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Human Amniotic Membrane Mesenchymal Stem Cells-derived Conditioned Medium Alleviates Myocardial Fibrosis

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Abstract:
Background: Lots of people die from heart failure (HF) because of fibrosis formation. As injured myocytes deregulated MMP-2, MMP-4, TIMP-2, Ang, plasma renin activity (PRA), and ACE leading to fibrosis, their regulation can improve HF. One of the most effective treatments for heart failure is the use of hAMSCs-CM, which has been shown to improve heart function and reduce symptoms. The study innovation was the investigation of the in vivo mode of action of hAMSCs-CM on HF fibrosis focusing on the mentioned proteins for the first time. We expected that this study partly fill the scientific gap in HF treatment.
Methods: Frothy rats were divided into 4 groups; Control, HF, culture medium, and CM. To induce HF, isoproterenol (ISO) was injected into all animals except for the control. CM were injected into the CM group and the culture medium group received culture medium. Then, cardiac functions were measured using echocardiography and serum fibrosis was evaluated by ELISA.
Results: HF model showed decreased MMP-2, MMP-4, Ang, PRA, and ACE and increased TIMP-2, whereas hAMSCs-CM therapy reversed them compared with controls.
Conclusion: Our result has partially filled the HF treatment's gap as hAMSCs-CM improved cardiac function and reduced cardiac fibrosis and the serum fibrogenic proteins.

INTRODUCTION

Annually, lots of people are dying or suffering from heart failure (HF) as the final stage of chronic heart disease all over the world (1). Ischemic heart diseases (IHD) are a leading cause of heart failure due to maladaptive cardiac remodeling (2, 3). Huge Loss of cardiomyocytes due to ischemic events activates excessive immune and inflammatory responses to protect the injured left ventricle (LV) and preserve ejection fraction (EF) (4).

As newly fibrotic tissue does not have contractile elements and interferes with normal cardiac contractile activity, it reduces ejection fraction (EF) and fraction shortening (FS) (5). It also disturbs cardiac electrical function and can cause life-threatening arrhythmia (6).

There are several treatments available to improve heart failure, but heart transplantation is the only definitive option. Pharmacological agents also failed to preserve the EF (3, 7). Therefore, an alternative strategy focusing on tissue regeneration and reducing fibrosis formation is desirable. One of the therapeutic means in regenerative medicine is stem cell therapy (8, 9).

In this regard, various cell types like mesenchymal stem cells (MSCs) and adult tissue-resident stem cells have been assessed in experimental and clinical research (8). Despite the widespread use of other cell sources, human amniotic membrane-derived MSCs (hAMSCs) are the most reliable cost-effective, and safest source of stem cells that can be used in heart diseases (10, 11). For instance, the in vitro and in vivo cardiomyogenic potency of amniotic fluid-derived

MSCs is more than bone marrow MSCs (BMMSCs) (12). In addition, the amniotic membrane is freely accessible post-parturition and hAMSCs show higher host immune system tolerance due to their unique immunological resources (13). They also possess higher proliferation and differentiation capacity, resulting from their embryonic origin such as OCT4 overexpression. MSCs produce and secrete paracrine factors known as conditioned medium (CM) which comprises a diverse range of cytokines, growth factors, and therapeutic peptides and proteins (14). A lot of evidence has shown that treatment with CM, instead of direct MSCs, makes up the MSCs-related drawbacks such as tumorigenicity and is more time and cost-effective (11).

The mechanism of LV remodeling which leads to ischemic-related HF has not been understood. However, studies showed that LV stiffens and remodeling is vastly associated with extracellular matrix (ECM) degradation (6). ECM is produced and released by injured myocytes and endothelial cells and is regulated by matrix metalloproteinases (MMPs) in which MMP-2 and MMP-9 are involved in LV changes related to acute myocardial infarction (AMI) (15). Based on evidence, screening the plasma MMP-9 can be a mortality predictor in Congenital heart disease (CHD) (16). MMP-2 can be also considered as an HF biomarker, as higher plasma MMP-2 was associated with congestive HF (17) and significantly predicted HF with Preserved Ejection Fraction(HF-PEF) with 91% sensitivity and 76% specificity (18).

