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Original Research Article

Adipose-derived mesenchymal stem cells mitigate doxorubicin-induced cardiomyopathy in rats

Demet Erciyes[1], Ejder Saylav Bora[2]*, Mustafa Agah Tekindal[3], Duygu Burcu Arda[4], Oytun Erbaş[5]

[1]Department of Cardiology, Faculty of Medicine, Demiroğlu Bilim University, Istanbul, [2]Department of Emergency Medicine, [3]Department of Basic Medical Sciences İzmir Katip Çelebi Unıversity, Faculty of Medicine, Izmir, [4]Department of Pediatrics, Gaziantep Faculty of Medicine Gaziantep, [5]Department of Physiology, Faculty of Medicine, Demiroğlu Bilim University, Istanbul, Türkiye

For correspondence:* ***Email:* *saylavbora@hotmail.com;* ***Tel:*** *0090-53245078 55*

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Abstract

Purpose: *To investigate the effect of adipose-derived mesenchymal stem cells (ADMSCs) on doxorubicin (DOX)-induced cardiomyopathy in rats.*

Methods: *A total of 30 male Sprague-Dawley rats were randomized into control (n = 10) and study groups (n = 20). Control group received no intervention while the study group received DOX administered intraperitoneally (i.p.) six times daily at a dose of 2.5 mg/kg/day. The study group was divided into 2 groups. One group received DOX + normal saline (0.9 %w/v) sodium chloride (NaCl) solution intraperitoneally at a dose of 1 mL/kg/day. Another group received DOX + ADMSC at a dose of 2.0 x 10^6 cells/kg intraperitoneally twice a week. Biochemical parameters and histopathological changes in blood and heart tissue samples were compared among groups.*

Results: *Caspase-3 immuno-expression, plasma malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), growth differentiation factor-15 (GDF-15), pro-brain natriuretic peptide (Pro-BNP), troponin, heart transforming growth factor–β (TGF-β) were significantly lower in DOX+ ADMSC compared to DOX + saline group (p < 0.05). However, caspase-3 immune expression and the number of regularly arranged cardiomyocytes significantly decreased in DOX + ADMSC group compared to DOX + saline group (p < 0.05).*

Conclusion: *Adipose-derived mesenchymal stem cells (ADMSCs) reduce caspase-3 immunoexpression, restore cardiac histology, and ameliorate DOX-induced cardiac injury. Further investigation and clinical trials are recommended, especially to determine the continued safety and efficacy of AD-MSC-based therapy in cardiac injury.*

Keywords: *Cardiotoxicity, Doxorubusin, ADMSc, Caspase-3, GDF-15*

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INTRODUCTION

Doxorubicin (DOX), a potent chemotherapeutic agent derived from Streptomyces, is extensively employed in treating different types of cancer, including breast cancer, leukemia, and lung cancer [1]. Cardiotoxic effects of DOX on cardiomyocytes vary depending on dosage, and this is a significant concern in clinical practice. Administration of DOX may lead to congestive

heart failure (CHF) if the total dosage exceeds 400 – 700 mg/m² in adults and 300 mg/m² in children [2]. Doxorubicin (DOX)-induced cardiotoxicity might occur at any point during the treatment process, be it chronic, acute or subacute cases [2]. Doxorubicin also induces cardiotoxicity through multiple mechanisms, including generating free radicals, elevating calcium levels, compromising mitochondrial function, modifying gene expression, apoptosis initiation, and impairment of the innate immune system [3].

Oxidative stress is a major causative agent of cardiac injury despite the multifactorial nature of doxorubicin-induced cardiotoxicity [4]. Doxorubicin elicits oxidative stress by generating free radicals in the body [5]. The enzymatic antioxidant defense system, consisting of catalase and glutathione peroxidase, effectively eliminates hydrogen peroxide (H_2O_2) due to its reduced toxicity. Also, the reduction of the carbonyl group in doxorubicin leads to the formation of a secondary metabolite known as doxorubicinol, which also contributes to heart toxicity [4,5].

Stem cells may generate identical copies of each other, self-sustaining, and differentiate into multiple types of cells. These unique characteristics make stem cells a promising option for managing congestive heart failure (CHF). Paracrine factors secreted by MSCs participate in myocardial injury response by ameliorating oxidative stress, exerting anti-inflammatory effects, and improving mitochondrial function [6]. However, it has been shown that MSCs from different sources have variable differentiation potential [6].

