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Original Article

Conditioned Medium from Human Amniotic Membrane-Derived Mesenchymal Stem Cells Modulates Inflammatory and Myofibrotic Factors in Vivo

Gazaleh Asgharnezhad, Ms[1], Sachli Mohamadi, Ms[1], Mahdieh Mehrab Mohseni, PhD[1], Neda Mousvi-Niri, PhD[2], Maryam Naseroleslami, PhD[1*]

[1]*Department of Cellular and Molecular Biology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.*
[2]*Department of Biotechnology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.*

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Abstract

Background: *Heart failure (HF) is a prevalent diagnosis with a significant mortality rate. Various therapeutic approaches exist for treating HF, and human adipose-derived mesenchymal stem cells-conditioned medium (hAMSCs-CM) therapy has emerged as a promising option. Despite its potential efficacy, the precise mechanism of action underlying hAMSCs-CM treatment remains unclear. To address this knowledge gap, we conducted a novel animal study to investigate the mechanism of action of hAMSCs-CM in an HF model, with a specific focus on transforming growth factor-β (TGF-β)/galectin-3, monocyte chemoattractant protein-1 (MCP1), B-type natriuretic peptide (BNP), and aldosterone (ALD).*

Methods: *Forty adult male Wistar rats were divided into 4 groups: control, HF, culture medium, and CM. All rats, except those in the control group, received an injection of isoproterenol to induce an animal model of HF. The CM group was administered the CM, while those in the culture medium group received standard culture media. Subsequently, serum levels of fibrotic factors, including TGF-β/galectin-3, MCP1, BNP, and ALD, were measured using ELISA. Statistical analysis was performed using one-way analysis of variance and the Tukey test.*

Results: *Serum levels of TGF-β/galectin-3, MCP1, BNP, and ALD were significantly elevated in the HF, CM, and culture medium groups compared with the control group ($P<0.001$). Additionally, these fibrotic factors were significantly reduced in the CM group compared with the HF group ($P<0.001$). Notably, CM therapy could not restore TGF-β/galectin-3, MCP1, BNP, or ALD levels to the normal range observed in the control group.*

Conclusion: *Our findings indicate that hAMSCs-CM modulates the expression of inflammatory and fibrotic cytokines, such as TGF-β/galectin-3, MCP1, BNP, and ALD, in isoproterenol-induced HF in male rats. These results contribute to a better understanding of the therapeutic mechanisms underlying hAMSCs-CM treatment for HF.*

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Keywords: *Mesenchymal stem cells; Heart failure; Inflammation; Fibrosis*

[*]**Corresponding Author:** ***Maryam Naseroleslami,*** *Assistant Professor of Cell & Molecular Biology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran. Zip code: 1949635881. Cell: +98 09122394618. Tel: +982126602642. Fax: +982126602642. E-mail: naseroleslamimaryam@gmail.com.*

Introduction

Heart failure (HF) is a significant global health concern, with a high prevalence and mortality rate. Annually, numerous individuals are diagnosed with HF, often as the final stage of a chronic heart condition, across various countries.[1] An estimated 26 million people worldwide are affected by HF.[2] In the United States alone, approximately 6.2 million adults are living with this condition, as reported by the Centers for Disease Control and Prevention (CDC).[3]

Transforming growth factor-β (TGF-β) has been identified as a key player in cardiac remodeling, particularly in the formation of fibrotic tissue. TGF-β contributes to this process by recruiting fibroblasts and initiating their transition to myofibroblasts.[4] Additionally, TGF-β promotes excessive collagen deposition through increased synthesis and release of the extracellular matrix.[5, 6]

Extensive research has demonstrated a strong association between TGF-β signaling and galectin-3, a well-known inflammatory marker of cardiac fibrosis. As a member of the galectin family, galectin-3 has a specific role in the formation of scar tissue and fibrosis.[7] Elevated galectin-3 levels have been observed in various fibrotic conditions, including chronic pancreatitis, renal fibrosis, and cardiac fibrosis.[8, 9] Furthermore, galectin-3 is known to play an active role in myofibroblast proliferation.[10] Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family. It is produced by endothelial and smooth muscle cells, as well as mononuclear immune cells, such as monocytes and macrophages, during the formation of atherosclerotic plaques. The production of MCP-1 is stimulated by various factors, including cytokines, angiotensin II, homocysteine, and other components associated with atherosclerosis. MCP-1 functions as a chemotactic factor by interacting with the C-C chemokine receptor type 2 (CCR-2) on monocytes, which attracts monocytes to the vascular wall. In patients suffering from stable coronary heart disease, as well as peripheral artery disease, MCP-1 plasma is overexpressed.[11]

