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Modulatory Effects of Estradiol-Treated Mesenchymal Stem Cells' Conditioned Medium on Impaired Cardiomyocytes in Nicotinamide/STZ-Induced Diabetic Male Rats: Study of PI3K/Akt-Atrogin-1 Signaling Axis

Saeideh Aghasi[1], Farrin Babaei-Balderlou ✉[1], Masoumeh Moradi-Ozarlou[2]

[1] *Department of Biology, Faculty of Sciences, Urmia University, Urmia, Iran.* [2] *Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.*

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ABSTRACT

Diabetes mellitus is a metabolic disorder caused by insulin resistance or lack of insulin secretion. This disease is associated with cardiovascular disorders caused by oxidative stress, inflammation, and apoptosis. Previously, the therapeutic effects of stem cells have been shown in diabetes. In the present study, the effects of substances secreted from mesenchymal stem cells extracted from bone marrow with or without treatment with estradiol were evaluated on some characteristics of the cardiovascular system in male diabetic rats. In this study, 22 male Wistar rats weighing 190±20 g were used. Two rats were used to extract the mesenchymal stem cells (MSCs) from bone marrow. Stem cells were exposed to 0 or 0.1 mM Estradiol, and then, the conditioned media (CM and ECM) of the cells were harvested. Diabetes was induced by intraperitoneal injection of nicotinamide (110 mg/kg) and then streptozotocin (65 mg/kg). Diabetic animals were divided into three subgroups intraperitoneally receiving saline, CM, or ECM (100 µl/rat) three times on days 7, 21, and 35 after the beginning of the study. On the fiftieth day, a part of the cardiac apex was transferred to -70 °C to evaluate the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), and the expression of PI3K, Akt, and Atrogin-1 genes by the qRT-PCR method. The rest of the cardiac tissue was placed in formalin for histopathologic study. Advanced glycation end products (AGEs) were measured in serum. Diabetes significantly ($p < 0.05$) caused to decrease of TAC and to increase of MDA, PI3K, Akt, and Atrogin-1 mRNA expression in the heart, and the serum AGEs levels compared to control rats, and led to cardiac tissue damage. Administration of CM or ECM significantly ($p < 0.05$) modulated all indices except Akt expression levels. In conclusion, conditioned media of bone marrow mesenchymal stem cells, especially ECM, improved some cardiovascular disorders in diabetic rats.

Introduction

Nicotinamide/streptozotocin-induced diabetes is a common laboratory model for inducing type 1 diabetes in animals. This method produces persistent hyperglycemia by selectively destroying pancreatic beta cells. The complications of diabetes result from the interaction between the direct and indirect metabolic consequences of insulin deficiency and genetic and environmental factors, and these complications include sleep disorders, cardiovascular disease, stroke, renal failure, cognitive impairment, hyperglycemic hyperosmolar syndrome, diabetic ketoacidosis, retinopathy, nephropathy, neuropathy, diabetic ulcers, and blindness.[1,2]

Diabetic cardiomyopathy (DCM) is a serious and prevalent complication of diabetes mellitus, leading to

✉ Corresponding authors. Email: f.babaei@urmia.ac.ir

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heart failure, left ventricular hypertrophy, and myocardial fibrosis.[3] Pathogenesis of this condition involves multiple contributing factors, including oxidative stress, chronic inflammation, insulin resistance, and the accumulation of advanced glycation end-products (AGEs).

Malondialdehyde (MDA), a well-established biomarker of oxidative stress, is significantly elevated in patients with diabetes and DCM. Increased MDA levels reflect lipid peroxidation of cardiac cell membranes, resulting in structural and functional damage to the myocardium. Conversely, total antioxidant capacity (TAC), represents the body's ability to neutralize free radicals, markedly reduced in these patients. The concurrent decrease in TAC and elevation in MDA shift the redox balance toward severe oxidative stress, thereby exacerbating cardiac injury.[4]

The PI3K/AKT signaling pathway plays a pivotal role in maintaining cardiomyocyte health and is profoundly impaired in the diabetic state. This pathway mediates growth factor signaling and is central to critical cellular processes such as glucose homeostasis, lipid metabolism, protein synthesis, and cell proliferation and survival. Activation of PI3K/AKT typically promotes cell survival, suppresses apoptosis, and enhances glucose metabolism. However, under conditions of chronic hyperglycemia, PI3K/AKT activity is often suppressed, directly contributing to the pathogenesis of diabetic cardiomyopathy.[5,6]

Concurrently, upregulation of Atrogin-1, a key ubiquitin ligase in the muscle proteolysis pathway, in diabetic cardiac tissue indicates activation of protein degradation pathways and myocardial atrophy. Atrogin-1 expression is induced by oxidative stress and diminished PI3K/AKT signaling, thereby promoting loss of cardiac muscle mass. Furthermore, AGEs, formed through non-enzymatic reactions between glucose and proteins, exacerbate inflammation, oxidative stress, and fibrosis upon binding to their receptor (RAGE). AGEs also inhibit the PI3K/AKT pathway and enhance Atrogin-1 expression. Thus, a complex interplay exists among oxidative stress (evidenced by elevated MDA and reduced TAC), dysregulation of the PI3K/AKT pathway, induction of Atrogin-1, and accumulation of AGEs, collectively playing a central role in the development and progression of diabetic cardiomyopathy. Targeting these interconnected pathways may offer novel therapeutic strategies for the prevention and management of this serious diabetic complication.[6,7]

Bone marrow mesenchymal stem cells (BM-MSCs) are multipotent cells that have the ability to differentiate into bone, cartilage, and adipocytes.[8] Their conditioned medium contains growth factors, cytokines, and exosomes that mediate paracrine effects of these cells. Bone marrow mesenchymal stem cells have attracted attention in the treatment of various diseases such as autoimmune diseases, muscle disorders, nerve damage, and inflammatory bowel diseases due to their anti-inflammatory, immunomodulatory, and regenerative properties.[9,10] These cells directly interact with the immune system and modulate the activity of lymphocytes, macrophages, and dendritic cells. However, today, most of the focus is on the conditioned medium (CM) of these cells, as CM contains a wide range of bioactive molecules that produce similar therapeutic effects without the need for live cell injection. This approach reduces the risks associated with cell transplantation (such as tumor formation or vascular occlusion) and allows for easier storage and standardization. Studies have shown that CM can reduce apoptosis, stimulate angiogenesis, and facilitate the regeneration of damaged tissues.[9,10] Treatment of BM-MSCs with various substances, including estradiol, can lead to changes in the secretion pattern of paracrine factors and produce a richer and more effective conditioned environment.

