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Research Article

Use of Mesenchymal Stem Cells in a Rat with Metabolic Syndrome Complicated by Cardiomyopathy

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Abstract

Objectives: Most obese patients develop metabolic syndrome, a cluster of clinical features characterized by hypertension, insulin resistance, and dyslipidemia, a pre-diabetic condition that is often complicated by diabetic cardiomyopathy (DC). A major effort is underway to develop therapies aimed at regenerating the myocardium or to stimulate endogenous repair. The application of mesenchymal stem cells (MSCs) in the treatment of DC in recent years offers promising results. This study was conducted to evaluate the effect of the intravenous administration of MSCs on the hearts of obese diabetic rats.

Methods: Sixty male rats were fed a regular diet until they reached 1 month of age. Then, 20 rats were kept on a regular diet (healthy) and 40 rats were switched to a high-fat diet (obese) until the end of the study (16 months of tested diet). Two months after the administration of MSCs, the following parameters were evaluated: blood pressure, blood glucose, glycated hemoglobin, insulin, triglycerides, and cholesterol. The gene expression of p300, atrial natriuretic peptide (ANP), and myocyte-enhancer factor 2 (MEF2A and MEF2C) as molecular markers of cardiac hypertrophy were assessed using real-time polymerase chain reaction. Sections from the heart were stained with hematoxylin and eosin and Masson's trichrome and histopathologically examined.

Results: Blood glucose, cholesterol, triglycerides, glycated hemoglobin, and insulin resistance measurements were significantly decreased in the MSC-injected group compared with the obese diabetic group ($p<0.001$ for all parameters except triglycerides). Gene expressions of p300, ANP, MEF2A, and MEF2C were significantly decreased ($p<0.01$). Cardiomyocyte hypertrophy and inflammatory cells were significantly decreased ($p<0.01$). The levels of all parameters were not normalized compared with the control group.

Conclusion: MSCs decreased the levels of blood glucose, glycated hemoglobin, insulin, triglycerides, and cholesterol. Gene expressions of p300, ANP, MEF2A, MEF2C, as well as the number of inflammatory cells and cardiomyocyte hypertrophy were also decreased in MSC-treated DC.

Keywords: Diabetic cardiomyopathy, metabolic syndrome, mesenchymal stem cells

Metabolic syndrome is a multiplex risk factors that results from obesity-related insulin resistance. In recent years, metabolic syndrome is considered a worldwide health problem.[1–3] Six risk factors in the metabolic syndrome are highly interrelated and predispose to cardiovascular complications, diabetes mellitus and diabetic cardiomyopathy. These risk factors are abdominal obesity, hypertension, atherogenic dyslipidemia, proinflammatory

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state, prothrombotic state, insulin resistance and/or glucose intolerance.[4] Heart disease is considered the major cause of morbidity and mortality worldwide. Despite the great advance in medical and surgical management of heart disease, current therapies are only directed to treat symptoms, delay the clinical deteriorations, elevate survival rate but are not effective in the repair of the damaged myocardium. Therefore, major efforts are currently conducted to develop therapies that target regeneration of the myocardium or stimulation of the endogenous repair programs.[5]

Diabetic cardiomyopathy (DC) is defined as development of ventricular dysfunction in diabetic patients without coronary artery disease (CAD), valvular heart disease or hypertension.[6] DC leads to changes in the myocardium such as cardiac hypertrophy, apoptosis, mitochondrial uncoupling and abnormal myocardial matrix deposition. There are changes in matrix metalloproteases MMP-2 and MMP-9 in DC. Decreased MMP-2 activity leads to increase in collagen accumulation and increased activity of pro-apoptotic MMP-9 leads subsequently to apoptosis of cardiomyocytes and poor myocardial perfusion. Other pathological defects include deficiency in microcirculation due to capillary density reduction and interstitial fibrosis.[7]

In recent years, mesenchymal stromal cells (MSCs) have gained great attention as an efficient tool in regenerative therapy. The therapeutic application of MSCs in the treatment of all cardiovascular disease including DC has been elaborated in both of preclinical and clinical studies. MSCs offer high potential of therapeutic benefits due to their direct differentiation into cardiomyocytes and also due to their secretion of paracrine mediators and potent trophic factors that are capable of inducing cardiac protection as well as cardiac regeneration.[8, 9]

The present study was conducted to evaluate whether administration of MSCs could modify cardiac dysfunction in obese diabetic rat by assessment of gene expressions of certain cardiac markers of myocardial hypertrophy as well as histopathological examination of cardiac tissue.

