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Human umbilical cord blood mesenchymal stem cells conditioned media inhibits hypoxia-induced apoptosis in H9c2 cells by activation of the survival protein Akt

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Abstract: This work aimed to study the beneficial role of human umbilical cord blood-derived mesenchymal stem cell-conditioned medium (MSC-CM) in hypoxia-induced apoptosis in H9c2 cardiomyoblasts, in which the serine/heroine kinases (Akt) pathway would be involved. For this, CM was collected by culturing MSCs in serum-free DMEM medium for 24 h, and paracrine factors were analyzed by protein chip. H9c2 cells were divided into the following groups: control group, hypoxia group, MSC-CM intervention group (CM group), MSC-CM + Akt phosphorylation inhibitor (LY294002) group (LY group). Apoptosis of the H9c2 cells was tested with chromatin dye Hoechst 33342 and FITC-conjugated Annexin V apoptosis detection kit by flow cytometer after a hypoxia/serum deprivation (H/SD) for 24 h. The apoptosis-related proteins were evaluated by Western blot. MSC-CM displayed significantly elevated levels of growth factors, anti-inflammatory, and anti-apoptosis cytokines. On Hoechst 33342 apoptosis staining, the H9c2 cell morphology displayed a lower proportion of apoptosis in the CM group than those in the hypoxia group, while apoptosis was increased in LY group. Flow cytometer analysis revealed the apoptosis ratio in the CM group was lower than the hypoxia group (12.34 ± 2.00% *vs.* 21.73 ± 2.58%; $p < 0.05$), while the LY group was significantly higher (22.54 ± 3.89%). Active caspase-3 expression was increased in hypoxia group than control group ($p < 0.05$), but decreased in CM group ($p < 0.01$). Umbilical cord blood-derived mesenchymal stem cell-conditioned media secrete multiple paracrine factors that are able to inhibit hypoxia-induced H9c2 cardiomyoblasts apoptosis, and in which the activation of Akt phosphorylation is involved to achieve the protective effect.

Introduction

Myocardial infarction is the leading cause of mortality in heart disease; the incidence gradually rises with the increase of age. Numerous studies have shown that mesenchymal stem cells (MSCs) transplanting improved heart function in ischemic myocardium (Colicchia *et al.*, 2019; Shafei *et al.*, 2017; Yu *et al.*, 2017). The mechanism leading to this improvement was unclear and complicated, including cell differentiation, paracrine function, and angiogenesis. However, most studies found no myocardial cell regeneration in the transplantation areas (Gnecchi *et al.*, 2016); moreover, the early beneficial effects of stem cell transplantation in acute myocardial infarctions were unlikely to be due to stem cell transdifferentiation to cardiac myocytes (Henning *et al.*, 2010). Hence, the paracrine mechanism mediated by biologically active factors plays an important role in cell transplantation.

MSCs can be harvested from many tissues, including bone marrow, umbilical cord, and adipose tissue (Najar *et al.*, 2014). Although the immunophenotypic profiles of MSCs from different tissues are similar, variation in the differentiation, function, and paracrine characteristics existed (Amable *et al.*, 2014). Many research suggested that MSCs from umbilical cord blood (UCB-MSCs) have stronger proliferation and myocardial differentiation ability (Jin *et al.*, 2013), low immunogenicity *in vitro* and *in vivo* (Lee *et al.*, 2014), make it emerging as the best option for clinical applications. Therefore, our results support the idea that UCB-MSCs may be more potential in paracrine effects. Moreover, animal-derived MSCs have been widely studied

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previously; little attention was given to the human MSCs, especially the more accessible UCB-MSCs.

Paracrine effects that promote survival and proliferation, account for cardiac benefits bestowed by stem cell, especially the Akt-overexpressing MSCs, have better myocardial repair function and more paracrine factors (Ranganath *et al.*, 2012). Moreover, most cytokine factors act through Akt to repress apoptosis. Therefore, using a cell culture model of myocardial infarction, we studied whether the factors released from UCB-MSCs can inhibit hypoxia/serum deprivation (H/SD) induced H9c2 cells apoptosis and the role of Akt pathway.

We hypothesize that UCB-MSCs secrete a broad repertoire of factors that significantly reduce H9c2 cell apoptosis. Therefore, in the present experiments, we determined the growth factors and anti-apoptosis cytokines secreted into cell culture media by MSCs, we then determined the effects of MSC-conditioned media on apoptosis; caspase-3 activity and activation of the cell survival proteins Akt in H9c2 cells during 24 h of H/SD. The present investigation demonstrates that UCB-MSCs secrete many biologically active factors, which significantly reduces H9c2 cell apoptosis by activation of the survival protein Akt during a severe H/SD environment.

Materials and Methods

UCB-MSCs culture

Human UCB was obtained from Fuxing Hospital, Beijing. All protocols involving human subjects obtained ethical approval of the local ethical committees and conformed the declaration of Helsinki. Isolation of UCB-MSCs was performed as our previously published paper by Ficoll density gradient centrifugation (Gong *et al.*, 2016). Cells were incubated in 37°C under 5% CO_2 in culture medium (Dulbecco's modified Eagle's medium/F12 (DMEM/F12), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin). The media was first completely replaced after five days in order to remove non-adherent cells. Hereafter the media was completely replaced every three days until putative MSC colonies were noted. All experiments involved three to five passages of MSCs.

