Human umbilical cord mesenchymal stem cells alleviate acute myocarditis by modulating endoplasmic reticulum stress and extracellular signal regulated 1/2-mediated apoptosis

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Abstract. Acute myocarditis is a non-ischemic inflammatory disease of the myocardium, and there is currently no standard treatment. Mesenchymal stem cells (MSCs) can alleviate myosin-induced myocarditis; however, the mechanism has not been clearly elucidated. In the present study, the authors investigated the ability of human umbilical cordMSCs (HuMSCs) to attenuate myocardial injury and dysfunction during the acute phase of experimental myocarditis. Male Lewis rats (aged 8 weeks) were injected with porcine myosin to induce myocarditis. Cultured HuMSCs ($1x10^6$ cells/rat) were intravenously injected 10 days following myosin injection. A total of 3 weeks following injection, this resulted in severe inflammation and significant deterioration of cardiac function. HuMSC transplantation attenuated infiltration of inflammatory cells and adverse cardiac remodeling, as well as reduced cardiomyocyte apoptosis. Furthermore, it was identified that HuMSC transplantation suppressed endoplasmic reticulum stress and extracellular signal-regulated kinase (ERK)1/2 signaling in experimental autoimmune myocarditis (EAM). The reduced number of TUNEL-positive apoptotic cells in myocardial sections from HuMSC-treated EAM rats compared with control demonstrates HuMSCs' anti-apoptotic function. Based on these data, the author suggested that treatment with HuMSCs inhibits myocardial apoptosis in EAM rats, ultimately protecting them from myocardial damage. The conclusion demonstrated that HuMSC transplantation attenuates myocardial injury and dysfunction in a rat model of acute myocarditis, potentially via regulation of ER stress, ERK1/2 signaling and induction of cardiomyocyte apoptosis.

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Introduction

Myocarditis is defined as an inflammatory infiltration of the myocardium with necrosis and/or degeneration of cardiomyocytes (1). Viruses are the primary cause of myocarditis; however, there are additional infectious causes of myocarditis, including *Borrelia burgdorferi* and *Trypanosoma cruzi* (2). There are a wide range of myocarditis symptoms, from mild dyspnea, arrhythmias and chest pain that resolves itself without specific therapy, to cardiogenic death (2). The major long-term consequence is dilated cardiomyopathy with chronic heart failure, for which appropriate treatment remains a significant clinical challenge.

A rat model of experimental autoimmune myocarditis (EAM) resembles human giant cell myocarditis and has been widely used in previous studies (3,4). Previous reports suggested that oxidative stress results in myocardial apoptosis, which serves an important role in the progression of EAM (5,6). Oxidative stress may activate mitogen-activated protein kinase (MAPK) signaling pathways and endoplasmic reticulum (ER) stress, both of which lead to myocardial apoptosis and myocardial damage (7). A previous study demonstrated that intravenous injection of bone marrow mesenchymal stem cells (BMSCs) may alleviate myosin-induced myocarditis (8). However, the invasiveness of obtaining bone marrow and the low numbers of MSCs yielded following processing limits its clinical potential. In previous years, human umbilical cord-derived mesenchymal stem cells (HuMSCs), which are generally discarded as medical waste following delivery, have become an alternate source of MSCs (9). Cluster of

differentiation (CD)29, CD44, CD59, CD90 and CD105 are expressed in HuMSCs. Markers of hematopoietic cells, including CD14, CD33, CD34, CD28, CD45 and CD117, and important graft-vs.-host disease (GVHD) markers, including CD80, CD86 and CD40, are not detectable or only weakly expressed in HuMSCs (10). Therefore, it has been hypothesized that HuMSCs may be broadly used in regenerative medicine without graft rejection reactions (11). However, the capacity of HuMSCs during EAM remains undetermined.

The current study investigated whether intravenous HuMSCs may improve cardiac function and alleviate myocardial inflammation in rats with myosin-induced myocarditis, as well as evaluating the potential underlying mechanisms.