Methods

Animals

The study was conducted on an inbred colony (Curl: HEL1) of adult rat weighing 100–150 g obtained from the Animal Experimental Unit, Faculty of Medicine, Cairo University. Care protocols of experimental animals were approved by the Institutional Animal Ethics Committee. Animals were housed at constant temperature (22±2°C) and humidity (60%), with a 12:12 hour light:dark cycle and unrestricted access to food and water. Sixty rats were included in the study. Animals were divided into 2 groups: 20 healthy control group and 40 tested rat with experimental induced obesity. The tested animals were fed on high fat diet for 16 months, then this tested obese rat group was subdivided into 2 subgroups: MSCs obese rat subgroup which was injected with MSCs intravenously and the obese rat subgroup which received the vehicle only. On the sacrifaction date, animals were deeply anesthetized and received an overdose of ketamine/xylazine (60/4 mg/Kg).

Obesity Induction

All rats were fed a regular diet up to one month of age. The control rat group was kept on a regular diet (healthy) and the tested rat group switched to a high-fat diet (obese) until the end of the study (16 months of tested diet). Regular diet consisted of to 10 cal% fats, 20 cal% proteins and 70 cal% carbohydrates. High-fat diet consisted of to 60 cal% fat, 20 cal% proteins and 20 cal% carbohydrates.[10, 11]

Mscs Isolation and Ex Vivo Expansion

Six to eight week-old male rat were sacrificed by cervical dislocation. Bone marrow cells were obtained by flushing femurs and tibias with sterile PBS. After centrifugation, cells were resuspended in alpha-MEM supplemented with 10% selected fetal bovine serum and 80 ug/mL gentamicin and plated at a density of 1x106 nucleated cells/cm^2. Non-adherent cells were removed after 72 hours by media change. When foci reached confluence, adherent cells were detached with 0.25% trypsin, 2.65 mM EDTA, centrifuged and subcultured at 7.000 cells/cm^2. After two subcultures, adherent cells were characterized and transplanted.[12]

Phenotype of Administrated Mscs

Immunophenotyping was performed by flowcytometry analysis after immunostaining with monoclonal antibodies against CD73 (FITC-conjugated) from BD Pharmingen, USA, and CD90 (PE-conjugated) (Fig. 1).[13]

Adipogenic and Osteogenic Differentiation Potential of Mscs

MSCs differentiation osteogenic and adipogenic potential was assessed after cell exposure to standard osteogenic and adipogenic differentiation protocol for 14 and 21 days as previously described.[14]

Msc Intravenous Administration

A total of 0.5x106 MSC were suspended in 0.2 mL of 5% rat plasma and administered via the tail vein to lightly anesthetized rat. Control animals received 0.2 mL of vehicle.

Biochemical Evaluation

Two months following MSC administration the following parameters were evaluated: Blood glucose, glycated hemoglobin, insulin, triglyceride and cholesterol were assessed