Estradiol, as a sex steroid, regulates the expression of genes related to tissue repair, anti-inflammation, and angiogenesis by binding to estrogen receptors. This improved conditioned medium could be more effective in therapeutic applications such as tissue repair, reducing inflammation, and supporting neurogenesis.[11,12]

The aim of this study was to investigate the effect of conditioned medium of bone marrow mesenchymal stem cells treated with estradiol on the extent of tissue changes and the involvement of the PI3K/Akt signaling pathway in these events in cardiomyocytes of streptozotocin-nicotinamide-induced diabetic rat model.

Materials and Methods

Animals

In this study, the animals (22 male Wistar rats weighing 190 ± 20 g), were randomly divided into four groups: control, diabetic, diabetic treated with CM derived from mesenchymal stem cells (MSCs), and diabetic treated with conditioned medium derived from mesenchymal stem cells treated with 0.1 mM estradiol (ECM). The initial number of animals used in this study was 30 rats. However, due to a 33% mortality rate during the diabetes induction phase (three rats from each diabetic group died, despite initial hypoglycemia management), the final sample size was adjusted to n = 5 per group.

Surgical Processes and MSCs Isolation

MSCs were harvested from the tibial and femoral bone marrow of rats following a protocol adapted from Zappia

et al.[13] Rats were initially anesthetized via intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. The femur and tibia were then aseptically excised and placed in sterile Petri dishes containing Dulbecco's Modified Eagle Medium (DMEM). To extract the marrow content, DMEM was flushed repeatedly through the medullary cavities of the bones using a syringe. Marrow suspensions collected from multiple animals were pooled, centrifuged, and re-suspended in phosphate-buffered saline (PBS, pH = 7.4) for double washing. The resulting cell suspension was plated at a density of 4×10^5 cells/cm^2 in T25 tissue culture flasks pre-filled with DMEM supplemented with 10% fetal bovine serum (FBS). The non-adherent cells were removed during medium replacement after 24 hours of incubation at 37 °C in 5% CO_2, and the adherent cells remained for further proliferation. The culture medium was refreshed every 48 hours thereafter. Upon reaching approximately 70% confluence, adherent cells were detached using a trypsin solution containing 0.02% EDTA, reseeded into new flasks, and designated as passage 1. This isolation strategy exploits the inherent adherence properties of MSCs to plastic surfaces. Through serial passaging the culture becomes progressively enriched. By the third passage, the cell population is considered highly purified for MSCs.[13]

Conditioned Media Preparation

Following the third passage, the MSCs were divided into two equal groups. One subset was subjected to treatment with 0.1 mM estradiol for duration of 48 hours.[14] Ethanol, as the vehicle for the estradiol, at the same final concentration, have been applied to the untreated MSCs generating CM for eliminating the effect of vehicle on harvested CM. Then, the culture medium was aspirated and the estradiol-exposed cells were washed three times with phosphate-buffered saline (PBS). Subsequently, they were maintained in serum-free DMEM for another 24 h. The resulting supernatant, designated as estradiol-conditioned medium (ECM), was harvested and processed by centrifugation at 3000 rpm for 10 minutes (Sigma, UK), followed by filtration through a 0.2 µm pore-size membrane to eliminate residual cellular fragments. An identical protocol was applied to untreated MSCs to generate standard CM.[14] For in vivo administration, the volume of CM or ECM administered to experimental animals was standardized relative to the secretome derived from 2×10^6 MSCs.[14] To assess the viability and proliferation index of MSCs after treatment with estradiol, the MTT assay was performed according to the method of Shushtari and Abtahi.[15] Spleen cells were isolated from healthy rat spleens under sterile conditions and stimulated with phytohemagglutinin (PHA) or culture medium alone, followed by 5 days of incubation. Suppression of polyclonal T-cell proliferation is a key indicator of the presence and function of mesenchymal stem cells.[15] The results of the MTT assay are presented as a proliferation index (PI) in Figure 1.

Diabetes Induction

In this study, the streptozotocin/nicotinamide (STZ/NA) method (Sigma Aldrich, USA) was used to induce diabetes in studied rats. Nicotinamide at a concentration of 110 mg/kg body weight of each rat dissolved in saline and STZ at a concentration of 65 mg/kg body weight of each rat dissolved in 0.1 M citrate buffer were injected intraperitoneally with a volume of 0.1 ml once in the fasting state with an interval of 20 minutes. [16] In this way, STZ was administered to the rat 20 minutes after the injection of NA. The non-diabetic group was injected with an equivalent volume of saline instead of STZ and NA. At this stage, after the end of the injections, 5% glucose solution was provided to the rat in the diabetic groups instead of water for 24 hours. On the third and seventh day after the injection of the aforementioned solutions, to ensure the induction of diabetes, the fasting blood glucose of the rats was checked and measured by a glucometer (Accu-chek, Performa, Germany) from the tail vein. Rat with blood glucose above 150 mg/dl were selected for the continuation of the experiment. [16] All solutions were prepared and freshly injected immediately before injection. The treatment period was 49 days. CM and ECM were prepared one week before the onset of diabetes induction. Conditioned media were injected in three sessions, on days 7 (at the same time as ensuring that the rat were diabetic), 21st, and 35th after STZ/NA injection, with a volume of 0.1 ml. At the end of the treatment period, dissections and sampling of rat tissues were performed. Animals were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood and tissue sampling was performed from these rat. To prepare serum, blood samples were centrifuged at 300 g for 10 minutes and placed at -20 °C until use. Tissue samples including the apex of the cardiac tissue were placed in a -80 °C freezer for further biochemical and molecular studies; TAC and MDA levels were evaluated using the kits from ArsamFarazist Co., Urmia, Iran, and the expression of PI3K, Akt, B-Actin, and Atrogin-1 genes were assessed using kits from Pars Tous and Denazist Asia, Mashhad, Iran. Remaining cardiac tissue was placed in formalin 10% solution for 2 weeks. The samples in formalin solution were used to examine changes in cardiac muscle tissue using hematoxylin-eosin (H&E) and Masson's trichrome (MTC) staining after undergoing tissue passage. The advanced glycation end products (AGEs) measured in sera using the semi-quantitative method of the Cusabio ELISA kit (CSB-E09413r) (detect range of 3.9-250 µg/ml and 0.97 µg/ml sensitivity).