Materials and methods

Animals. Male Lewis rats (n=24; aged 8 weeks; weight 180-200 g) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China) and were maintained in our animal facilities with air-conditioning at Shantou University Medical College, under constant temperature and humidity conditions with a 12:12-h light-dark cycle. All rats had free access to food and water. Throughout the studies, all animals were treated in accordance with the institutional guidelines for animal experiments. Ethical approval was obtained from the Institutional Review Board of Shantou University Medical College (Shantou, China).

Preparation of HuMSCs. Ethical approval was obtained from the Institutional Review Board of Shantou University Medical College (Shantou, China). HuMSCs were prepared as previously described (10). A total of 5 patients were involved in the study, and written informed consent was obtained from all patients. Human umbilical cords from consenting patients who underwent full-term Caesarian sections were collected immediately into a sterilized 50 ml tubes, washed with phosphate-buffered saline (PBS), and cut into 2- to 3-cm thick sections. Following dissection of the arteries and veins, the remaining tissue, the Wharton's jelly, was sectioned into smaller fragments and transferred to 75 cm^2 flasks containing Dulbecco's Modified Eagle's medium/F12 media (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml penicillin/streptomycin (Beyotime Institute of Biotechnology, Haimen, China), 1 g/ml amphotericin B (Gilead Sciences, Inc., Foster City, CA, USA), 5 ng/ml epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific, Inc.), and 5 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich; Merck KGaA). Cultures remained undisturbed for 5-7 days at 37˚C in 5% CO_2 to allow migration of cells from the explants. Subsequently, the media was replaced.

EAM induction. Purified porcine cardiac myosin (Sigma-Aldrich; Merck KGaA) was dissolved in 0.01 M PBS and emulsified with an equal volume of complete Freund's adjuvant supplemented with10 mg/ml *Mycobacterium tuberculosis* (Sigma-Aldrich; Merck KGaA). On day 0, rats received a single immunization at two subcutaneous sites (both footpads) with a total of 0.2 ml emulsion per rat. Rats not subjected to immunization were included as a control-group (n=8).

A total of 16immunized rats were randomly divided into two groups. Rats were randomly assigned into the following two groups: Control group, 0.2 ml PBS only (n=8);HuMSCs group, 0.2 ml HuMSCs ($1x10^6$ cells/animal; n=8). A total of 10 days following myosin injection, HuMSCs or vehicle (PBS) were administered intravenously via the tail vein.

Echocardiographic studies. Echocardiography was performed 21 days following the myosin injection. Rats were anesthetized with 1.5-2.0% volume of isoflurane in air (Sigma-Aldrich; Merck KGaA). A 13-MHz probe was placed at the left fourth intercostal space for imaging using two-dimensional echocardiography (AcusonAntares, Siemens AG, Munich, Germany). Left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), interventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW) and fractional shortening (FS%) were measured and recorded as the mean for three beats. Fractional shortening (%) was calculated as [(LVDd-LVDs)/LVDd]x100%. Investigators blinded to the treatment group performed the echocardiography studies and all analyses were performed offline.

Histopathological studies. Following echocardiographic analyses, rats were sacrificed by cervical dislocation. Hearts were excised above the origin of the great vessels 21 days after the myosin injection. Hearts were fixed in 4% paraformaldehyde for 6 h at 4˚C, embedded in paraffin, sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E). A cardiovascular pathologist with no knowledge of the experimental groups evaluated H&E-stained sections. Myocardial injury and inflammation were characterized by assigning histoscores to every fifth cross section, according to a previously published 6-tier scoring system (grade 0, no inflammation; grade 1, cardiac infiltration in <5% of the cardiac sections; grade 2, 6-10% infiltration; grade 3,11-30%infiltration; grade 4, 31-50% infiltration; and grade 5, infiltration in >50% of cardiac sections) (12,13).