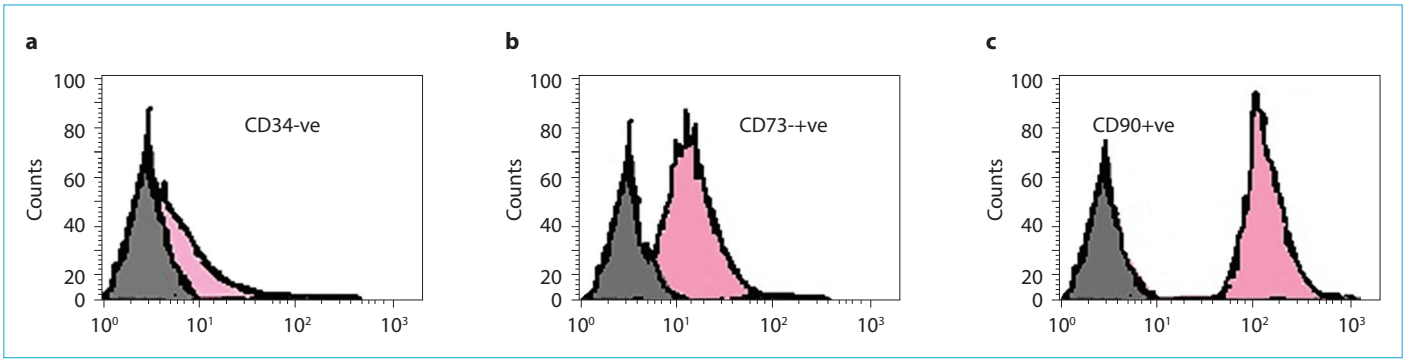

Figure 1. Flowcytometry analysis of MSCs.

by standard routine laboratory technique protocols. Blood insulin level was assessed by Insulin Rat Elisa (DRG International, Inc., USA, Catalogue No. EIA-2048). Hemoglobin A1C was determined by quantitative colorimetric determination using Stanbio™ Glycohemoglobin Test, Catalogue No SB-0350-060 (Thermo Fisher Scientific, USA).

Gene expression of p300, atrial natriuretic peptide (ANP) and myocytes enhancer factor 2 (MEF2A and MEF2C) was assessed by real time PCR as molecular markers of cardiac hypertrophy. Genomic RNA was extracted using QIAamp DNA kit (QIAGEN, USA, Catalogue No. 51104). After extraction, genes were determined by real time PCR using PCR primers set (Table 1). Kit was supplied by Qiagen, QuantiTect SYBR® Green RT-PCR Kit, Cat No./ID: 204243

Histological and Morphometric Evaluations

On the planned time of the end of the study, rats were sacrificed by cervical dislocation and dissected to expose the hearts that were fixed in 10% formalin. After fixation longitudinal sections involving both ventricles of the myocardium were randomly obtained, processed, embedded in paraffin and were cut at 5 micron thickness for histopathological examination with light field microscopy. The specimens were stained by Hematoxyline and Eosin (H&E) and Masson's trichrome and examined using an Olympus CX41 RF microscope connected to a computer through Olympus digital Camera E-330. Photomicrographs were obtained and processed using cell^B software. Morphometry was conducted using the same software.

To detect hypertrophy of cardiomyocytes the transverse trans-nuclear widths of randomly selected cardiomyocytes were measured after calibrating the system in (H&E) stained sections. The mean value of 100 LV cardiomyocytes represents each sample.[15] The number of inflammatory cells was determined by counting them in 10 fields (400×) per heart in hematoxylin-and eosin-stained sections after calibrating the system.[16] To assess fibrosis, the sections were stained with Masson's trichrome and 20 randomly selected fields per section were analyzed (200×). After each field was scanned and computerized with a digital image analyzer, collagen volume fraction was calculated as the sum of all areas containing connective tissue divided by the total area of the image.[17]

Statistical Analysis

Data was analyzed using SPSS computer program version 22.0. Quantitative data was expressed as means±standard

Table 1. Effect of Endosulfan on the activity of Monoamine Oxidase (MAO) and Glutamate Dehydrogenase (GDH)

	Primer sequence
p300 gene >XM_006242146.1:Rattus norvegicus E1A binding protein p300 (Ep300)	Forward primer 1 CTGGACAGCAGATTGGAGCA 20 Reverse primer 1 AAGCTGCTGCTGGATGAGTT 20
Atrial natriuretic peptide (ANP) PCR primer NPPA natriureticpeptide A>NM_012612.2 Rattus norvegicus natriuretic peptide A (Nppa),	Forward primer 1 CGTATACAGTGCGGTGTCCA 20 Reverse primer 1 ATCTATCGGAGGGGTCCCAG 20
Myocytes enhancer factor 2 (MEF2A and MEF2C) PCR primer>NM_001014035.1 Rattus norvegicus myocyte enhancer factor 2a (Mef2a),	Forward primer 1 TGCATCTTGTGGAAAAGGAACAA 23 Reverse primer 1 GTATCAGGGTCTGGGCTGTC 20
Myocyte enhancer factor 2C (Mef2c), transcript variant X13, mRNA>XM_006231743.1:	Forward primer 1 GCAGCAAGAACACAATGCCA 20 Reverse primer 1 TGTGGGTATCTCGATGGGGT 20

deviation, median and range. Qualitative data was expressed as number and percentage. The data were tested for normality using Shapiro-Wilk test. The nonparametric Mann–Whitney test and Kruskal–Wallis test were used for data which wasn't normally distributed. Independent Samples t-test and One-way analysis of variance test were used for normally distributed data. Spearman's correlation was used for testing of correlation between different quantitative variables. Chi-Square test was used for comparison between qualitative variables. A 5% level was chosen as a level of significance in all statistical tests used in the study.