In recent years, adipose-derived MSCs (ADMSCs) have become the focus of interest because they could be easily obtained from subcutaneous fat tissue with minimal donor-site morbidity. Most studies evaluating stem cells on doxorubicin-induced cardiomyopathy used bone marrow and human umbilical cord blood MSCs [6]. However, relatively few studies have examined the effect of ADMSCs on doxorubicin-induced cardiomyopathy [7]. Treatment options for congestive heart failure induced by doxorubicin are limited to supportive measures. Currently, there is no available treatment that effectively addresses established doxorubicin cardiomyopathy.

Therefore, this study investigated the effect of ADMSCs on doxorubicin (DOX) induced cardiomyopathy in rats.

EXPERIMENTAL

Animals

A total of 30 male Sprague Dawley rats (Alfasan International B.V) weighing 150 - 200 g and aged 10 - 12 weeks were randomly assigned to control (n = 10) and study groups (n = 20). The study group was equally divided into 2 groups comprising 10 animals each. This study followed the Principles of Laboratory Animal Care and approved by the Institutional Ethics Committee of Demiroğlu Science University (approval no. 2323072802). The rats were kept in stainless steel enclosures under a 12-period light/dark cycle and were provided with unrestricted food and water under regulated circumstances (22 ± 2 °C). The study was conducted in accordance with ARRIVE guidelines for pre-clinical animal trials [8].

Treatment

A total of 30 Sprague Dawley rats were assigned to control (n = 10) and study groups (n = 20). Control group did not receive any intervention, while study group received DOX (intraperitoneally) 2.5 mg/kg six times per day [9] resulting in a cumulative dosage of 15 mg/kg. Following DOX administration, the study group was randomly and equally divided into two groups consisting of 10 rats in each group. One group received intraperitoneal injection of 1 mL/kg normal saline (0.9 %w/v) solution. Another group received 2.0 × 10 ADMSCs twice a week, resulting in a total dose of 8 × 10 cells/kg. Treatment duration was 15 days. After treatment, blood samples taken from the tail veins of each rat were subjected to biochemical analysis. The animals were terminated by performing cervical dislocation using a mixture of ketamine (100 mg/kg, Ketasol, Richter Pharma AG, Austria) with xylazine (50 mg/kg, Rompun, Bayer, Germany). Heart tissue specimens were harvested for histopathological investigation.

Isolation of mesenchymal stem cells (MSC) from adipose tissue

Mesenchymal stem cells (MSCs) were taken from the flank area of the adipose tissue. Rats were given dosages of 50 mg/kg Ketasol and 10 mg/kg Rompun. Thereafter, the fatty tissue was separated and kept on ice in a sterile surrounding, before it was dispatched to the stem cell lab. Small pieces of adipose tissue were cut, and incubated at 37 °C with 0.2 % collagenase type II for 40 min under continuous shaking (Gibco, USA). The disturbed tissue was centrifuged at 1500 rpm for 5 min. The residue

was evenly distributed into 3 mL Dulbecco's Modified Eagles Medium (DMEM; Gibco, USA), supplemented with 10 % fetal bovine serum (FBS, Gibco, USA), 1 % penicillin, and 1 % streptomycin in 3 mL culture bottles. Thereafter, 2 mM L-glutamine (Invitrogen, Netherlands) and DMEM were added, and the culture vials were placed in a 5 % CO_2, and high humidity incubator set at 37 °C. The surroundings converged to an 85 % level over 3 days. After 0.25 % trypsin (Gibco, USA) was used for propagation until passage 4, the cells were seeded with an equal quantity of DMEM for inactivation. Fourth passage mesenchymal stem cells (MSCs) were kept at very low temperatures for use in the next cell transplants. Thereafter, 2 x 10 cells/mL were placed in a solution of 50 % DMEM medium, 40 % FBS, and 10 % dimethyl sulfoxide (DMSO; M.P. Bio) for cryopreservation in labeled sterile cryovials kept in a nitrogen tank at -196 ^oC. The shape and growth of the cells were examined under an inverted microscope. The cells were removed from the nitrogen tank and thawed in a water bath at 37 °C, centrifuged at 1500 rpm for 5 min, and the tiny cell cluster was mixed with DMEM solution. The samples were then kept in a CO_2 at 37 °C and ideal humidity until conditions fit for use were obtained.