Western blotting. Heart tissues were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Following centrifugation (12,000 x *g* for 10 min at 4˚C), supernatants were collected and the total protein concentration in samples was measured by use of a BCA Protein Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. For western blotting assays, 30 μg of total protein was separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Filters were blocked with 5% non-fat dry milk in TBST (20 mM Tris, pH 6.8, 137 mM NaCl, 0.1% Tween-20) overnight at 4˚C, washed, and incubated overnight at 4˚C with a 1:1,000 dilution of primary antibodies in blocking solution. Washing was conducted four times with TBST, for 10 min each, with constant shaking. The following primary antibodies were used: GAPDH (catalog no. D4C6R), extracellular signal-regulated kinase (ERK)-1/2 (catalog no. 9258), phosphorylated (p)-ERK-1/2 (catalog no. 4668), p38 MAPK

(catalog no. 8690) and p-p38 MAPK (catalog no. 4511), which were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Glucose-regulated protein 78 (GRP78) (catalog no. SC-13968) and caspase 12 (catalog no. SC-5627) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Membranes were subsequently washed and incubated for 1 h at room temperature with a 1:2,000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (catalog no. 4050-05; Southern Biotechnology Associates, Inc. USA). Protein bands were visualized using the ECL Plus chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden).

Detection of apoptosis. Paraffin-embedded heart tissues were cut into 4-μm thick sections at room temperature. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed using an *in situ* apoptosis detection kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology). Sections were mounted and examined using light microscopy. For each animal, five sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were considered.

Statistical analysis. Data are expressed as the mean ± standard deviation. Analyses of differences between groups were performed using one-way analyses of variance, followed by Tukey's multiple comparison tests using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

HuMSC treatment improves cardiac structure and function, and cardiomyocyte apoptosis. A significant suppression of cardiac function in EAM rats 21 days following cardiac myosin injection was observed. There was significant impairment in the systolic and diastolic components of cardiac contraction as presented in echocardiographic analyses compared with normal rats (P<0.01; Fig. 1A). HuMSC treatment significantly reversed cardiac remodeling with reduced LVDs and LVDd, and increased FS compared with vehicle-treated EAM rats (Table I). There was marked inflammatory cellular infiltration in the control group as identified by H&E staining, whereas HuMSCs-treated rats exhibited much less inflammatory cellular infiltration (Fig. 1A and B). Cardiomyocyte apoptosis was confirmed by TUNEL staining of myocardial tissue slices. Control animals demonstrated numerous TUNEL-positive apoptotic nuclei whereas HuMSCs-treated animals exhibited fewer apoptotic cells (Fig. 1A and C). Additionally, control rats demonstrated LV remodeling with increased LVDd and LVDs, and reduced FS in vehicle-treated EAM rats, when compared with untreated rats (normal group), indicating impaired myocardial function (Fig. 1D).

HuMSCs treatment modulates the ERK1/2 pathway. As assessed by western blot analysis (Fig. 2A), p-ERK1/2 expression was significantly increased in control rats compared with normal rats, suggesting that oxidative stress mediates stimulation of ERK1/2 signaling. HuMSC treatment significantly ameliorated protein expression levels of ERK 1/2 ($^{\#\#}$P<0.05 vs. Normal, *P<0.05 vs. Control) and p-ERK1/2 in EAM rats ($^{\#\#}$P<0.05 vs. Normal, *P<0.05 vs. Control) (Fig. 2B). p38-MAPK and p-p38MAPK levels did not differ between the three groups (data not shown).

Table I. Alterations to echocardiographic parameters 3 weeks following treatment with HuMSCs in EAM rats.

Parameter	Normal	Control	HuMSCs
LVDd (mm)	4.75±0.25	6.80±0.46[a]	5.04±0.55[b]
LVDs (mm)	2.72±0.16	4.60±0.90[a]	3.08±0.40[b]
IVS (mm)	1.88±0.83	1.94±0.18	1.74±0.21
LVPW (mm)	2.0±0.12	1.64±0.13	1.78±0.20
FS (%)	0.44±0.04	0.26±0.07[a]	0.39±0.05[b]

LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; IVS, interventricular septal thickness; LVPW, left ventricular posterior wall thickness; FS, fractional shortening; Normal, age-matched untreated rats; Control, EAM rats treated with vehicle; HuMSCs, EAM rats treated with HuMSCs. Data are expressed as the mean ± standard deviation. [a]P<0.01 vs. Normal; [b]P<0.01 vs. Control.