Results

Flowcytometry analysis of MSCs demonstrate that MSCs were negative for CD34 and positive for CD73 and CD90 (Fig. 1). Regarding biochemical parameters in MSCs rat subgroup, there was a significant decrease in blood glucose, glycated hemoglobin, insulin, triglycerides and cholesterol levels in comparison to the obese rat subgroup ($p<0.0001$ for all parameters except triglycerides $p<0.01$), whereas, all of the above mentioned parameters were significantly higher in MSCs obese rat subgroup in comparison to the healthy control group ($p<0.001$ for insulin and $p<0.0001$ for the other parameters) (Table 2.)

As regards molecular markers of cardiac hypertrophy, gene expression levels of p300, ANP, MEF2A and MEF2C were significantly increased in obese diabetic group when compared to the control group ($p<0.0001$), also significantly increased in MSCs injected group in comparison to the control group ($p<0.0001$) but significantly decreased in MSCs injected group when compared to obese diabetic group ($p<0.01$) (Table 2).

Histopathology of H&E stained cardiac tissue of the control group showed single, oval and centrally located nuclei of cardiomyocytes with regularly arranged cardiac myofibrils (Fig. 2) in comparison to the diabetic group where the nuclei were hypertrophied and deformed in sizes and shapes and the myofibrils were found to be in disarrayed, fragmented, degenerated with decrease in their staining intensity and vacuolization and infiltrated by inflammatory cells (lymphocytes and histiocytes) compared to the control group (Fig. 3-5). The MScs injected group showed the same changes observed in obesity induced diabetic cardiomyopathy group but to a lesser extent (Fig. 6). Fat cells were observed inside the cardiomyocytes and in between the muscle bundles forming aggregates much in diabetic group compared to the MSCs treated group but they were nearly absent in the control group (Fig. 4, 6).

Mean cardiomyocyte width, as a marker of cardiomyocyte

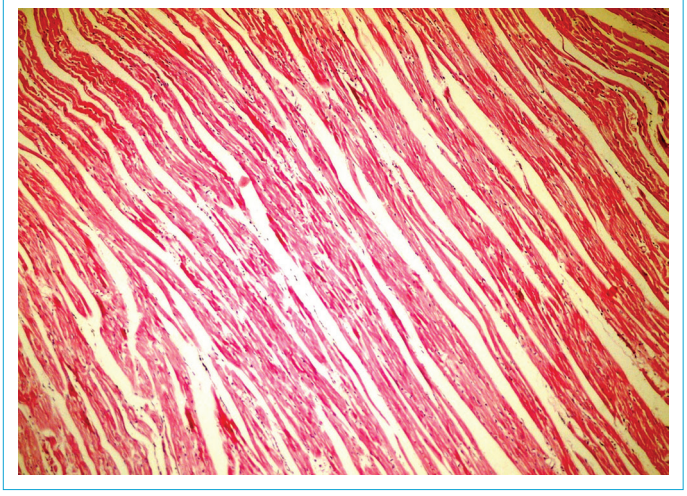

Figure 2. Cardiac tissue of control rat group.

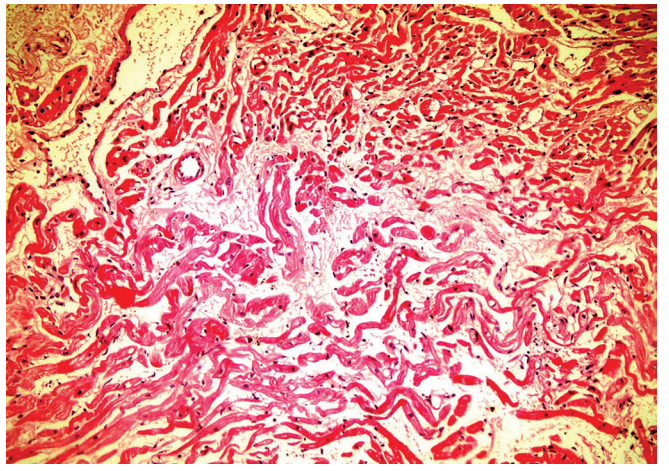

Figure 3. Cardiac tissue of obese diabetic rat group showing nuclei with hypertrophy and deformity in sizes and shapes, disarrayed, fragmented and degenerated myofibrils.