B-type natriuretic peptide (BNP) is a peptide hormone primarily stored in granules within the ventricles and released in significant quantities upon stimulation. In patients with HF, serum BNP levels can be up to 100 times higher than that in healthy individuals, with a half-life of around 20 minutes. The synthesis of BNP involves the processing of pro-BNP into biologically active BNP and the inactive counterpart, N-terminal pro-BNP (NT-proBNP).[12]

Aldosterone (ALD) is a mineralocorticoid hormone responsible for regulating blood pressure and electrolyte balance via the intracellular mineralocorticoid receptor. Further, ALD plays a role in cardiovascular remodeling and disease progression by influencing cardiac hypertrophy, arterial stiffness, fibrosis, inflammation, and oxidative stress.[13]

Given that heart transplantation remains the only definitive treatment for HF, researchers continue to explore alternative therapeutic options. Regenerative medicine, with its focus on tissue regeneration and reducing fibrosis, offers potential alternatives. Stem cell therapy is one such approach that has garnered significant attention due to its potential benefits.[14, 15]

Among the various types of stem cells, human amniotic membrane-derived mesenchymal stem cells (hAMSCs) have garnered significant trust.[16, 17] Additionally, hAMSCs exhibit favorable characteristics, such as an appropriate proliferation rate, differentiation capacity, and immunogenic properties due to their low expression of major histocompatibility complex class I (MHC-I) molecules.[18] Moreover, hAMSCs can secrete paracrine-conditioned medium (CM), which contains a rich mix of therapeutic agents, including cytokines and growth factors.[19, 20] Studies have shown that CM does not have the potential risks associated with MSCs, such as tumorigenicity.[17]

While hAMSCs-CM has been employed in the treatment of various diseases, the precise mechanisms underlying its therapeutic effects remain unclear. To address this knowledge gap, we conducted a novel study using an animal model of HF to investigate the mode of action of hAMSCs-CM. Our primary focus was on key fibrotic and inflammatory markers, including TGF-β/galectin-3, MCP1, BNP, and ALD.

Our previous research focused on elucidating the mode of action of hAMSCs-CM in treating HF at the tissue level. Recognizing the need for a more comprehensive understanding of the effects of hAMSCs-CM on serum factors, we designed the present study to explore this aspect. The findings of our investigation are summarized in Figure 1, which provides a graphical abstract to facilitate comprehension of the key outcomes.

Methods

Amniotic membranes were obtained from postpartum volunteers at Shahid Akbar Abadi Hospital after obtaining informed consent, following the ethical guidelines approved by the Ethics Committee of the Iran University of Medical Sciences (IR.IAU.PS.REC.1400.018). To ensure the amniotic origin of the isolated cells, we performed fluorescence-activated cell sorting, following the protocol described in our previous work.21 The isolated cells were cultured in α-MEM supplemented with 10% fetal bovine serum (Gibco, Australia), 100 U/mL penicillin, 2 M L-glutamine, and 100 µg/mL streptomycin for 48 hours. The cells were then washed with phosphate-buffered saline and replaced with serum-free α-MEM. CM was harvested after incubating the MSCs under hypoxic conditions for 48 hours. The CM was centrifuged at 1200 rpm, filtered through a 0.22 µm filter, and stored at −80 °C for further use.

Forty adult male Wistar rats were provided by the Iran

University of Medical Sciences and randomly assigned to 4 groups: 1) control: rats without any intervention; 2) HF: rats treated with 170 mg/kg isoproterenol (Sigma, Aldrich, USA) via subcutaneous injection for 4 consecutive days; 3) culture media: rats anesthetized with 80 mg/kg ketamine and 5 mg/kg xylazine received injections of 150 µL cell-free Dulbecco's modified eagle medium into 4 points of the myocardium using a 31-gauge needle, 28 days after the last isoproterenol injection; and 4) CM: rats were administered 150 µL of CM under the same conditions as the culture media group.

Following the treatments, blood samples were collected from all rats and centrifuged at 600g for 10 minutes at 4 °C. The serum was used to evaluate TGF-β/galectin-3, MCP1, BNP, and ALD levels using ELISA kits according to the manufacturer's instructions (RayBiotech, Inc). The ELISA reactions were measured at 450 nm using an ELISA Reader (Synergy MX BioTek). All concentration results were reported in pg/mL.