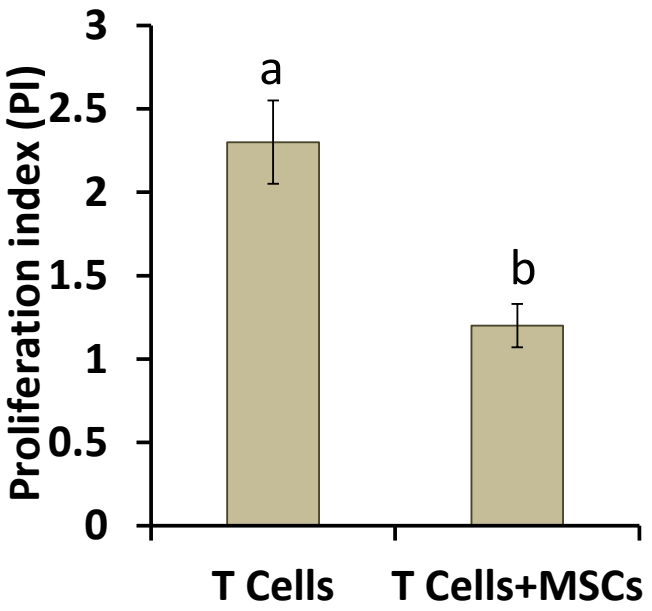

Figure 1. MTT assay results expressed as a proliferation index (PI) of T-Lymphocytes co-cultured with MSCs and without MSCs. Data were shown as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

Biochemical Evaluations in Cardiac Muscle Tissue

Total Antioxidant Capacity (TAC). To evaluate this index, the ArsamFarazist kit containing thiobarbituric acid (ABTS) 10×, reference antioxidant, lysis buffer, potassium persulfate 10× was used. In this method, ABTS is oxidized to green $ABTS^{+}$ in the presence of a suitable oxidant, which is inhibited in the presence of an antioxidant. TAC of the sample was evaluated by measuring the absorbance of $ABTS^{+}$ at 414 nm after following the steps according to the kit protocol.

Malondialdehyde (MDA). The TBARS assay as a direct quantitative method for measuring MDA in biological samples was used to evaluate the levels of MDA based on the ArsamFarazist kit containing thiobarbituric acid (ABTS), reference MDA, lysis buffer, TCA solution, 100× BHT solution. Samples containing MDA and MDA standards were first reacted with TBA at 95 °C. After a few minutes of incubation, the samples and standards were measured spectrophotometrically. The MDA content of the unknown samples was determined by comparing it with the MDA standard curve.

Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a sensitive and accurate method for measuring gene expression that begins with RNA extraction. In the first step, total RNA was extracted from cardiac tissue using a DNAzyst Asia RNA extraction column kit. Briefly, 2 g of tissue were pulverized in liquid nitrogen, and then 50 mg of that was homogenized with 500 µl of TRIzol reagent. Then, 250 µl of chloroform and 400 µl of isopropanol were added to it, respectively, and in between each solution, the solutions were centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the precipitated RNA was washed with 75% alcohol and centrifuged. The precipitated material was placed in a freezer at -80 °C. In this process, it is of great importance to maintain the integrity of RNA and prevent its degradation by ribonucleases (RNase); therefore, all tools must be RNase-free and the work must be carried out under sterile conditions. After extraction, the purity and concentration of RNA were checked with a NanoDrop device. The obtained numbers, if between 1.8 and 2, indicated the absence of DNA contamination and the purity of the samples. Then, the amount of RNA present in 1 microliter of sample was obtained in nanograms. In the next step, the extracted RNA was treated with DNase to remove any genomic contamination. The volume of solutions and temperature conditions were applied according to the instructions of the cDNA synthesis kit from Pars Toos Company. After cDNA synthesis, the qPCR step was started. In this step, cDNA was used as a template, specific forward and reverse primers for each target gene including PI3K, Akt, and Atrogin I, and reference genes (Housekeeping genes) including Actinβ, and a fluorescent dye (SYBR Green). Table 1 shows the characteristics of the primers.[17,18] The qPCR reaction was performed in thermal cycles (35 cycles) including denaturation (95 °C) for 30 seconds, primer annealing (60 °C) for 31 seconds, and primer extension (72 °C) for 30 seconds. During each cycle, the fluorescence intensity recorded was proportional to the amount of amplified product. The data obtained from the real-time analyzer were used to calculate the final expression levels of the genes for each group using the $2^{-\Delta\Delta Ct}$ method.[19]

Table 1. Sequences of the primers used in the present study.

Primer	Sequence 5' ◊ 3'	Gene product (bp)	References No.
Atrogin-1	F: GAACAGCAAAACCAAAACTCAGTA R: GCTCCTTAGTACTCCCTTTGTGAA	74	17
PI3K	F: TTCTCTGATCCATTAACCTT R: TCTTTGACAACTTGATCCTG	143	18
Akt	F: TCACCTCTGAGACCGACACC R: ACTGGCTGAGTAGGAGAACTGG	146	18
Actb	F: GGCACAGTCAAGGCTGAGAATG R: ATGGTGGTGAAGACGCCAGTA	143	18

F: Forward; R: Reverse; PI3K: phosphatidylinositol-3-kinase; Akt, protein kinase B; Actb: beta actin.