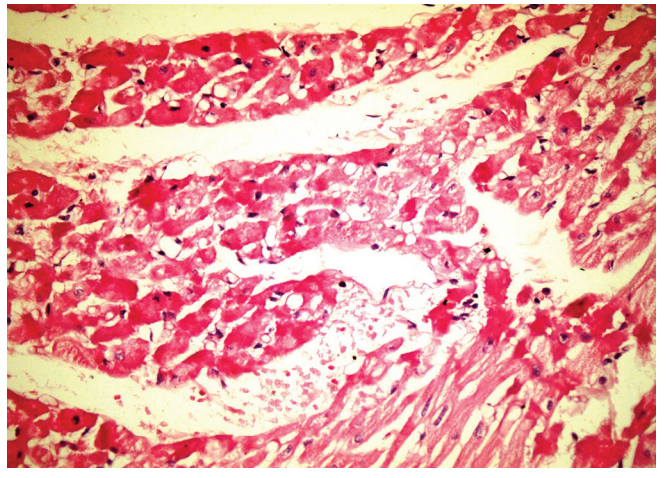

Figure 4. Cardiac tissue of obese diabetic rat group showing cardiomycytes with vacuolization and many fat cells.

Table 2. Histopathologic and biochemical parameters in the studied groups

Parameter , Mean± SD, Median (Range)	Metabolic syndrome complicated with cardiomyopathy (A)	MScs treated (B)	Control (C)	p	p1	p2	p3
Glucose (mg/dl)	229.3±35.8 218 (192–293)	150.9±21.5 145 (124–180)	90.6±9.9 87 (81–112)	0.000*	0.000*	0.000*	0.000*
Insulin (ng/ml)	3.7±0.9 4.03 (2.5–4.7)	2.4±0.3 2.4 (2.02–3.01)	1.9±0.3 1.9 (1.4–2.5)	0.000*	0.000*	0.001*	0.000*
Cholesterol (mg/dl)	238.9±34.8 226 (201–312)	177.9±15.4 180 (153–204)	153±16.5 151 (132–181)	0.000*	0.000*	0.000*	0.000*
Triglycerides (mg/dl)	103.3±17.5 109 (73–121)	89.1±9.9 91 (75–103)	71.6±12.09 73 (48–91)	0.000*	0.000*	0.016*	0.000*
HBA1c (%)	5.5±1.4 4.9 (4.1–8.2)	4.04±0.3 4.03 (3.7–4.6)	3.9±0.2 4.03 (3.6–4.3)	0.000*	0.000*	0.000*	0.000*
P300	10.3±4.4 11.5 (4.2–14.9)	3.5±0.89 3.01 (2.3–4.8)	1.2±0.28 1.04 (1–1.9)	0.000*	0.000*	0.000*	0.01*
ANP	11.04±2.8 10.7 (5.4–13.6)	4.1±1.06 3.7 (3.1–6)	1.9±0.9 2 (1–3.2)	0.000*	0.000*	0.000*	0.01*
MEF2A	10.7±3.2 10.2 (5.9–16.2)	3.3±1.3 2.6 (1.8–6.4)	1.1±0.2 1.04 (1–1.5)	0.000*	0.000*	0.000*	0.01*
MEF2C	8.02±4.3 8.1 (1.8–12.7)	3.4±1.3 3.01 (2.05–5.03)	1.2±0.3 1.03 (0.9–1.7)	0.000*	0.000*	0.000*	0.01*
Mean cardiomyocyte width	17.6±.94 18 (16–19)	15.3±1.4 15 (13–17)	10.8±1.2 11 (9–13)	0.000*	0.000*	0.000*	0.01*
Inflammatory cells	573.6±197.1 610 (270–915)	180.6±50.9 185 (80–250)	16.8±.7.9 16 (8–34)	0.000*	0.000*	0.000*	0.01*
Collagen volume	0.18±0.04 0.19 (0.13–0.21)	0.14±0.04 0.16 (0.07–0.19)	0.001±.002 .00 (.00–.007)	0.000*	0.000*	0.000*	0.069

*p Significant p value comparing three groups together by Analysis of Variance.
*p1Significant p value comparing Obese untreated with Control.
*p2 Significant p value comparing MSCs treated with Control.
*p3 Significant p value comparing MSCs treated with untreated obese.