MSC characterization

Second-passage immunofluorescence staining of CD 13, CD 29, and CD 105 molecules helped to identify mesenchymal stem cells [10]. After rinsing cells grown in culture dishes with phosphate-buffered saline (PBS), they were treated with methanol at −10 °C for 5 min to initiate immunostaining. Methanol was removed and dried once fixation was over. The cells were incubated for 10 min under blocking serum (normal goat serum). Following the removal of the blocking serum, cells were rinsed with PBS thrice. Simple methods were used in culturing, characterizing, and isolating mesenchymal stem cells from rat adipose tissue. The cells were incubated for 1 h under a primary antibody specific for CD13, CD29, and CD105 molecules. After washing for 5 min in PBS, the cells were incubated for 45 min using a secondary antibody before being three times washed in PBS. The cells were put in a mounting media once the knockdown was completed and viewed under fluorescence microscopy.

Histopathological examination of heart tissue

The animals were terminated by cervical dislocation using a mixture of xylazine (8 mg/kg, Alfazyne®, Ege Vet, Alfasan International B.V., Netherlands) and ketamine (80 mg/kg, Alfamine®, Ege Vet, Alfasan International B.V., Netherlands). The animals were perfused with 200 mL of 4 % formaldehyde solution in 0.1 M PBS following anesthesia. Hematoxylin and Eosin (H&E) stains were applied to formally fixed heart sections. Sections were photographed using an Olympus BX51 microscope and an Olympus C-5050 digital camera. Morphological analysis was assessed using computerized picture analysis. Shape and thickness of cardiac muscle cells were assessed using a light microscope.

Evaluation of parameters/indices

Caspase-3 immunoexpression

The sections were treated with 10 % H_2O_2 for 30 min to eradicate inherent peroxidase activity. The cells were obstructed using a solution of 10 % normal goat serum (Invitrogen) at room temperature, subjected to primary antibodies (caspase-3, Santa Cruz Biotechnology) and incubated at 4 °C for 24 h. The Histostain-Plus Bulk kit by Invitrogen contains antibodies that specifically target rabbit IgG, and the final product [11] utilized 3,3' diaminobenzidine (DAB). Photographs were captured using an Olympus C-5050 (digital camera that was connected to an Olympus BX51 microscope). Detection of brown cytoplasmic staining indicated the presence of caspase-3. Identification of caspase-3 (+) cells was conducted objectively in which at least 100 cardiomyocytes per field were systematically evaluated in ten fields of tissue pieces, repeated 40 times.

Levels of TNF-α, GDF-15, pro-BNP, and troponin

Levels of TNF-α, GDF-15, pro-BNP, and Troponin were measured using readily accessible enzyme-linked immunosorbent assay (ELISA) kits.

Heart biochemical analysis

The hearts were harvested and promptly kept at -20 ^oC for biochemical examination. Whole heart tissues were analyzed five times at the original pH of 7.4 PBS via a glass homogenizer. The mixture was centrifuged at 5000 G for 15 min. The Bradford method was used to measure protein in the heart mixtures after collecting the supernatant using bovine serum albumin as standard [12]. Commercially available rat ELISA kits were used to measure TGF-β levels in heart supernatants. Determination was done in duplicate following manufacturer instructions, and the absorbance was read with a microplate

reader (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, USA).

MDA levels

Once applied to the tissue samples, a mixture of the trichloroacetic acid and TBARS solution was heated to 100 °C for 60 min. After being chilled on ice, the samples were centrifuged at 3000 rpm. The absorbance of the supernatant was measured at 535 nm [13].

Statistical analysis

Data were analyzed using Statistical Packages for Social Sciences 22.0 software (SPSS, IBM, Armonk, NY, USA). Categorical data are presented as frequency and percentages and analyzed using Chi-square test. Measurement data are displayed as mean ± standard error of the mean (SEM) and parametric component was compared using analysis of variance (ANOVA) and Students t-test. The non-parametric component was compared using the Mann-Whitney U test. following a normality test, while Students t-test was used for comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Caspase-3 immunoexpression

The DOX + saline group exhibited a significant increase in caspase-3 immunoexpression compared to control group ($p < 0.05$). However, the DOX + ADMSC group showed significantly lower caspase-3 expression compared to DOX + saline group ($p < 0.05$) (Table 1).