Histopathological Evaluations

The samples fixed on formalin underwent standard histological processing, were embedded in paraffin, and sectioned using an automatic rotary microtome (LKB, UK). Sections were stained with hematoxylin-eosin and Masson's trichrome staining. The desired parameters, including cardiomyocyte hypertrophy (size and diameter), hyperchromacy of nuclei, degeneration, vacuolation, and necrosis of the cells, infiltration of inflammatory cells, and interstitial space fibrosis, were evaluated by light microscope (Olympus, BX60) and compared between groups.

Statistical Analysis

In this study, the normality of the data distribution was determined using the Shapiro-Wilk test, and the data were compared for significant differences using the One-Way Analysis of Variance (ANOVA) test and the Tukey's test. The data were presented as mean ± standard deviation. Values of $p < 0.05$ were considered significant.

Results

Biochemical and Molecular Assessments

Figure 2 shows the levels of AGEs measured in the blood serum of the studied animal models. According to this graph, the levels of AGEs in the serum of diabetic rat were significantly increased compared to healthy control rat ($p < 0.05$). Treatment of diabetic rat with simple conditioned media also caused a significant decrease in the level of AGEs compared to diabetic rat ($p < 0.05$). Treatment of diabetic rat with estradiol conditioned media also caused a significant decrease in the level of AGEs compared to diabetic rat ($p < 0.05$). Comparison of rat receiving conditioned media with each other showed that estradiol conditioned media did not cause a significant difference in the serum AGEs index compared to simple conditioned media ($p > 0.05$).

Figure 3, which shows the TAC levels in the cardiac tissue of the animal models used in this study, a significant decrease in TAC levels in the hearts of diabetic rat compared to healthy control rat is clearly visible ($p < 0.05$). However, treatment of diabetic rat with a CM significantly increased TAC levels compared to diabetic rat ($p < 0.05$). Treatment of diabetic rat with an ECM also led to a significant increase in TAC levels compared to diabetic rat ($p < 0.05$). The observational study showed that the ECM had a more significant, but greater, increasing effect on TAC levels in the cardiac tissue compared to the simple conditioned environment.

The results of the study of MDA levels in tissue samples obtained from the hearts of the animal models used in this study are shown in Figure 4. Based on this graph, it was observed that the MDA level in the cardiac

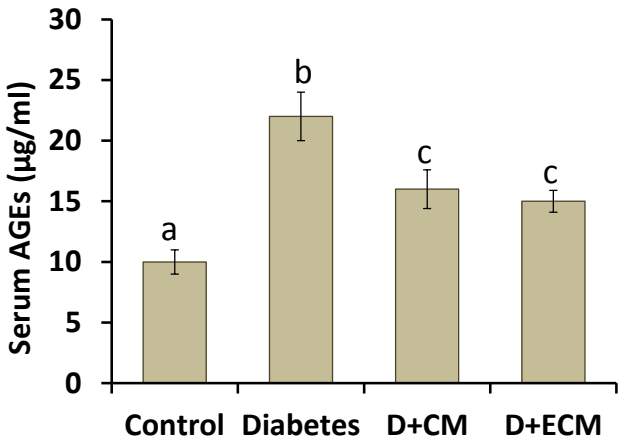

Figure 2. The levels of advanced glycation end products (AGEs) in the serum of the studied animals. CM: CM obtained from the culture of bone marrow mesenchymal stem cells, ECM: conditioned medium obtained from the culture of bone marrow mesenchymal stem cells treated with estradiol. Data were shown as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

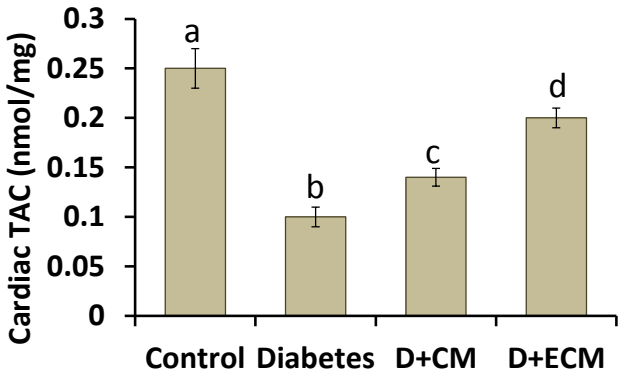

Figure 3. Total antioxidant levels (TAC) in the myocardium of the studied animals. CM: CM obtained from the culture of bone marrow mesenchymal stem cells, ECM: conditioned medium obtained from the culture of bone marrow mesenchymal stem cells treated with caffeine. Data were shown as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

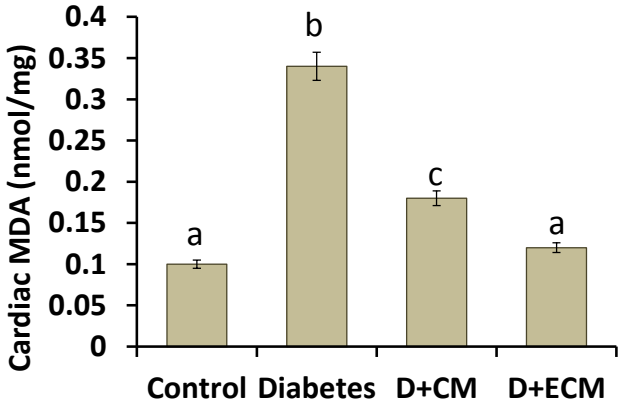

Figure 4. Malondialdehyde (MDA) levels in the cardiac tissue of the studied animals. CM: CM obtained from the culture of bone marrow mesenchymal stem cells, ECM: conditioned medium obtained from the culture of bone marrow mesenchymal stem cells treated with caffeine. Data were shown as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