Flow cytometry analysis and differentiation assays

Cell surface antigen phenotyping of MSCs was performed at passage 3. The following cell-surface epitopes were marked with the anti-human CD34-FITC, CD44-PE, CD45-Percp, CD105-APC (Miltenyi Biotec, Germany). Mouse isotype antibodies served as a control, and they were analyzed using a FACS system.

To induce adipogenic and osteogenic differentiation, MSCs were seeded at a density of 10^3 cells/cm^2 in six plates and cultured for up to three weeks in differentiation culture medium. The differentiation capacity was verified by morphology changes and staining for specific markers, which is alizarin red for osteoblast and oil o for adipocytes.

Preparation of MSC-conditioned medium

MSCs were passaged and maintained in DMEM/F12 containing 10% fetal bovine serum (Gibco). We obtained rat cardiomyocyte-derived cell line H9c2 cells from the cell library of Union Medical College; cells were used at the third passage for all experiments.

MSCs at 5×10^3 cells/cm^2 were grown in T25 cell culture flask containing DMEM/F12 with serum. The following day the cultures were serum-starved with DMEM without serum. After 24 h, the supernatants were collected and centrifuged (2000 rpm, 10 min), then filtered (0.22 μm cellulose syringe filter) and labeled as MSC-conditioned medium (MSC-CM), which was used to determine the effects on hypoxia-induced apoptosis in H9c2 cells.

Induction of hypoxia in H9c2 cells

To detect the protective role of MSC-CM on H9c2 cells, the cells were washed with PBS twice, and then cultured by a replacement with fresh DMEM or MSC-CM or MSC-CM +LY294002 (40 μM). H9c2 cells were subjected to 24 h of hypoxia (a sealed, hypoxic GEN box jar fitted with a catalyst); the normal cultured H9c2 cells served as the control group.

Assessment of morphological changes

Chromosomal condensation was assessed using the chromatin dye Hoechst 33342. Cells were fixed for 30 min in 4% paraformaldehyde at room temperature, washed twice with PBS, and then exposed to 5 μg/mL Hoechst 33342 for 30 min at room temperature. Finally, all samples were washed with PBS and observed using a fluorescence microscope. Apoptotic cells were characterized by condensed chromatin and cell shrinkage.

Cell survival assessment

After hypoxia for 24 h, H9c2 cells in the different groups were harvested and resuspended in 50 μL PBS per 5×10^5 cell, then added 450 μL Muse Count and Viability reagent (Millipore, Darmstadt, Germany), incubated for 5 min at room temperature in the dark. Finally, the cell suspensions were analyzed by Muse Cell Analyzer.

Flow cytometric analysis of apoptosis

H9c2 cells were subjected to hypoxia for 24 h, after exposure, the cells were harvested through trypsinization and washed twice with cold PBS, 5 μL of Annexin V and propidium iodide (PI) were added to the cells, which were resuspended in 500 μL buffer and then analyzed by flow cytometry. The percentage of apoptotic cells was assessed based on the fluorescein isothiocyanate (FITC) Annexin V-binding assay.

Western blot analysis

Western blot analysis for caspase-3 and the phosphorylation of Akt (p-Akt) were performed with each group. Proteins were separated by 12% SDS-PAGE, transferred onto a polyvinyl denedifluoride membrane. After blockage with 0.1% Tween in Tris-buffered saline containing 5% skim milk at room temperature for 2 h, the membranes were detected by primary antibodies p-Akt and caspase-3 (1:1000, CST) overnight at 4°C. The next day, after washing, the membranes were incubated for 2 h at room temperature with peroxidase-conjugated secondary antibodies. Finally, protein bands were detected using a chemiluminescence detection system (FluorChem M, USA); β-actin was used as a standard control.

Protein chip assay

In this study, to determine whether MSCs secrete paracrine factors, we used Biotin label-based Human Antibody Array I (RayBiotech, Inc., Norcross, GA) to compare the differences in protein expression levels between the DMEM culture and MSC-CM. This antibody array membrane is marked with 507 specific antibodies toward cytokines, chemokines, growth factors, angiogenic factors, and other proteins, which can comprehensively analyze the differences in protein expression level. The culture medium was harvested for protein chip assay according to the manufacturer's instructions. The measurements of MSC-CM were then expressed as a percentage of the DMEM culture measurements.

Moreover, cytokine profiles of serum from STEMI patients and healthy controls were analyzed with a semi-quantitative human antibody array (RayBiotech, Inc., Norcross, GA) for 43 proteins, then the pathways of differential protein genes were analyzed, and we focused on the Akt signal path changes in these two groups of patients.

Statistical analysis

Statistical analysis was performed with SPSS 20.0 software (SPSS Inc., USA). Data were expressed as mean ± standard deviation (SD). Univariate comparisons of the means were evaluated using the Student's *t*-test or one-way analysis of variance with Tukey's *post hoc* adjustment for multiple comparisons when appropriate. Statistical significance was assigned when $p < 0.05$.