Biochemical parameters

Levels of MDA, TNF-α, GDF-15, pro-BNP levels, and TGF-β were significantly higher in DOX + saline-treated group compared to control group ($p < 0.05$). However, DOX + ADMSC treated group showed significantly lower MDA, TNF-α, GDF-15, and pro-BNP levels, compared to DOX + saline group ($p < 0.05$, Table 2).

Histopathological features

Control group showed normal cardiomyocytes in the control group (A - B), DOX + saline group exhibited increased caspase-3 immunoexpression (asterisk) and disintegration of damaged cardiomyocytes (arrow). Furthermore, DOX + ADMSC group exhibited decreased caspase-3 immunoexpression and regular cardiomyocyte sequence (Figure 1).

DISCUSSION

For patients with a weak heart requiring transplantation, there are currently no options other than finding a suitable donor. For this reason, experimental studies on the protection and regeneration of cardiac muscle are ongoing, and one of the most promising options is MSCs. Doxorubicin is often used especially for cancer and lymphoma patients. However, the undesirable side effect of cardiotoxicity attributed to DOX due to the absence of available alternatives requires attention. A study by Pınarlı *et al* [14] demonstrated the effectiveness of resveratrol and adipose-derived MSCs in preventing and treating doxorubicin cardiotoxicity revealed that MSCs reduced the effects of doxorubicin-induced cellular senescence through the VEGF/Notch/TGF β signaling pathway. Also, Garbade *et al* [15] revealed that MSCs prevent doxorubicin-induced cardiac senescence by inhibiting microRNA-34a. This suggests that autologous bone marrow MSCs improve the contractility, capillary density, and collagen content in doxorubicin-induced heart failure, and administering bone marrow MSCs through subepicardial injection significantly improves ventricular function [15].

Table 1: Caspase-3 immunoexpression (n = 10 in each group, mean ± SEM)

Parameter	Control group	DOX+ Saline group	DOX+ ADMSC group
Caspase-3 immuno expression	1.4±0.5	23.5±1.7*	9.6±0.8 $^{\#}$

Note: *$P < 0.01$ vs control group, $^{\#}p < 0.05$ vs DOX + saline group

Table 2: Biochemical results (N = 10 in each group, mean ± SEM)

Parameter	Control group	DOX + saline group	DOX + ADMSC group
MDA (nmol/mg)	5.4±0.9	109.2±8.6**	57.5±7.1$^{\#\#}$
TNF-α (pg/mL)	12.4±1.5	52.7±6.3*	25.7±2.2$^{\#}$
GDF-15 (pg/mL)	322.4±5.9	618.2±7.5**	516.1±9.9$^{\#}$
pro-BNP (pg/mL)	4.08±0.3	24.2±4.1**	8.3±1.3$^{\#}$
Troponin (pg/mL)	0.96±0.1	4.05±0.8*	1.8±0.2$^{\#}$
Heart TGF-β (pg/g)	35.1±2.2	264.8±3.5**	91.03±5.7$^{\#\#}$

Note: *$P < 0.01$, **$p < 0.001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs DOX + saline group