Additionally, Tissue inhibitors of metalloproteinases (TIMPs) are important regulators of the MMPs as ECM-degrading enzymes (19). TIMP-2 levels are altered in patients with certain heart conditions, such as pressure overload and atrial fibrillation, but not in those with ischemic or idiopathic dilated cardiomyopathy (19), In end-stage dilated cardiomyopathy, TIMP-2 levels were found to be elevated, suggesting a potential role in disease progression (20). TIMP-2 is the most important TIMPs because it prohibits as well as activates some MMPs. For instance, TIMP-2 is necessary for cell surface activation of MMP-2 via converting pro-MMP-2 to active MMP-2 with lower molecular weight. MMP-2 also is associated with several cardiomyopathies (19). So, TIMP-2 overexpression in HF is very important because of its dual biochemical function.

In addition, plasma renin activity (PRA) levels have been associated with an increased risk of cardiovascular events (21). PRA is also introduced as a prognosticator in HF patients with LVEF. Acute decompensated heart failure (ADHF) people with elevated PRA on admission represent poor prognosis. PRA significantly fluctuates with alteration in fluid volume and renal blood flow after HF therapy (22). The baseline PRA at the admission of acute HF people was an independent prognostic marker for their readmission as well as mortality (21).

Furthermore, angiotensin-converting enzyme 2 (ACE2) plays an important role as a counterfactor in adjustment of the renin-angiotensin-aldosterone system (RAAS). Renin cleaves angiotensinogen to AngI, which is more catabolized by ACE to AngII (23).

AngII can lead to some conditions such as hypertension and fibrosis. ACE2 is also considered a key mediator in human HF, hypertension, and different cardiovascular problems (23). Higher levels of ACE2 is associated with more vulnerability of elderlies suffering from cardiovascular problem accompanied by related disease and ACE2 is elevated in HF, too (24).

Although the therapeutic effects of hAMSCs-CM have been reported, the mode of action by which hAMSCs-CM exerts its effects has remained to be understood. The current investigation was innovative in focusing on the mode of action of hAMSCs-CM, in the HF model of male Wistar rats.

We expected that this study would fill a part of the scientific gap in the treatment of fatal HF disorder. A graphical abstract of this study has been presented in Fig. 1.

MATERIALS AND METHODS

The hAMSCs-CM preparation

The amniotic membranes were provided by post-partum donors in Shahid Akbar Abadi Hospital who signed written consent. The amniotic origin of isolated cells was confirmed using fluorescence-activated cell sorting (FACS) like our previous job (25). In short, provided cells were cultivated for 48h in a mixture of α-MEM, containing 10% FBS (Gibco, Australia), 100U/mL penicillin, 2mM L-glutamine, and 100µg/mL streptomycin. Phosphate-buffered saline (PBS) was used to wash the cells 3 times. Then, the medium was swapped with a serum-free α-MEM to harvest CM. The MSCs were placed in an incubator under hypoxic conditions (94%N2, 5%CO2, and 1%O2) for 48h, to yield CM. Finally, samples were centrifuged (1200 rpm) and were filtered through a 0.22 m filter and stored at -80°C.

Animal Models

Forty Wistar male rats(180–230g) were purchased from the Iran University of Medical Sciences and randomly divided into four groups; 1) Control: animals with no treatment, 2) HF: animals received 170mg/kg isoproterenol(Sigma, Aldrich, USA) subcutaneously for four successive days, 3) Culture Media: under deep anesthesia with a mixture of ketamine (80mg/kg) and Xylazine (5mg/kg), HF animals were injected 150µl cell-free DMEM into four points of the myocardium with a 31gauge needle along the left