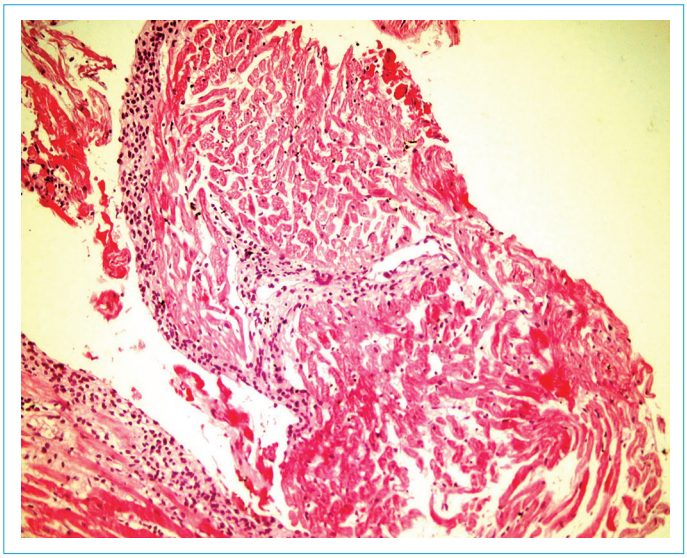

Figure 5. Cardiac tissue of obese diabetic rat group showing infiltration with inflammatory cells and lymphocytes.

hypertrophy was significantly increased in diabetic group when compared to the control group, also significantly increased in MScs injected group in comparison to the control group but significantly decreased in MSCs injected group when compared to diabetic group P<0. 0.01 (Table 2).

The number of inflammatory cells was significantly increased in diabetic group when compared to the control group, also significantly increased in MScs injected group in comparison to the control group but significantly decreased in MScs injected group when compared to diabetic group P<0. 0.01 (Table 2).

Masson's trichrome was used to stain fibrosis (stained green). Fibrosis was assessed by collagen volume fraction in perivascular area, between muscle bundles and in subendocardial region. Fibrosis was absent in the control group but was detected in both the diabetic and the MScs treated groups. Collagen volume fraction was higher in di-

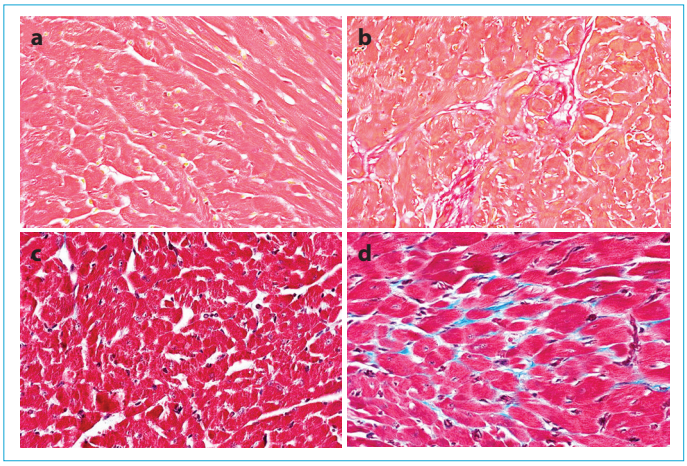

Figure 6. Cardiac tissue of MSCs treated obese diabetic rat group: **(a)** normal array of myofibrils, **(b)** small extent of degeneration with little number of fat cells, **(c)** small extent of inflammatory cells infiltration, **(d)** some degree of fibrosis.