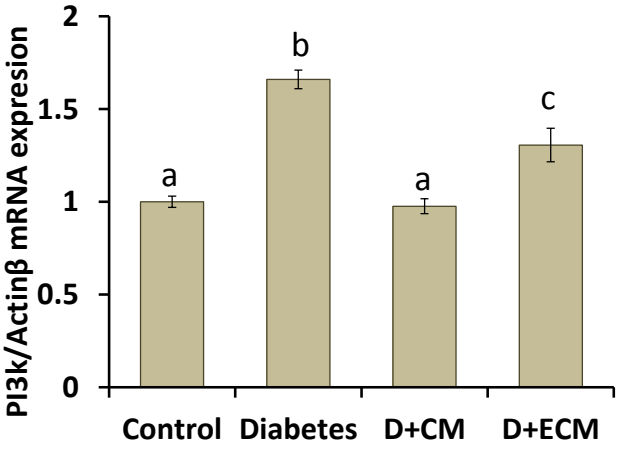

Figure 5. PI3K mRNA expression levels in cardiomyocytes of the studied animals. HG: hyperglycemia induced by diabetes, CM: CM derived from bone marrow mesenchymal stem cell culture, ECM: conditioned medium derived from bone marrow mesenchymal stem cell culture treated with estradiol. Data were expressed as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

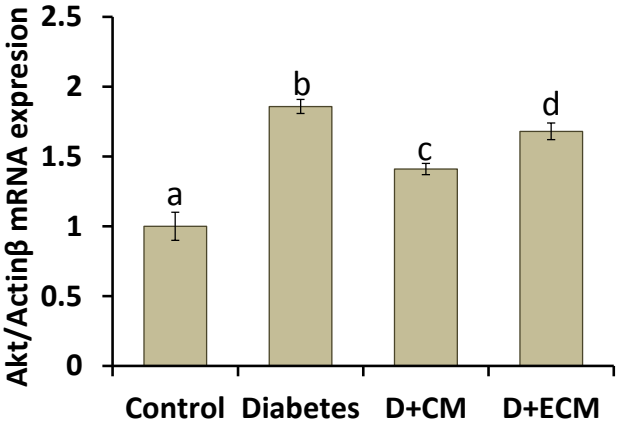

Figure 6. Akt mRNA expression levels in the hearts of the studied animals. HG: hyperglycemia induced by diabetes, CM: CM obtained from bone marrow mesenchymal stem cell culture, ECM: conditioned medium obtained from bone marrow mesenchymal stem cell culture treated with estradiol. Data were expressed as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

tissue samples of diabetic rat was significantly increased compared to healthy control rat ($p < 0.05$). While the treatment of diabetic rat with CM caused the MDA levels to decrease significantly compared to diabetic rat ($p < 0.05$). Also, the treatment of diabetic rat with ECM also resulted in a significant decrease in MDA compared to diabetic rat ($p < 0.05$). Comparison of the obtained results showed that the ECM had significantly greater reducing effects on the MDA level of cardiac tissue samples compared to the CM ($p < 0.05$).

In the analysis of the results obtained from real-time PCR, it was found that the expression level of PI3K mRNA in cardiomyocytes of diabetic rat was significantly increased compared to healthy control rat ($p < 0.05$) (according to Figure 5). While the treatment of diabetic rat with a CM was able to significantly reduce the expression index of PI3K mRNA compared to the diabetic group ($p < 0.05$). Also, the treatment of diabetic rat with an ECM had a similar result and led to a significant decrease in the expression of this gene compared to the diabetic group ($p < 0.05$). However, the reducing effects of the CM on PI3K gene expression were significantly greater than the effects of the ECM ($p < 0.05$).

Further analysis of the real-time PCR data showed that the expression of Akt mRNA in the cardiac tissue samples of diabetic rat showed a significant increase ($p < 0.05$) compared to healthy control rat (Figure 6). Also, treatment of diabetic rat with ECM was able to significantly decrease the expression of this gene compared to diabetic group ($p < 0.05$). Treatment of diabetic rat with CM was able to significantly reduce this index compared to the diabetic group ($p < 0.05$). Therefore, a significant functional difference ($P<0.05$) was observed between the groups treated with conditioned medium, so that CM caused more decrease in Akt gene expression compare to ECM in diabetic rats.

In addition to what was mentioned above, in the studies conducted on the data obtained from real-time PCR, the expression of Atrogin-1 mRNA in cardiac tissue samples of rat showed a significant increase in the expression of this gene in diabetic rat compared to healthy control rat ($p < 0.05$). While the treatment of diabetic rat with a CM was able to significantly reduce this index compared to the diabetic group ($p < 0.05$). Also, the treatment of diabetic rat with an ECM was able to significantly reduce the expression of this gene compared to the diabetic group ($p < 0.05$). However, a significant difference was observed between the groups treated with both conditioned media (simple and estradiol) ($p < 0.05$), such that the CM had greater reducing effects compared to the ECM (Figure 7).