HuMSCs protect against ER stress. GRP78 is a marker of ER stress. ER stress leads to activation of caspase 12, resulting in cardiomyocyte apoptosis. Significant increases were observed in myocardial GRP78 and caspase 12 expression in EAM animals, indicating the involvement of ER stress. HuMSC-treated animals exhibited significant attenuation of these ER stress markers compared with the control group (Fig. 2B).

Discussion

In the present study, the therapeutic potential of HuMSCs in the acute phase of myocarditis was investigated. In a rat model of acute myocarditis, intravenous administration of HuMSCs 10 days following myosin injection significantly improved cardiac function. Echocardiographic analyses demonstrated LV remodeling with increased LVDs, LVDd and reduced FS in vehicle-treated EAM rats compared with normal rats, indicating impaired systolic and diastolic function of the myocardium. HuMSC treatment positively affected LV remodeling by significantly reversing the increased LVDs and LVDd, and reducing FS. Pathological findings in the heart 21 days following myosin injection indicated that EAM rats in the control group suffered extensive inflammatory cellular infiltration, whereas rats treated with HuMSCs exhibited significantly less infiltration. Based on these results, HuMSCs demonstrated protective effects in myocardial inflammation.

Myosin-induced EAM contributes to a model that is similar to giant cell myocarditis in humans with three phrases: Antigen-priming (from days 0 to 14), autoimmune response (from days 14 to 21), and a chronic phase featuring cardiac remodeling and fibrosis (3). Although EAM pathogenesis has remains to be fully elucidated, MAPK signaling pathways