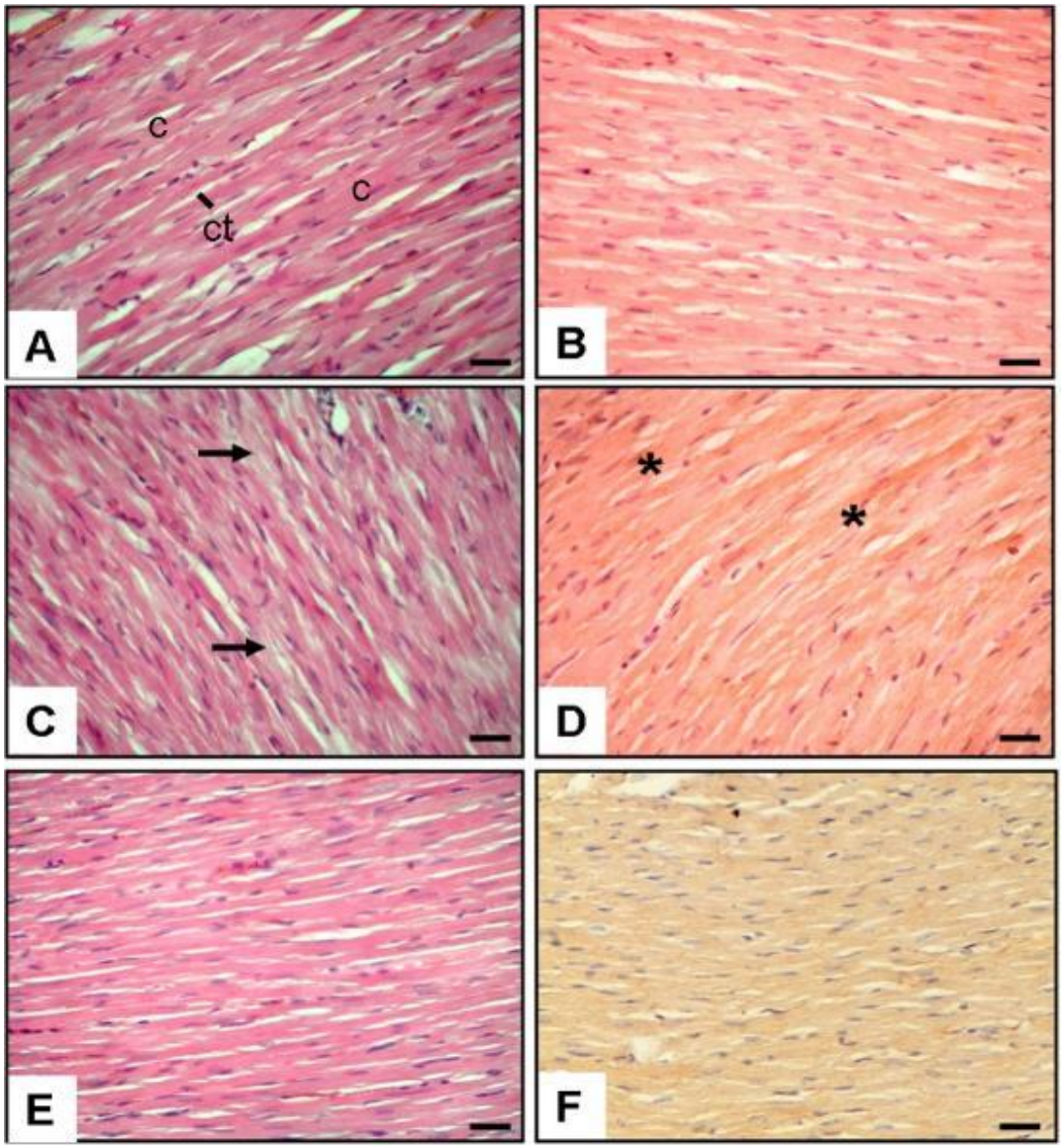

Figure 1: Heart histopathology. Hematoxylin and eosin stain and caspase-3 immunoexpression (x 40). A-B: Normal cardiomyocyte. C-D: DOX + saline group showed increased caspase-3 immunoexpression (asterisk) and disintegration of damaged cardiomyocytes (arrow). E-F: DOX + ADMSC group showed decreased caspase-3 immunoexpression and regular sequence of cardiomyocyte

Furthermore, ADMSCs are significantly more convenient than embryonic and bone marrow mesenchymal stem cells due to their abundance, ease of acquisition through minimally invasive procedures, and absence of ethical concerns. Additionally, it possesses the benefit of autologous transplantation. Recent studies have demonstrated that ADMSCs exhibit more excellent resistance to chemotherapy-induced cell death and apoptosis than bone marrow MSCs [16].