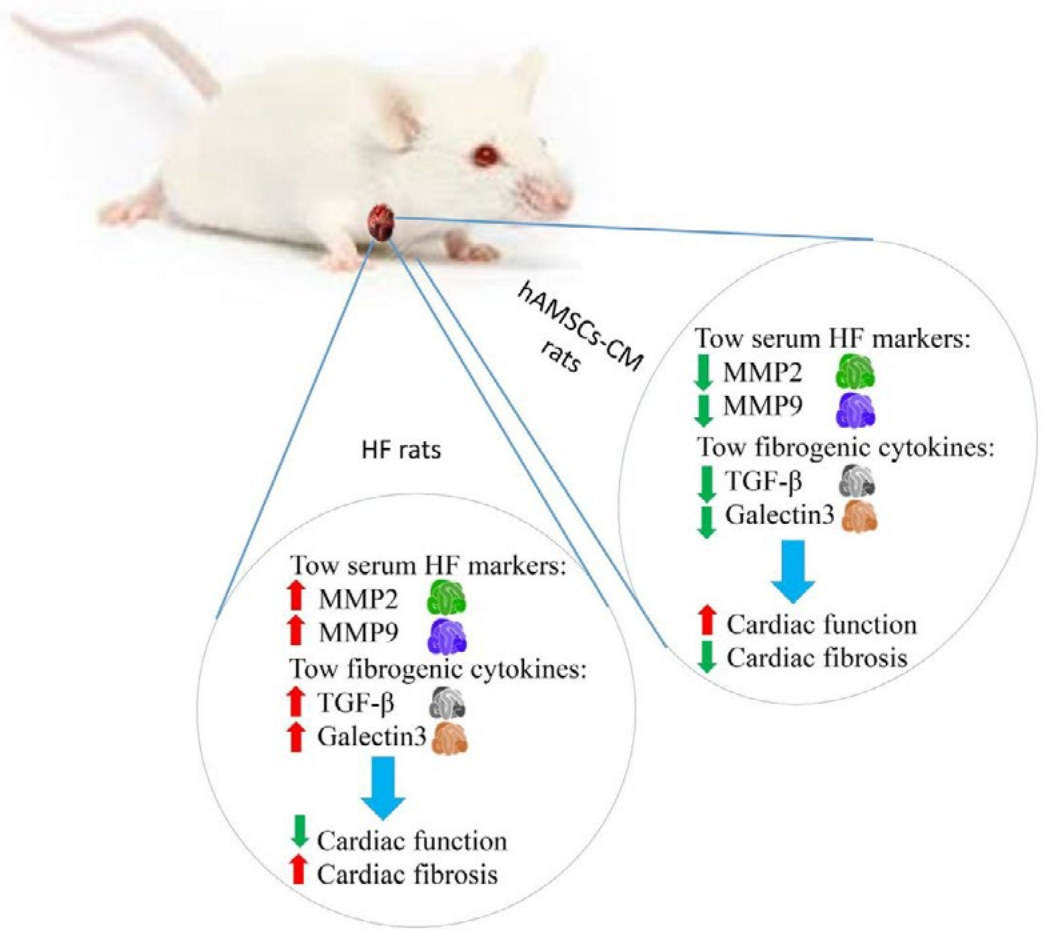

Fig 1. A graphical abstract: heart failure (HF) model rats treated with hAMSCs-CM controlled several factors related to cardiac fibrosis resulted in cardiac function improvement

anterior descending artery four weeks after the last ISO injection, and 4) Conditioned Medium (CM): HF animals were injected 150µl CM with the same condition of Culture Media group.

Echocardiography assessment

After anaesthetizing and shaving the chest and applying an acoustic coupling gel, the echocardiography test was accomplished using a VIVID-7 echocardiography device (GE Vigmed Ultrasound Norway) equipped with a 10s cardiac phase array transverse. A parasternal 2D short-axis view at the level of papillary muscles was selected to record LVDd and LVDs. Then, EF% and FS% were measured.

ELISA assay

After echocardiography, a blood sample was collected and was centrifuged(600 g for 10min at 4^{o}c) to detach serum. The levels of all factors in the serum were assessed using ELISA kits from RayBiotech, Inc. The ELISA plates were then analyzed at a wavelength of 450 nm using an ELISA Reader called Synergy MX BioTek. The results were reported in picograms per millilitre (pg/ml).

STATISTICAL ANALYSIS

All values were presented as mean ± SEM. The one-way analysis of variance and the Tukey test on Prism v5.0 (GraphPad Software, La Jolla, USA) was used for data analysis. P-values less than 0.05 were regarded as significant.