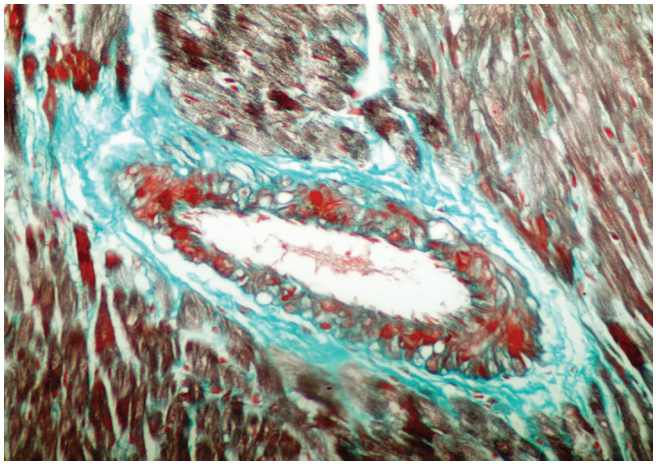

Figure 7. Cardiac tissue of obese diabetic rat group showing intense perivascular fibrosis.

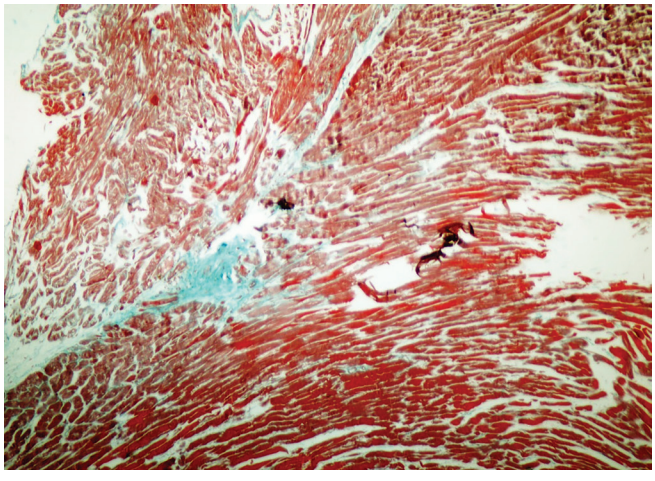

Figure 8. Cardiac tissue of obese diabetic rat group showing fibrous tissue between muscle bundles.

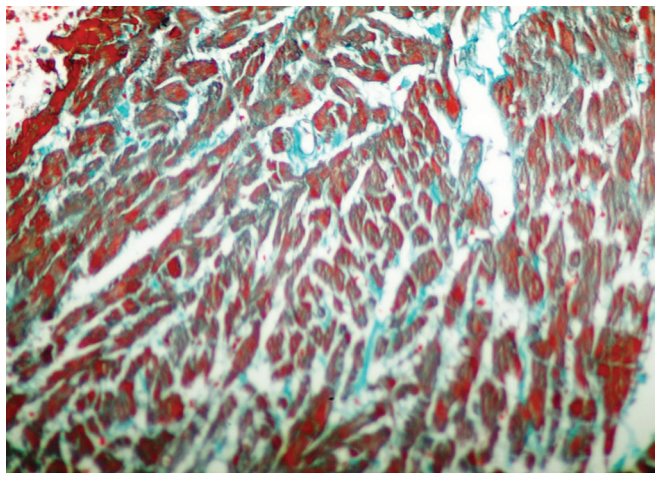

Figure 9. Cardiac tissue of obese diabetic rat group showing fibrosis between cardiomyocytes.

abetic group than in the MSCs injected group but this was statistically insignificant P<0.069 (Table 2) (Fig 6-9).

Discussion

Diabetic cardiomyopathy is characterized by myocardial cell damage, apoptosis, ventricular hypertrophy, interstitial fibrosis, functional and structural alterations of the small coronary vessels, disturbance of the metabolic management of the cardiovascular stress load and the presence of autonomic neuropathy.[18] In the present study, cardiomyopathy was confirmed in the obese diabetic rat group by histopathological detection of cardiomyocyte hypertrophy, inflammatory cells infiltration and the presence of intense fibrosis in the perivascular area, between muscle bundles and damaged cardiac myofibrils. Moreover, there was a significant elevation in gene expression levels of markers of cardiac hypertrophy: p300, ANP, MEF2A and MEF2C. Similar findings were reported in a previous study which stated that hyperglycemia induced an increase in mRNA and protein expression levels of the transcriptional coactivator p300, MEF2A and MEF2C.[19] Interstitial fibrosis and myocyte hypertrophy are characteristic features of diabetic cardiomyopathy and these pathological features lead to decreased diastolic compliance and ventricular hypertrophy.[20] These facts agree with our histopathology findings of the ventricular tissue hypertrophy and the elevated gene expression levels of markers of cardiac hypertrophy. Moreover, another study confirmed the presence of several interacting pathways for reactive oxygen species (ROS) production and their injurious effects on diabetic cardiomyocytes.[20] These facts could explain our results of the beneficial therapeutic role of the use of MSCs in obese diabetic rat. MSCs was proved to have significant anti-oxidant role in several studies.[21, 22]