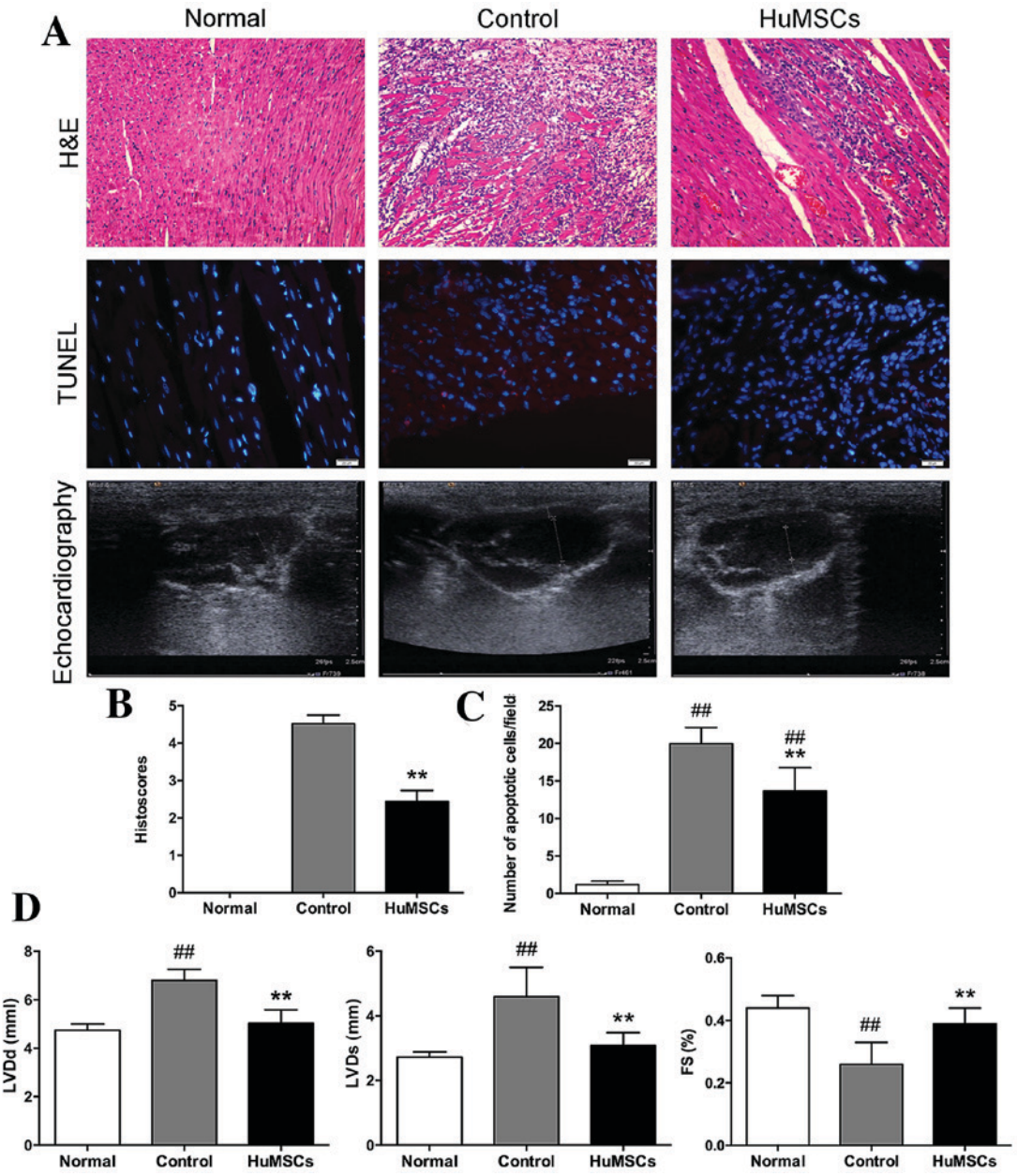

Figure 1. Experiments comparing cellular infiltration, levels of apoptosis and systolic and diastolic components in normal cells, control cells and HuMSCs. (A) H&E staining of left ventricular tissue slices (magnification, x100). TUNEL staining of left ventricular tissue slices depicting apoptotic nuclei (magnification, x400). Echocardiography in four chambers. (B) Histoscores of H&E staining reflecting levels of cardiac infiltration. (C) Bar graphs presenting the average number of TUNEL-positive cells per field. (D) Quantification of LVDs, LVDd and FS. Data are expressed as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. Normal, **P<0.01 vs. Control. HuMSCs, human umbilical-derived mesenchymal stem cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; FS, fractional shortening.

and ER stress-induced myocardial apoptosis are likely to be involved.

MAPKs are a group of serine/threonine protein kinases. There are three MAPK subfamilies: ERK, c-Jun N-terminal kinase and p38-MAPK. Overexpression of mitogen activated protein kinase kinase (MEK) 1, the upstream activator of ERK 1/2, results in cardiomyocyte hypertrophy *in vitro* (14). The MEK/ERK cascade serves a role in fibrotic diseases (15,16) and affects lymphocyte activation and differentiation (17-19). In addition, the MEK/ERK signaling pathway enhances the production of diverse proinflammatory cytokines, including interleukin (IL)-β, tumor necrosis factor-α and IL-6 (20-22). The MEK/ERK signaling pathway is targeted for the treatment of rheumatoid arthritis (23), and is involved in IL-17-mediated cardiac fibrosis in EAM (24). Activation of ERK1/2 serves a role in cardiomyocyte apoptosis caused by doxorubicin (25). Proteomic and biochemical analyses further revealed that ERK-1/2 signaling and ER stress mediates cardiomyocyte apoptosis in EAM (26).