RESULTS

Effects of hAMSCs-CM on cardiac function

Four weeks after CM administration, EF and FS significantly decreased in the HF and Culture Media group compared with the control group ($p<0.001$). EF and FS were significantly improved in CM groups compared with HF groups ($p<0.001$). Although, EF and FS comparison of CM with control revealed a significant difference ($p<0.05$). In other words, treatment with hAMSCs-CM could not return cardiac function up to a normal range (Fig2 and Fig3, A and B).

Effects of hAMSCs-CM on serum level of MMP-2, MMP-4, TIMP, ANG, PRA, and ACE

The ELISA assay revealed that 4 weeks after

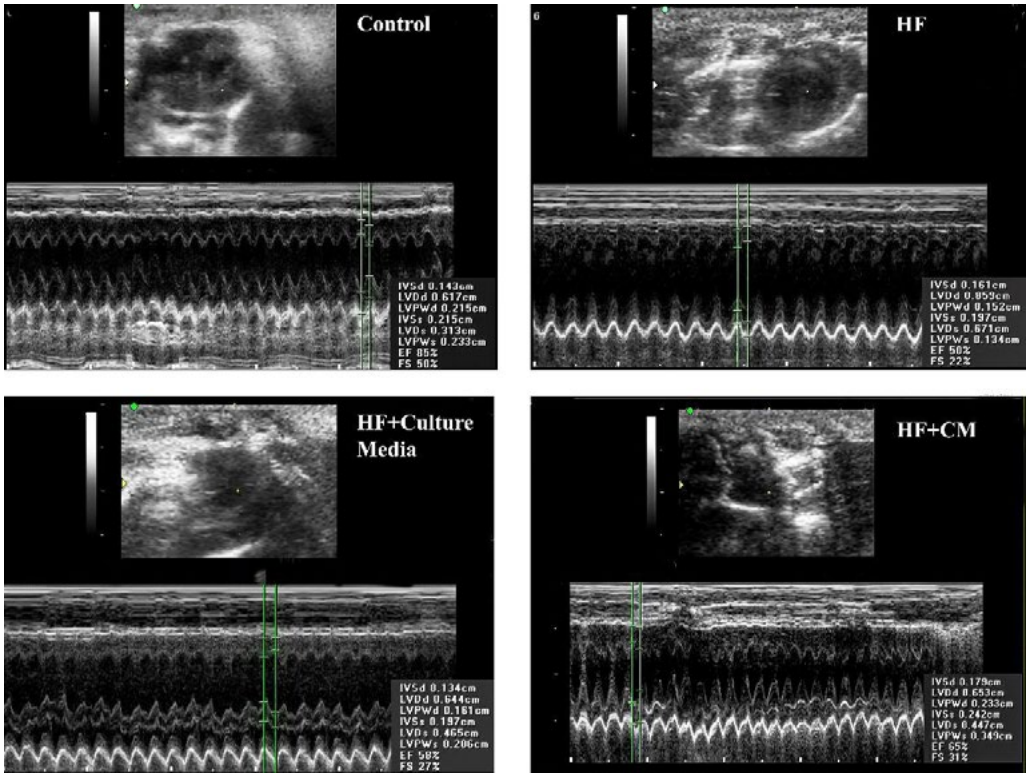

Fig 2. Echocardiographic images of all experimental groups. Ejection fraction (EF) was reached from 50% (HF group) to 65% (CM group) after hAMSCs-CM treatment.