The results of this present study revealed that ADMSC reverses the cardiotoxicity of doxorubicin through histological and biochemical investigations. Oxidative stress and mitochondrial damage in the heart are the primary factors contributing to doxorubicin-induced cardiotoxicity. Impairment of cardiac mitochondria occurs shortly after the drug is given, typically within a few hours, and elevated calcium levels play a pivotal role in damaging cardiomyocytes. Also, doxorubicin-induced cardiotoxicity occurs due to iron accumulation in the mitochondria. As a result, reduction in mitochondrial iron levels is fundamental in reversing doxorubicin-induced cardiomyopathy [17]. Another study specifically investigated the role of oxidative stress in doxorubicin-induced cardiotoxicity and revealed that decrease in plasma MDA levels provides evidence that ADMSCs mitigate doxorubicin-induced oxidative stress [18]. This is consistent with the overarching concept of oxidative stress playing a

significant role in the process of doxorubicin-induced cardiomyopathy. This study also evaluated the inflammatory and oxidative markers linked to cardiac toxicity and revealed that ADMSCs reverse inflammation and exert antioxidant effects.

Growth differentiation factor-15 (GDF-15) shows great potential as a biomarker for evaluating treatment efficacy and predicting prognosis for patients with cardiovascular diseases. It is used alongside traditional prognostic factors to enhance its predictive capabilities. An elevated GDF-15 is a strong and reliable indicator of myocardial injury. There may be a correlation between GDF-15 levels and the development of late-onset cardiotoxicity in Hodgkin lymphoma patients who undergo DOX-containing chemotherapy. Also, GDF15 is protective against bacterial, and viral infections, and sepsis [19]. In this study, GDF-15 increased following administration of DOX which is reversed by ADMSc. Doxorubicin affects various biomarkers, and levels of troponins and pro-BNP serve as early predictors of doxorubicin-induced cardiotoxicity. Although GDF-15 is more specific than NT-proBNP, it is an important marker of death or heart failure in patients after acute myocardial infarction [19]. This suggests that GDF-15 is statistically more specific to myocardial tissues. Doxorubicin triggers programmed cell death in heart muscle cells by activating caspase-3, potentially contributing to cardiomyopathy.

The results of this present study revealed that the level of caspase-3 increased following administration of DOX which was reversed after treatment with ADMSc. This is consistent with previous studies in which ADMSc significantly reversed the increase in caspase-3 following DOX administration [20]. Also, TGF-β signaling is essential for infarct healing and cardiac remodeling in the cardiovascular system. This is achieved by inhibiting inflammation, controlling the properties of fibroblasts, promoting the accumulation of extracellular matrix, and inducing interstitial fibrosis [21].

Research has demonstrated that blocking the TGF-β pathway may reduce the restructuring of the left ventricle and improve both contraction and relaxation of the heart. This helps in reducing the adverse effects of doxorubicin on endothelial cells [21]. The present study showed that treatment with ADMSCs led to a significant decrease in caspase-3, TNF-α, GDF-15, pro-BNP, and Troponin compared to control and DOX + saline groups. It has been established that GDF15, a protein belonging to the TGF-super-family, has cardioprotective properties. It may prevent cardiac hypertrophy and failure by promoting scar tissue formation and facilitating the differentiation of adult hematopoietic stem cells into cardiomyocytes [22]. Pınarlı *et al* [14] conducted a histopathological analysis of the rat myocardium treated with doxorubicin and revealed that ADMSCs effectively enhanced left ventricular functions and improved myocardial histology in doxorubicin-induced cardio-myopathy [14]. This is consistent with the findings of this present study that ADMSCs effectively reverse doxorubicin-induced cardiomyopathy.

Limitations of the study

The study has some limitations. Variability in findings may be attributed to the differences in the specific type of stem cells used, administration methods, and treatment duration. Also, reliance on animal experimentation may have different ethical implications for humans. Future studies would require a larger sample size, and other MSCs may need to be assessed to cover a broader range of factors.

CONCLUSİON

Adipose-derived mesenchymal stem cells (ADMSCs) significantly lower caspase-3 immunoexpression, and reverses inflammation and cardiac injury in doxorubicin-induced cardiomyopathy. Further investigation and clinical studies are required to determine the continued safety and efficacy of AD-MSC-based therapy in cardiac injury.

DECLARATIONS

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Ethical approval

It was granted by the Institutional Ethics Committee of Demiroğlu Science University (2323072802).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Oytun Erbaş (0000-0002-2515-2946), Ejder Saylav Bora (0000-0002-2448-2337), Demet Erciyes (0000-0003-1331-8221), Duygu Burcu Arda (0000-0002-4384-1323) and Mustafa Agah Tekindal (0000-0002-4060-7048), contributed equally to this study and they each made critical revisions related to the relevant intellectual content of the manuscript.

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