Statistical analysis was performed using one-way analysis of variance and the Tukey test for post-hoc comparisons on Prism v5.0 (GraphPad Software, La Jolla, USA). All concentrations were expressed as mean±SEM, and a P value of less than 0.001 was considered statistically significant.

Results

Effects of hAMSCs-CM on serum levels of fibrogenic markers

The results of the ELISA assay showed that 4 weeks after isoproterenol administration, serum levels of TGF-β significantly increased in HF (P<0.001), culture media (P<0.001), and CM (P<0.05) groups compared with the control group (P<0.001). In contrast, after hAMSCs-CM administration, TGF-β significantly decreased in the conditioned medium group compared with the HF group, although it was significantly higher than that of the control group (P<0.001) (Figure 2, A).

Isoproterenol-induced HF caused a significant rise in serum levels of galectin-3 in HF, isoproterenol, and culture media groups (P<0.001). However, treatment with hAMSCs-CM significantly decreased the level of galectin-3 compared with the HF group (P<0.001) and the culture media group (Figure 2, B).

Serum MCP1 was significantly increased in the HF, CM, and culture media groups compared with the control group (P<0.001); additionally, it significantly decreased in the CM group compared with the HF group (P<0.001) (Figure 2, C).

Serum BNP was significantly elevated in the HF, CM, and culture media groups compared with the control group (P<0.001); in addition, it significantly dropped in the CM rodents compared with the HF group (P<0.001) (Figure 2, D).

Serum ALD also exhibited significant overexpression in the HF, CM, and culture media groups compared with the control group (P<0.001); moreover, it was significantly underexpressed in the CM group compared with the HF group (P<0.001) (Figure 2, E). Nonetheless, CM therapy failed to return TGF-β/galectin-3, MCP1, BNP, and ALD to

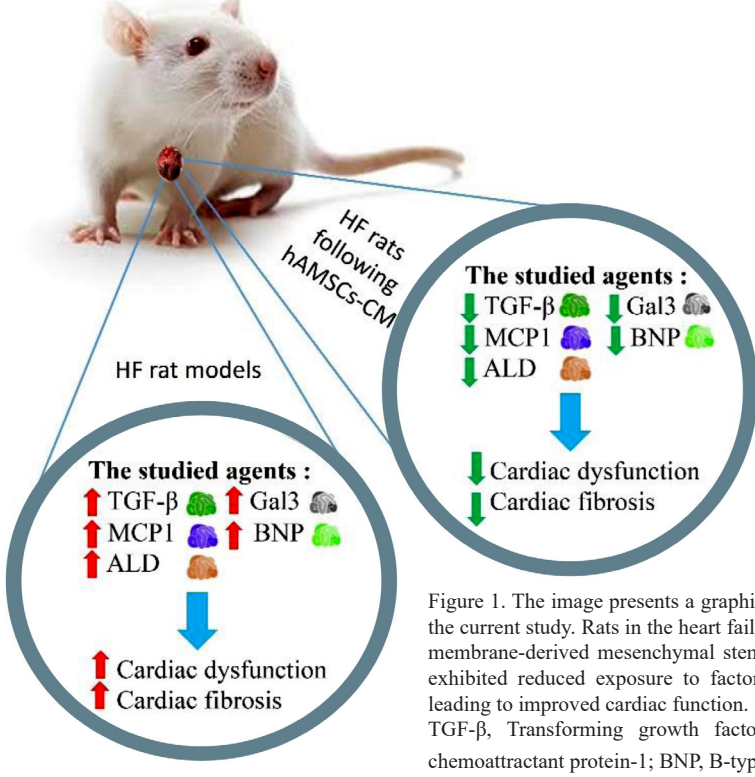

Figure 1. The image presents a graphical abstract summarizing the key findings of the current study. Rats in the heart failure (HF) model treated with human amniotic membrane-derived mesenchymal stem cells-conditioned medium (hAMSCs-CM) exhibited reduced exposure to factors related to cardiac ferroptosis, ultimately leading to improved cardiac function.
TGF-β, Transforming growth factor-β; Gal3, Galectin-3; MCP1, Monocyte chemoattractant protein-1; BNP, B-type natriuretic peptide; ALD, Aldosterone