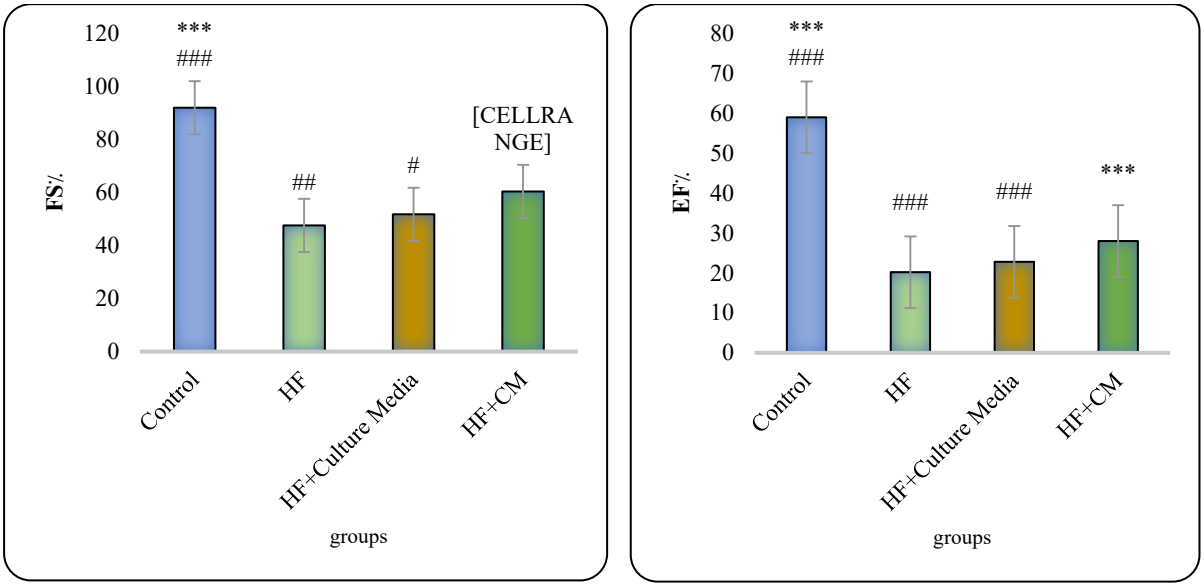

Fig 3. Echocardiographic evaluation of Cardiac function. Cardiac function parameters 4weeks after the intramyocardial administration of hAMSCs-CM(n=10). A) EF% and B) FS%. ###P<0.001, ##P<0.01, #P<0.05 vs. control; ***P<0.001, **P<0.01 vs. HF. Data are presented as mean ± SEM.

intramyocardial delivery of conditioned medium, serum level of MMP2 significantly rose in HF and Culture Media (p<0.01), as well as the CM group (p<0.05) compared with the control. Nevertheless, MMP2 in the CM group significantly declined compared with HF(p<0.01) (Fig5, A).

Serum MMP9 was elevated in HF and culture media(p<0.001), as well as CM group (p<0.05) in comparison with control.

Like MMP2, the level of MMP9 decreased after CM treatment compared with HF (p<0.001) (Fig5, B). CM treatment could not return MMP2 or MMP9 down to a normal range.