In the present study, p-ERK1/2 expression was significantly increased in EAM rats treated with the vehicle. However, HuMSC supplementation protected against cardiac damage as evidenced by decreased levels of p-ERK1/2 proteins. However, p38-MAPK levels did not differ among the three groups. These results confirmed the involvement of ERK1/2 signaling in EAM, as well as indicating that HuMSC treatment may

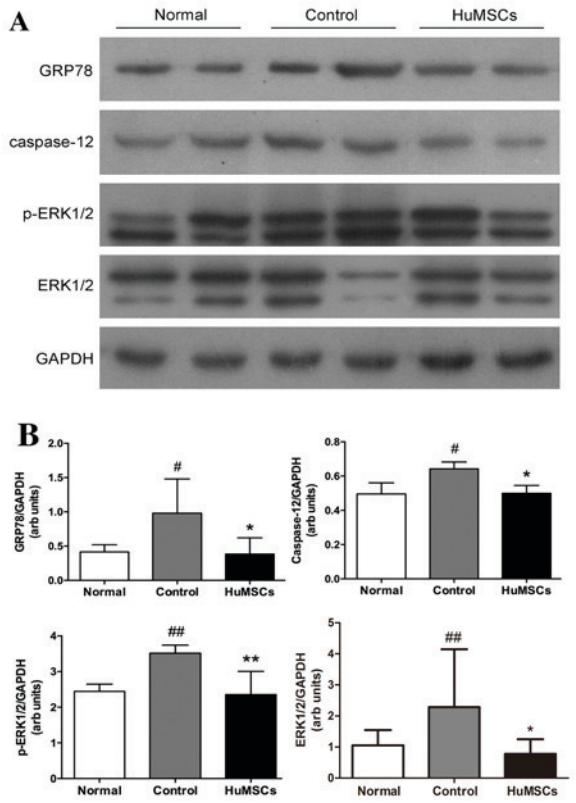

Figure 2. Protein expression levels of GRP78, caspase-12, ERK1/2 and p-ERK1/2. (A) Representative western blot images and (B) quantification of GRP78, caspase-12 and p-ERK1/2 protein expression levels. GAPDH served as an internal control. Data are expressed as the mean ± standard deviation. $^{\#}$P<0.05 vs. Normal, $^{\#\#}$P<0.01 vs. Normal, *P<0.05 vs. Control, **P<0.01 vs. Control. Normal, age-matched untreated rats; Control, immunized rats treated with vehicle; HuMSCs, immunized rats treated with HuMSCs; GRP78, glucose-regulated protein 78; ERK, extracellular-signal regulated kinase; HuMSCs, human umbilical cord-derived mesenchymal stem cells; p, phosphorylated.

protect animals from cardiac damage by avoiding activation of the ERK1/2 signaling pathway.

The ER is generally defined as an organelle that participates in folding of membrane and secretory proteins (27). ER stress, caused by stimuli such as ischemia, hypoxia, heat shock, genetic mutation and oxidative stress, can lead to ER dysfunction (27). ER stress is becoming a focus of research because it causes numerous inflammatory disorders (27). In response to ER stress, there is a marked upregulation of ER chaperones including GRP78. Cell apoptosis may occur via caspase 12, which is localized in the ER and activated by ER stress (7,28). Studies have revealed that quercetin and edaravone improve autoimmune myocarditis and decrease cardiomyoctye apoptosis by relieving ER stress (29,30). In the present study, HuMSC treatment was demonstrated to protect EAM rats from ER stress, as demonstrated by downregulated expression of GRP78 and caspase 12 (Fig. 2B and C).

Identification of TUNEL-positive apoptotic cells in the myocardium is useful for examining the intensity of damage to myocardial cells (31). In addition, the anti-apoptotic role of HuMSCs was confirmed by reduced numbers of TUNEL-positive apoptotic cells in the myocardial sections of EAM rats treated with HuMSCs. Based on these data, the authors suggested that treatment with HuMSCs is effective for the prevention of myocardial apoptosis in EAM rats and may protect them from myocardial damage.

In conclusion, the present study demonstrated that the protective effects of intravenously administered HuMSCs in EAM result from regulation of ER stress and ERK1/2 signaling-mediated apoptosis. AsHuMSCs are available in large numbers using non-invasive procedures, these findings provide a novel perspective for the treatment of acute myocarditis.

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