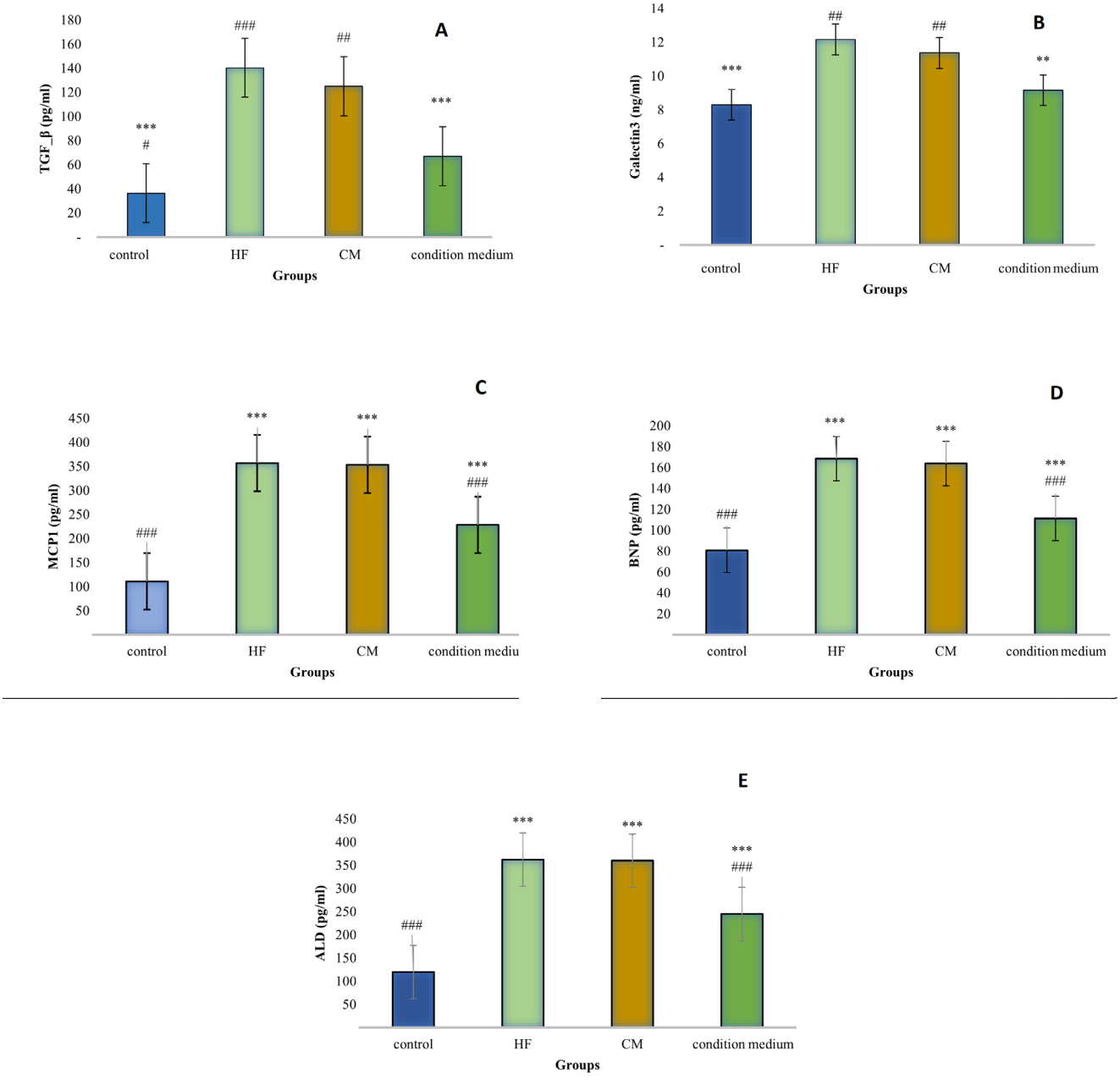

Figure 2. The images illustrate the results of the ELISA assay of the serum levels of A) TGF-β, B) galectin-3, C) MCP1, D) BNP, and E) ALD 4 weeks after intramyocardial administration of hAMSCs-CM (n=10).
Data are presented as mean±SEM.
HF, Heart failure; CM, Conditioned medium; hAMSCs-CM, Human amniotic membrane-derived mesenchymal stem cells-conditioned medium; TGF-β, Transforming growth factor-β; Gal3, Galectin-3; MCP1, Monocyte chemoattractant protein-1; BNP, B-type natriuretic peptide; ALD, Aldosterone
$^{###}P<0.001$, $^{##}P<0.01$, and $^{#}P<0.05$ vs the control group
$^{***}P<0.001$ and $^{**}P<0.01$ vs the HF group