The results of the ELISA assay showed that serum

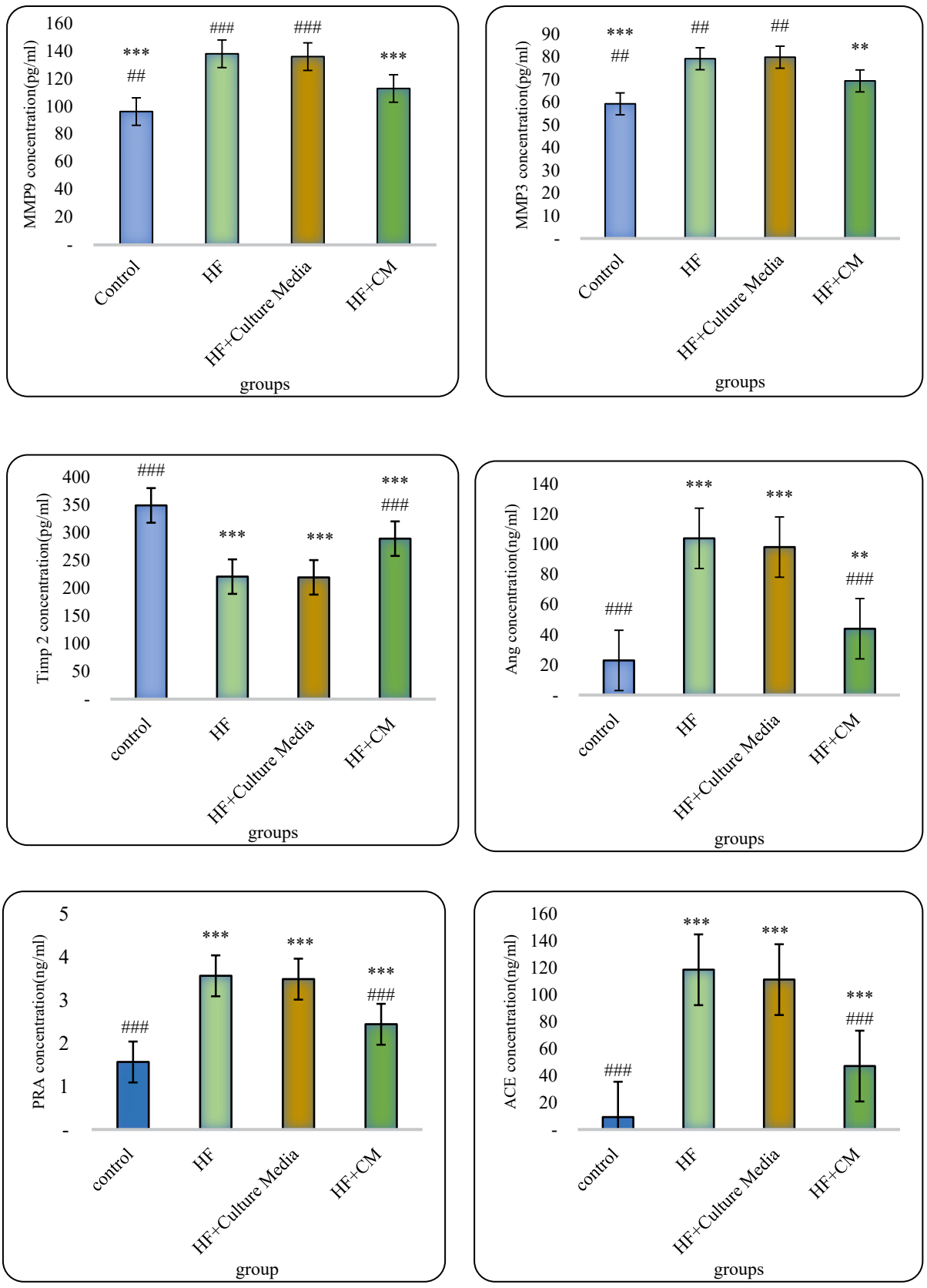

Fig4. ELISA Assay of serum level of A) MMP-2, B) MMP-9, C) TIMP-2, D) ANG, E) PRA, and F) ACE 4weeks after intramyocardial administration of hAMSCs-CM(n=10). ###P<0.001, ##P<0.01, #P<0.05 vs. control; ***P<0.001, **P<0.01 vs. HF. Data are presented as mean ± SEM.

TIMP-2, ANG, PRA, and ACE significantly increased in HF, Culture Media, as well as CM groups compared with the control group ($p<0.001$) while after hAMSCs-CM administration, they were reduced in CM group compare with HF group(all $p<0.001$) but all of them were significantly higher than the controls ($p<0.001$) expect for ANG that was significantly lower than the controls ($p<0.01$)(Fig4, A).

DISCUSSION

Maladaptive cardiac remodeling is the most underlying reason for the death of patients with developing HF. It occurs when an ischemic event triggers the cell death pathways such as apoptosis and necroptosis. The phenomena further cause cardiac fibrosis formation (26).

Fibrotic myocardium impairs cardiac function and eventually, gives rise to HF and even cardiac sudden death. There is a positive correlation between the size of the fibrotic area and developing HF (27, 28). Therefore, preventing cell death and fibrosis formation has emerged as the main point of HF studies (29).

In recent decades, to treat cardiac diseases Mesenchymal Stem cells have been widely used and, hAMSCs have the most tendency to differentiate into cardiomyocytes. Moreover, they are inexpensive, and are easily accessible (8, 10, 30). However, the risk of tumorigenicity and the need to long-term follow up restricted the application of MSCs in clinical trials. As paracrine secretion of MSCs is rich in various growth factors, cytokines, nuclear acids, and therapeutic peptides without the aforementioned drawbacks (9, 11). We hypothesized that hAMSCs-CM could improve HF condition.