Histopathological Evaluation of Cardiac Tissue

Histopathological analysis of cardiac tissue section revealed, the control group exhibited normal cardiac structure, with healthy cardiomyocytes, regularity and uniformity in fiber dimensions and fiber interconnection are evident (Figure 8A). Histopathological observations of cardiac tissue sections in the diabetic group included cardiomyocyte hypertrophy (increased diameter and larger, hyperchromatic nuclei), myofibril degeneration, vacuolated cytoplasm and necrosis with pyknosis of the cell nucleus, and scattered infiltration of inflammatory cells and fibrosis in the interstitial space (grade: +3) (Figure 8B). Figures 8C and 8D show cardiac slices from diabetic rat that received CM (C) and ECM (D), respectively. A reduction in lesion severity was observed in the treated groups (CM group, garde: +2; ECM group, grade: +1) compared to the diabetic group (reduction in

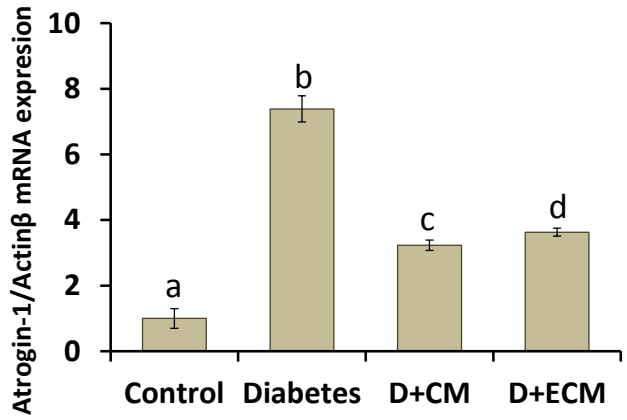

Figure 7. Atrogin-1 mRNA expression levels in the hearts of the studied animals. HG: hyperglycemia induced by diabetes, CM: CM obtained from bone marrow mesenchymal stem cell culture, ECM: conditioned medium obtained from bone marrow mesenchymal stem cell culture treated with estradiol. Data were expressed as mean ± standard deviation. Different letters indicate significant differences between groups at the 95% confidence level.

the number of pyknotic nuclei and vacuolation of the cytoplasm), which were more similar to the control group.

Mason's Trichrome Staining

Figure 9 shows cardiac tissue samples stained with Masson's trichrome in different groups. This type of tissue staining shows changes associated with fibrosis. The blue color and, subsequently, the bluish-purple color indicate the accumulation of elastic collagen fibers in the tissue. Figure 9A shows that collagen levels in the cardiac tissue appear within the normal range; consequently, the pathological condition of fibrosis is not observed in the cardiac tissue of rats in the healthy control group. However, Figure 9B shows a relative but not significant interstitial fibrosis in the myocardial tissue of diabetic rat. Fibrosis resulting from increased collagen deposition in the interstitial spaces between cardiomyocytes can lead to impaired cardiac function. Figures 9C and 8D show sections of cardiac tissue in the diabetic groups receiving simple and estradiol-treated conditioned media, respectively. As shown, no significant fibrosis was observed in the cardiac tissue of either group. It was more similar to the myocardium of the healthy control group, and complications of diabetes were not evident in these tissues. Also, the density of collagen fibers around dilated blood vessels was more evident in the hearts of the diabetic group than in the other groups. In addition, the integrity of the diabetic myocardial tissue was altered and local disruption was observed in the diabetic tissue. These changes were not observed in the control and treatment groups.

Discussion

In the present study, the effects of CM derived from BM-SCs, pretreated with either 0 or 0.1 mM estradiol, on certain characteristics of cardiomyocytes in male rats were investigated. According to the obtained results, administration of either CM or ECM to diabetic rats led to a significant reduction in serum levels of AGEs. This amelioration appears to be attributable to the restoration of redox homeostasis through modulation of pro-oxidant and antioxidant pathways. Specifically, our findings demonstrated that treatment with CM or ECM significantly increased TAC and markedly decreased MDA levels. Furthermore, quantitative assessment of mRNA expression levels of PI3K, AKT, and Atrogin-1 in cardiac tissue revealed their significant upregulation in diabetic rats. Notably, CM treatment exerted a more pronounced

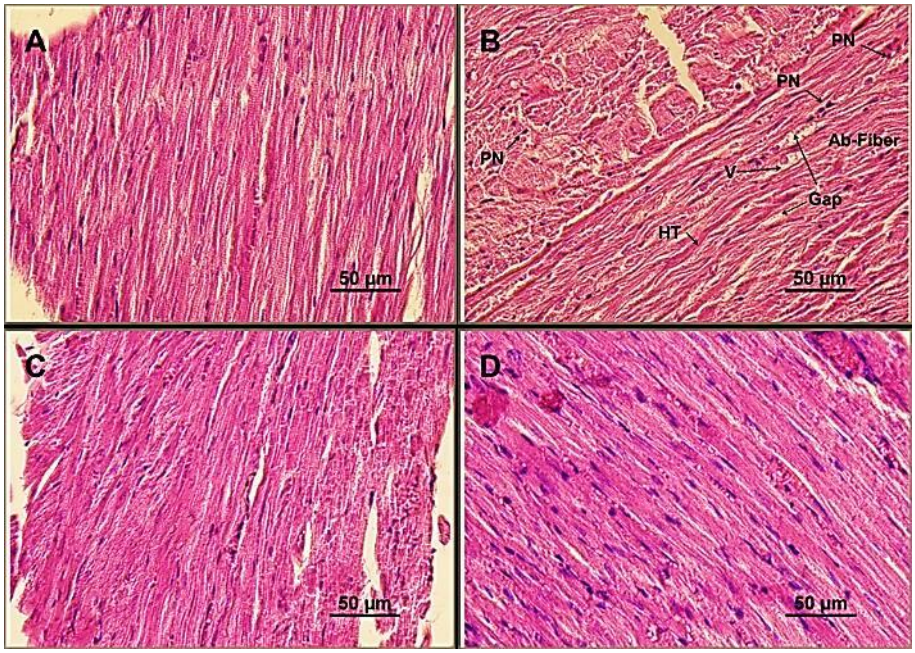

Figure 8. Rat cardiac tissue sections stained with hematoxylin-eosin. Cardiomyocytes of healthy Control group (A), Diabetic group (B), Diabetic groups receiving CM (C) and diabetic groups receiving ECM (D). Hypertrophy (HT), abnormal fibers with distorted structure (Ab-Fiber), pyknotic nuclei (PN), wide spaces between fibers and vacuolation (V).

