.62	193.65±8.44 A	188.88±7.06 A	191.66±4.13 A	190.74±5.14 A	183.31±2.90 A	182±5.97 A	158.08±8.49 A

Data are represented as mean±SE. Statistically significant ($p<0.05$) compared to corresponding value in A: Control negative, B: Control positive, C, E, G: COL treated after 1,2,3, weeks after last ISO injection respectively, D, F: MSC treated after 1,2 weeks respectively. HR: Heart rate, SBP: Systolic blood pressure, MMPII: Matrix Metalloproteinase, LDH: Lactatedehydrogenase, CPK: Creatininephosphokinase

of tubulin impeding the formation of microtubules in vitro[37] preventing their migration toward the chemotactic factors. Colchicine can also stimulate the activities of MMPs. It arrests mitosis and reduces DNA synthesis.[38] It is a good inhibitor of fibroblast proliferation and of collagen synthesis.

Treatment at the 3 time intervals shows a statistically significant decrease in the expression of *collagen* while only treatment after 3 weeks shows a statistically significant decrease in the expression of the *MMPII*. This may explain at least in part that the difference in improvement of cardiac functions after 3 weeks compared to that after 1 and 2 weeks since MMPs degrade the normal collagens and synthesize poorly cross-linked collagens that may cause cardiac dysfunction by means of dilation of the ventricles,[39,40] thus increasing expression of MMPs decreases cardiac function[41] while inhibiting them ameliorate heart functions inhibiting the progression of left ventricular remodeling.[42]

MSC treatment at the 3 time intervals shows a statistically significant decrease in the expression of the MMPII compared to con+ve group while only treatment after 3 weeks shows no significant difference compared to con-ve group in the expression of Collagen I and this may explain at least in part that the difference in improvement of cardiac functions after 3 weeks compared to that after 1 and 2 weeks of last ISO injection. The anti-fibrotic effect of MSCs is thought to be related to MMPs production.[21,22] Another postulated mechanism is through paracrine factors decreasing collagen expression by cardiac fibroblasts.[23]

Accordingly, Xu et al[43] showed that, in infarcted control hearts, the expression of collagen types I and III, tissue inhibitor of metalloproteinase and transforming growth factor were increased and that this increase is attenuated by MSCs transplantation. Also, Nagaya et al.[13] found that the antifibrotic effects of MSCs is by the inhibition of the proliferation of cardiac fibroblast and type I and III collagen synthesis. Furthermore, they[13] maintained that the injection of MSCs decreased LV end-diastolic pressure, increased LV contractility, increased capillary density and decreased the collagen deposition in the myocardium of rat model of induced dilated cardiomyopathy.

After 2 weeks of last ISO injection, there is a significant change in HR, SBP, Collagen and MMP-II between the MSC group, the Con-ve group and Con+ve group. This may indicate that the diffuse myocardial cell death caused by isoproterenol[44,45] persists for 2 weeks affecting the transplanted cells. This effect is minimal after 3 weeks. Thus, treatment after 3 weeks of the last ISO injection either by COL or by MSC showed the best results in the different time intervals of the same treatment group. This is why we recommend performing in-vivo imaging of the transplanted cells and their quantification.

Noticeably, CPK and LDH levels are still significantly higher than the negative control with both treatments (Col and MSC) and this indicates that still there is an active cardiac muscle injury thus the process of fibrosis will go further. This cannot be attributed to ISO since its half-life time is very short.[46] This points to the possibility of the need to repeated interference by stem cell therapy in order to combat CF which will eventually happen.

CONCLUSION

From the current study, MSC transplantation can attenuate cardiac fibrosis and improve heart functions by means of inhibiting normal collagen degradation and poorly cross-linked collagens formation. In addition, this impact can persist for at least 3 weeks.

Furthermore, the time of injection of stem cells is a crucial factor since the injection 1 week and 2 weeks after last ISO injection shows less improvement compared to that after 3 weeks. Till now, there is no evidence attributing this improvement to regeneration and other paracrine mechanisms are also believed to contribute in the improvement of heart function.

We recommend performing in-vivo imaging of the transplanted cells to confirm or deny the postulation of the death of the transplanted cells due to the effect of Isoproterenol on it.

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DISCLOSURE

No competing financial interests exist.

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