Our study provided evidence that administering hAMSCs-CM directly into the heart increased EF and FS. In a study Timmers L et al. reported that hMSCs-CM therapy led to angiogenesis. Their results showed that treatment with hMSCs' secretions increased capillary density, and resulted in a rise in myocardial perfusion which gave rise to decreased infarct area and preserved EF and FS in an I/RI pig model (31). Importantly, reduced infarct size is negatively associated with developing post-MI HF (26, 32).

In the current study, we reported that hAMSCs-CM increased TIMP-2 fibrogenic factor as well as reduced serum levels of MMP-2, MMP-9, ANG, PRA, and ACE.

Our study is consistent with the findings of Daltro and colleagues, who showed that treatment with either BMMSCs or their CM led to a significant decrease in cardiac fibrosis, improvement in cardiac function, and reduction in arrhythmia incidence in a mice model of HFD-induced obesity. However, they stated that only MSCs could reduce the cardiac tissue level of MMP9, and TIMP1 but not the CM, which conveyed that the CM improved cardiac function independent of MMP9. The discrepancy probably is due to different cell lines, animal models, and sampling (1).

Moreover, targeting therenin–angiotensin–aldosterone system (RAAS) significantly increased survival in chronic HF with decreased EF. Activated RAAS leads to chronic HF development via retention of salt and water and systemic vasoconstriction (33). In response to reduced cardiac output it also improves organ perfusion through reforming and maintaining intravascular volume but long-term RAAS activation causes inappropriate ventricular remodeling as well as volume (34). RAAS activation plays a similar role in acute HF(AHF) (35). RAAS is a key system in HF because its inhibition is one of the HF treatments (33).

Furthermore, in a retrospective assessment from the BLAST-AHF trial, overexpressed PRA was related to a higher risk of rehospitalization or mortality during 30 days and baseline PRA was elevated despite more use of aldosterone receptor antagonists. So, PRA activation predicts adverse AHF outcomes. Moreover, PRA may act as an AHF target. However, it is not obvious whether the relation between elevated PRA and adverse AHF outcomes is because of renin-induced overactivation of the RAAS or the increased PRA is because of the greater severity of baseline HF. In a clinical trial, the administration of Aliskiren, as a direct renin inhibitor with the standard AHF therapeutics did not affect rehospitalization or cardiovascular mortality at 6 or 12 months after discharge (36). According to Ueda et al. study, higher PRA is associated with more advanced disease in AHF (37)

Ueda et al divided a cohort study on clinical outcomes of PRA on AHF patients based on average PRA (3.4 ng/mL/h) and reported that patients with higher PRA had greater cardiovascular death during 29 months. In the ASTRONAUT trial, in HF patients with decreased EF who were administered Aliskiren, PRA was reduced early and persistently. However lower baseline PRA was accompanied by better outcomes, Aliskiren treatment did not improve outcomes during one year (38).

Moreover, Chirinos et al. evaluated the clinical and proteomic associates of plasma ACE2 protein in a large cohort of HF people. According to this study, the ACE2 shows carboxypeptidase activity, which leads to ACE-associated AngII production as a result of the AngI degradation to Ang1 to 9 and the AngII degradation decreases its effectiveness. Moreover, the Ang1 to 7 production shows protective effects. Mentioned mechanisms opposed several negative effects of AngII, which is important in pathological situations with the overstimulated RAAS. Ang 1 to 7, shows a kind of biological effects, which are against AngII(23). Furthermore, ACE2 works independently on RAAS to adjust the intestinal microbiome as well as amino acid

homeostasis (39).

CONCLUSION

Our study has yielded valuable insights into the treatment of heart failure, as we have demonstrated that intramyocardial delivery of hAMSCs-CM can reduce cardiac fibrosis induced by ISO. Treatment with hAMSCs-CM also resulted in improved cardiac function and adjusted the serum level of MMP-2, MMP-4, TIMP, ANG, PRA, and ACE. In general, our results pointed out the therapeutic properties of hAMSCs-CM in male rats subjected to ISO-induced HF.

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