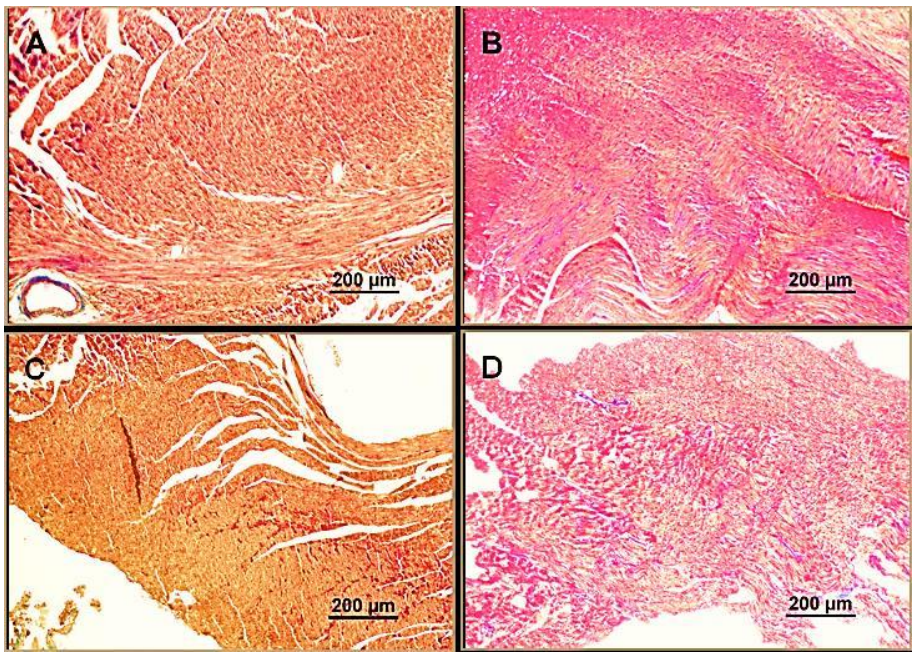

Figure 9. Rat cardiac tissue sections stained with Mason's trichrome. Cardiomyocytes of healthy control group, no collagenous areas and without fibrosis (A) Diabetic group, a scattered and, although small and insignificant increase in collagen fibers (B). Diabetic groups receiving CM (C) and diabetic groups receiving ECM (D) it was more similar to the myocardium of the healthy control group.

modulatory effect, whereas ECM treatment showed a comparatively milder impact, both leading to a significant downregulation of these markers. Increased mRNA expression of PI3K/AKT in chronic diabetes may arise from multiple physiological and pathophysiological mechanisms. The PI3K/AKT signaling pathway is one of the most critical intracellular cascades involved in glucose metabolism, cell survival, growth, and inflammation.[20] Alteration in this pathway is prevalent in diabetes, and several explanations exist for the observed upregulation of PI3K/AKT mRNA expression. Under conditions of insulin deficiency or insulin resistance, the body may develop a compensatory response by enhancing insulin signaling sensitivity. Given that the PI3K/AKT pathway is a major effector downstream of the insulin receptor, compensatory upregulation of genes in this pathway may occur in an attempt to maintain insulin responsiveness.[21] Cardiomyocytes, in particular, may activate the PI3K/AKT pathway under stress conditions to promote cell survival, as this cascade ultimately suppresses apoptotic processes. [22] Although in advanced stages of diabetes, this pathway usually exhibits reduced activity due to aberrant phosphorylation events or protein degradation at early stages or under certain pathological conditions (such as chronic inflammation or oxidative stress), mRNA expression of PI3K/AKT components may paradoxically increase, even when the activity of downstream proteins is impaired. [23] Diabetes is closely associated with systemic oxidative stress and chronic low-grade inflammation. Pro-inflammatory cytokines such as TNF-α and IL-6 can indirectly modulate the transcription of PI3K/AKT-related genes. In certain settings, the PI3K/AKT pathway is activated as a protective mechanism to counteract apoptosis in vulnerable cell populations, including pancreatic β-cells and cardiac or skeletal myocytes.[22,23] Moreover, in the present study, Atrogin-1 expression was significantly upregulated in cardiomyocytes of diabetic rats. In recent years, accumulating evidence has demonstrated that type 2 diabetes mellitus acts as a major risk factor for cardiovascular diseases and can lead to diabetic cardiomyopathy. A key mechanism implicated in this pathological process involves dysregulation of signaling pathways governing protein metabolism and cellular survival.[24] Among these, Atrogin-1 (also known as MAFbx) is a muscle-specific E3 ubiquitin ligase and a core component of the ubiquitin-proteasome system, which selectively targets contractile and regulatory proteins for degradation. Its expression is markedly elevated under catabolic conditions such as diabetes, muscle atrophy, and oxidative stress.[25] The observed upregulation of Atrogin-1 in the current study is therefore consistent with prior findings in the literature. In this investigation, diabetic rats exhibited oxidative stress (evidenced by increased MDA levels and decreased TAC), accumulation of AGEs, and dysregulation of the PI3K/Akt-Atrogin-1 signaling axis in cardiac tissue. Notably, treatment with either CM or estradiol-preconditioned BM-SCs' conditioned medium effectively modulated the mRNA expression of key genes in this pathway, suggesting a potential therapeutic role for stem cell-derived secretomes in mitigating diabetic cardiomyopathy through restoration of proteostasis and redox balance. The impact of CM derived from BM-SCs on the PI3K/Akt-Atrogin-1 signaling axis in diabetes has not been previously investigated. However, several studies have reported significant modulatory effects of BMSC transplantation on various diabetes-induced