Table 1. Analytical analysis of MCP1, ALD, TGF-β, galectin-3, and BNP by group

Group	MCP1	ALD	TGF-β	Galectin-3	BNP
Control	111±2.18	119.78±2.51	36.22±6.29	8.27±0.61	80.78±3.08
HF	356.56±1.84	362.11±3.47	146.22±19.16	11.86±0.44	168.44±5.74
CM	353.55±4.91	359.67±2.91	126±7.82	11.50±0.75	163.78±6.47
Conditioned medium	228.35±6.75	245±5.86	68.11±6.38	9.17±0.38	111.11±0.84

TGF-β, Transforming growth factor-β; Gal3, Galectin-3; MCP1, Monocyte chemoattractant protein-1; BNP, B-type natriuretic peptide; ALD, Aldosterone

the normal range. (Since the plots of MCP1 and ALD are similar, the mean±SD of MCP1 and ALD in all categories is presented in Table 1).

Discussion

Cardiac remodeling is the primary cause of morbidity in HF, which ultimately leads to cardiac fibrosis.[22] Therefore, preventing cell damage and fibrosis is a key area of research in the field of HF.[23-25]

MSCs have recently emerged as a potential therapeutic option for treating cardiac diseases. Among MSCs, hAMSCs have gained particular attention due to their high potential to differentiate into cardiomyocytes, cost-effectiveness, and ease of procurement.[14, 16, 26] Nevertheless, the use of hAMSCs has been associated with the potential risk of tumor formation. To overcome this limitation, several researchers have utilized hAMSCs-CM, which contains paracrine secretions such as growth factors, cytokines, and proteins.[15, 20] Additionally, hAMSCs exhibit minimal expression of MHC-I, which reduces the likelihood of unintended immune reactions during transplantation.[27, 28]

Building on these findings, we hypothesized that hAMSCs-CM could be effective in improving HF in rats.

Isoproterenol-induced HF animal models are widely employed in experimental studies due to their ability to closely mimic human HF conditions.[22] Isoproterenol, a β-adrenergic agonist, exerts acute positive chronotropic and inotropic effects, making it a suitable choice for inducing HF in experimental settings.[25, 29, 30] In this study, we used an isoproterenol-induced HF rat model to investigate the effects of hAMSCs-CM on the serum levels of TGF-β/galectin-3, MCP1, BNP, and ALD. These biomarkers were chosen to evaluate the potential therapeutic effects of hAMSCs-CM on HF. Our results revealed that intramyocardial injection of hAMSCs-CM elevated fibrogenic cytokines, including galectin-3 and TGF-β.

In a related study, we investigated the protective effects of hAMSCs labeled with superparamagnetic iron oxide nanoparticles (SPIONs) against isoproterenol-induced myocardial injury, both in the presence and absence of a magnetic field. We subsequently assessed myocardial fibrosis, heart function, characterization of hAMSCs, and histopathological changes using Masson's trichrome, echocardiography, flow cytometry, and hematoxylin and eosin staining, respectively. Using ELISA, we also measured the levels of pro-inflammatory cytokines. Our findings consistently demonstrated that the application of SPION-labeled MSCs in the presence of a magnetic field holds the promising potential to effectively mitigate the detrimental factors associated with isoproterenol-induced myocardial injury.[28]

In a separate study, we explored the cardioprotective effects of hAMSCs-CM using a rat model of isoproterenol-induced myocardial damage. We aimed to elucidate the impact of hAMSCs-CM on cardiac tissue. Isoproterenol was administered subcutaneously at 170 mg/kg/day for 4 consecutive days to establish the model. Echocardiography, immunohistochemistry assays, and Masson's trichrome staining were employed to assess the function of hAMSCs-CM. Intramyocardial post-treatment with 150 µL of hAMSCs-CM led to significant improvements in the evaluated parameters.[21]

Although galectin-3 has been identified as a clinical biomarker for acute and chronic HF due to its upregulation in patients,[31] the precise mechanisms through which galectin-3 contributes to cardiac remodeling and fibrosis remain incompletely understood. Some studies propose that its expression is connected to the TGF-β/Smad signaling pathway and inflammatory conditions. Substantial evidence indicates that elevated serum levels of galectin-3 are associated with overactivation of fibroblasts and macrophages, which play crucial roles in adverse cardiac remodeling.[7, 32]