Findings of the present study also demonstrated that MSCs use in obese diabetic rat led to a significant decrease in p300, MEF2A and MEF2C gene expression levels denoting amelioration of cardiac hypertrophy in comparison to the untreated rat group. Similar study stated that MSCs ameliorate cardiac remodeling and improve cardiac functions in diabetic cardiomyopathy.[23] MSCs induce angiogenesis and myogenesis by secretion of several angiogenic, antiapoptotic and mitogenic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1).[24] These findings could explain our results that exhibited amelioration of cardiac hypertrophy in MSCs treated obese diabetic rat. As regards collagen deposition in diabetic cardiomyopathy, our results showed that there was a significant elevation of collagen volume fraction in the perivascular area, between muscle bundles and in the subendocardial region of the obese diabetic rat, whereas, use of MSCs led to a significant decrease in the collagen volume deposition in the cardiac tissues. Similar findings was reported in a similar study.[23] The authors stated that in diabetic cardiomyopathy there was an increase in the quantity of the extracellular matrix (ECM) with subsequent increase in collagen deposition. Whereas, after MSCs transplantation there was a significant increase in MMP-2 activity and a significant decrease in MMP-9 activity.[24] This phenomenon leads to an increase in myocardial arteriolar density and a decrease in collagen deposition with subsequent amelioration of the cardiac remodeling and improvement of myocardial function.[24] The therapeutic benefits of MSCs in diabetic cardiomyopathy was confirmed by assessment of left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-systolic dimension (LVDs), left ventricular end-diastolic dimension (LVDd), left ventricular posterior wall thickness (LVPW) and interventricular septum thickness (IVS) all of which revealed that these parameters of cardiac functions were significantly improved by the use of MSCs in diabetic cardiomyopathy.[8]

Previous studies conducted on rodent models of diabetic cardiomyopathy and dilated cardiomyopathy reported that MSCs transplantation resulted in induction of angiogenesis, myogenesis, and secretion of angiogenic factors as well as anti-apoptotic factors (IGF-1, HGF, VEGF and adrenomedulin (AM)).[25, 26] In another experimental models of heart failure and dilated cardiomyopathy, it was proved that transplanted MSCs differentiated into cardiomyocytes, smooth muscle cells, vascular endothelial cells with improvement in myocardial functions, decrease in ventricular remodeling, decrease in collagen volume and a significant reduction in myocardial fibrosis as compared to untreated animal groups.[27-30]

Conclusion

In obese diabetic rat MSCs decrease blood glucose, glycated hemoglobin, insulin, triglyceride and cholesterol levels. Gene expressions of p300, ANP, MEF2A, MEF2C, the number of inflammatory cells and cardiomyocyte hypertrophy were also decreased in MSCs treated rat group in comparison to the untreated obese diabetic rat group.

Disclosures

Ethics Committee Approval: The study was conducted on an inbred colony (Curl: HEL1) of adult rat weighing 100–150 g obtained from the Animal Experimental Unit, Faculty of Medicine, Cairo University. Care protocols of experimental animals were approved by the Institutional Animal Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship contributions: Concept – N.S.A., T.H.S.; Design – N.S.A., T.H.S.; Supervision – N.S.A., T.H.S., H.F.; Materials – E.A.A.A.; Data collection &/or processing – E.A.A.A., H.F.; Analysis and/or interpretation – N.S.A., T.H.S., H.F.; Literature search – E.A.A.A.; Writing – E.A.A.A., N.S.A.; Critical review – H.F., T.H.S.

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