complications in rodent models.[26,27] Despite these promising findings, the direct use of stem cells to treat diabetes and other chronic diseases accompanied with potential adverse effects such as tumorigenesis, immunogenicity, or inconsistent engraftment.[28,29] Consequently, recent research efforts have increasingly prioritized the therapeutic application of cell-free approaches, particularly the use of conditioned media or extracellular vesicles, which recapitulate many of the paracrine benefits of stem cells while minimizing safety risks.[30,31] Type 2 diabetes mellitus is characterized by profound metabolic disturbances that establish a high-risk pathophysiological milieu within cardiac tissue. Among the key contributors to diabetic cardiomyopathy, oxidative stress and the accumulation of AGEs have been consistently identified as central pathogenic factors. Our findings demonstrate that, in a rat model of diabetes, MDA, reliable biomarker of lipid peroxidation, was significantly elevated in cardiomyocytes, concomitant with a marked reduction in TAC. Furthermore, serum levels of AGEs were significantly higher in diabetic animals compared to controls. These biochemical alterations not only reflect a state of chronic pro-oxidant imbalance in the myocardium, but are also directly linked to dysregulation of the PI3K/Akt signaling pathway and subsequent upregulation of Atrogin-1.Oxidative stress in diabetes primarily stems from chronic hyperglycemia, which drives excessive production of reactive oxygen species (ROS) through multiple mechanisms; this redox imbalance disrupts cardiomyocyte homeostasis, promotes proteolytic activation via ubiquitin ligases such as Atrogin-1, and contributes to the structural and functional deterioration characteristic of diabetic cardiomyopathy. Elevated MDA levels reflect structural damage to cellular membranes and intracellular organelles in cardiomyocytes, which can profoundly impair cellular function. Concurrently, the observed reduction in TAC indicates a diminished intrinsic ability of the cell to neutralize free radicals, thereby rendering cardiomyocytes more vulnerable to oxidative injury. Under such redox-imbalanced conditions, probably, the PI3K/Akt signaling pathway, normally activated in response to insulin and growth factors, becomes functionally impaired due to oxidative modification of key signaling proteins, including Akt itself and insulin receptor substrate 1 (IRS1). Consequently, increased oxidative stress, marked by elevated MDA and reduced TAC, directly contributes to the upregulation of Atrogin-1, leading to enhanced proteasomal degradation of contractile and regulatory cardiac proteins and ultimately resulting in myocardial atrophy. However, it is important to emphasize that increased mRNA expression does not necessarily equate to increased protein activity or abundance; post-transcriptional and post-translational regulatory mechanisms may uncouple transcriptional levels from functional protein output. In the present study, an unexpected increase in mRNA expression of the PI3K/Akt pathway was observed in cardiomyocytes of diabetic rat. However, the functional activity of PI3K and Akt proteins may be significantly reduced due to post-translational mechanisms despite the increased transcript levels. Therefore, it is likely that the observed increase in PI3K/Akt mRNA expression in this context likely represents a compensatory cellular response aimed at counteracting the reduction in downstream signaling, rather than indicating actual activation of the pathway. The observed increase in PI3K/Akt mRNA expression is a novel preliminary finding. Therefore, characterizing the exact impact of CM and ECM on the PI3K/Akt signaling axis in diabetes requires future investigations focused on protein expression analysis.

A key limitation of the present study was the inability to assess protein expression levels and phosphorylation status of key signaling molecules, which prevented definitive conclusions about the functional status of the PI3K/Akt pathway in diabetic cardiomyocytes. Previously, CM of BM-MSCs treated with estradiol (E2) prior to collection of the conditioned medium has been proposed as a promising therapeutic approach. Estradiol is known to have anti-inflammatory, antioxidant, and protective effects on cardiac cells through estrogen receptors (ERα and ERβ).[32,33] Treatment of BM-MSCs with estradiol leads to a change in the secretory profile of these cells, such that the resulting conditioned medium is enriched with protective paracrine factors such as VEGF (vascular endothelial growth factor),[34] IGF-1 (insulin-like growth factor type 1) (31), HGF (hepatocyte growth factor),[35] SOD and catalase (antioxidant enzymes),[36] and regulatory miRNAs (such as miR-21, miR-146a).[37] When conditioned media, including CM and ECM, are applied to cardiomyocytes under diabetic stress, several protective mechanisms are likely to be activated, including the following: Antioxidant factors present in CM or ECM reduce ROS levels and increase intracellular antioxidant capacity. This prevents damage to mitochondria, DNA, and sarcomeric proteins and reduces oxidative stress. IGF-1 and other growth factors in CM activate the PI3K/Akt pathway through binding to tyrosine kinase receptors. Akt activation not only enhances survival, but also represses Atrogin-1 expression through phosphorylation and inhibition of FoxO (a key transcription factor). Collectively, these mechanisms contribute to the restoration of redox homeostasis, suppression of proteolytic pathways, and preservation of cardiomyocyte structure and function, highlighting the therapeutic potential of estradiol-preconditioned MSC-derived secretomes in the management of diabetic cardiomyopathy. Also, in the present study, the CM

modulated the hyperactivity of the PI3K/Akt pathway caused by diabetes. Probably, they modulate the balance of muscle catabolism/anabolism. By reducing Atrogin-1, the degradation of cardiac proteins is reduced and the structure and function of the sarcomere are preserved. Taken together, the conditioned medium of estradiol-treated BM-MSCs both reduces oxidative stress in diabetic cardiomyocytes and restores the balance of the PI3K/Akt–Atrogin-1 pathway through a complex network of paracrine signals. These findings suggest that combining cell and hormonal therapies may be an effective strategy to combat diabetic cardiomyopathy, without the need for direct cell injection and with reduced associated risks. This approach also highlights the importance of priming stem cells with physiological factors, as this may enhance their therapeutic potential.

Collectively, the present data suggest that oxidative stress (decreased TAC and increased MDA) and AGEs accumulation in diabetes not only serve as indicators of cellular damage but are also actively involved in the disruption of PI3K/Akt signaling and Atrogin-1 induction. These mechanisms explain why in diabetes, despite the presence of compensatory signals at the transcriptional level, cardiomyocytes continue to undergo atrophy and functional failure. Therefore, simultaneously targeting oxidative stress and AGEs accumulation with antioxidants or inhibitors of AGEs formation and other factors present in the conditioned medium and restoring PI3K/Akt signaling and Atrogin-1 levels to normal values could be an effective therapeutic strategy to prevent or reduce the progression of diabetic cardiomyopathy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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