Studies have reported the overexpression of galectin-3 in the left ventricular tissue, suggesting its involvement in left ventricular remodeling.[33] In another study, it was demonstrated that galectin-3 activated liver myofibroblasts via TGF-β, directly linking it to liver and kidney fibrosis. Moreover, that study reported that TGF-β activation of galectin-3 was implicated in the inflammatory response during cardiac remodeling.[26] Intriguingly, galectin-3 is also associated with the turnover of several extracellular matrix proteins, including procollagen types I and III, as well as MMP-2. Considering these findings, our results demonstrating reduced serum levels of galectin-3 in rats treated with hAMSCs-conditioned medium hold great significance.[7]

Our findings chime with a recent study by Tang et al,[8] demonstrating that bone marrow-derived MSCs could attenuate renal interstitial fibrosis and reduce the expression of TGF-β1 and galectin-3. Their study further unveiled a direct correlation between galectin-3 downregulation and diminished renal fibrotic tissue formation, likely mediated through the galectin-3/Akt/GSK3β/Snail signaling pathway.

In a related study, treatment with hMSCs-CM effectively inhibited heart tissue apoptosis by reducing oxidative stress and downregulating TGF-β signaling. It is known that TGF-β overexpression in injured cardiomyocytes modulates fibroblast function.5 Moreover, TGF-β regulates connective tissue growth factor, a potent fibrogenic factor, further contributing to fibrosis.[23, 34] The infarcted myocardium heavily depends on TGF-β for the regulation of inflammation; thus, complete inhibition of TGF-β could have detrimental effects in this context.6 Consequently, the observation that hAMSCs-CM administration in our study did not reduce TGF-β levels to those seen in healthy animals may be advantageous in maintaining the delicate balance between the beneficial and detrimental effects of TGF-β in the infarcted heart.

In our study, serum levels of MCP-1 and BNP were elevated in rats with HF, and CM treatment effectively reduced these levels. Consistent with our findings, Luis et al[35] investigated plasma MCP-1, NT-proBNP, and Gal-3 as predictive factors for recurrent cardiovascular events, such as acute ischemic heart disease, HF, and death in patients with stable coronary artery disease and persistent or low inflammation. They reported higher plasma levels of MCP-1 and NT-proBNP in patients with persistent inflammation, whereas Gal-3 levels remained unchanged. Plasma MCP-1 and NT-proBNP were associated with worse outcomes in patients with persistent inflammation. Moreover, NT-proBNP was linked to a higher incidence of HF or death in patients with both persistent and low inflammation.

In our current study, serum ALD levels were increased in rats with HF, and CM treatment effectively reversed this elevation. According to a study by Messaoudi et al,[37] ALD is the primary ligand of the mineralocorticoid receptor. Accumulating experimental and clinical evidence has demonstrated the detrimental effects of mineralocorticoid receptor activation in cardiovascular diseases. The blockade of the mineralocorticoid receptor in patients with HF further emphasizes the importance of this receptor in cardiac and vascular tissues. Experimental models have also shed light on the specific effects of ALD on the heart tissue.[36]

In a related study, Cha et al[37] demonstrated that ALD induced the expression of galectin-3. A clinical trial further revealed that serum galectin-3 levels were significantly elevated in patients with ALD-producing adenoma, and both myocardial fibrosis and serum galectin-3 levels were reversed to normal levels following adrenalectomy.[38] Additionally, in mice and rats with ALD-induced cardiac fibrosis, galectin-3 knockout attenuated fibrotic changes and cardiac dysfunction.[39] These findings suggest a strong link between ALD and galectin-3 in the context of fibrosis and HF. Moreover, ALD upregulation has been implicated in the transition from inflammation to fibrosis, highlighting its crucial role in the pathogenesis of fibrotic diseases.[40]

Conclusion

We hope that our study aids in filling the knowledge gap regarding HF treatment by demonstrating the potential therapeutic benefits of hAMSCs-CM in mitigating cardiac fibrosis in a rat model of isoproterenol-induced HF. The administration of hAMSCs-CM led to a modulation of serum levels of TGF-β/galectin-3, MCP1, BNP, and ALD.

The findings of this study underscore the potential of hAMSCs-CM as an effective therapeutic option for managing HF, warranting further investigation into its underlying mechanisms and clinical applications.

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