Results

The characteristics of UCB-MSCs

The number of adherent cells increased with time and became spindle-shaped within 10 days. After 15–20 days, a number of classical MSCs colonies appeared, similar to the MSCs isolated from bone marrow (Fig. 1A). The cells with fat drops can uptake the Oil red o after adipocyte differentiation (Fig. 1B). Moreover, after exploring with an osteogenic medium, cells with an osteoblast-like phenotype by staining with alizarin red (Fig. 1C). Flow cytometry showed that UCB-MSCs were positive for CD44, CD105, but not CD34 and CD45, further confirmed the characteristic of MSCs isolated from UCB (Fig. 1D).

Factors secreted by UCB-MSC into media

Levels of growth factors, cytokines, and angiogenic factors were measured in DMEM media and MSC-CM using protein chip. The results showed that there was a significant augmentation of angiogenic, anti-inflammatory and anti-apoptotic factors, including Ang-1, VEGF, HGF, MCP-1, TIMP-1, MIG, IGF-1, IGF-BP-7, and G-CSF, in MSC-CM; however, these paracrine factors were absent in DMEM media (Tab. 1).

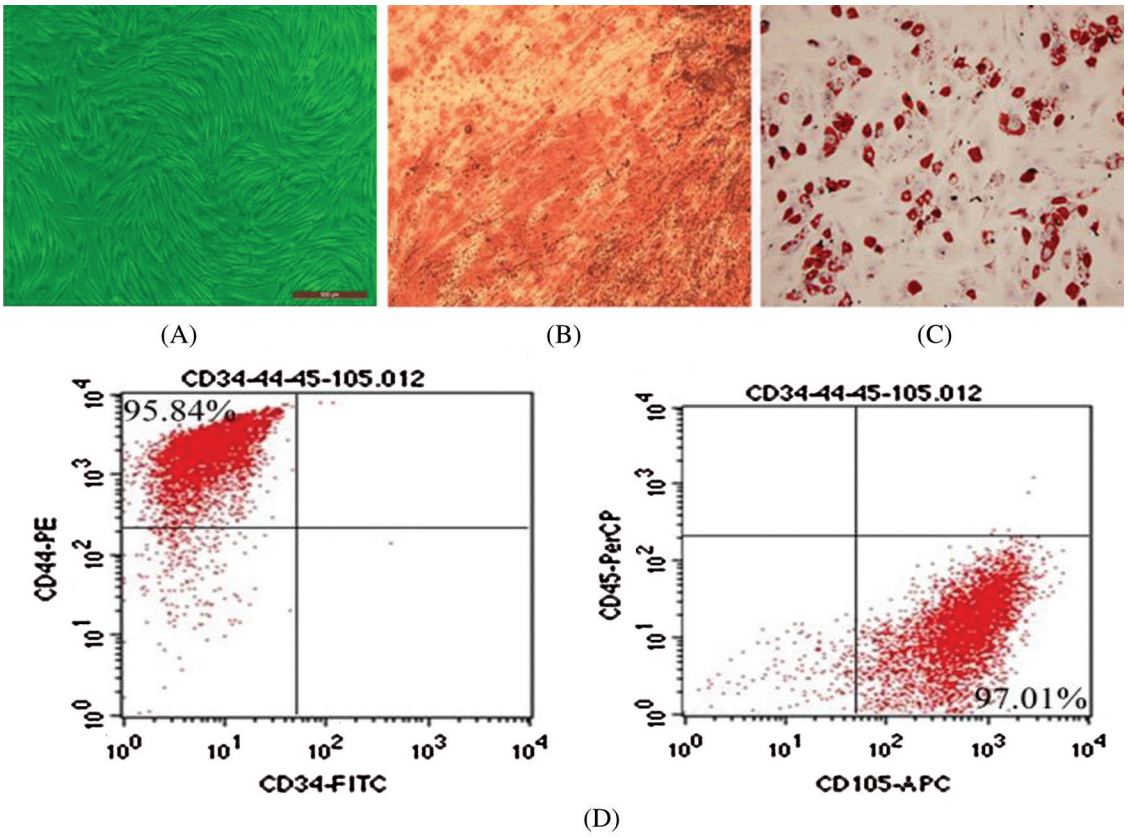

FIGURE 1. Characterization of human mesenchymal stem cells (MSCs) from UCB. (Figs. B C D quoted from Gong *et al.*, 2016). (A) Morphology of cultured MSCs at passages 3, all MSCs exhibited spindle-shaped morphology. (B) Differentiation capability of MSCs, osteogenic differentiation demonstrated by deposition of mineralized matrix detected by alizarin red staining, 100X. (C) Adipocytic differentiation was evidenced by oil-red O staining, 200X. (D) Surface marker expression of UCB-MSCs, MSCs at passage 3 by flow cytometry analysis demonstrated positive for mesenchymal markers CD105 and CD44, but negative for hematopoietic markers CD34 and CD45

TABLE 1

Paracrine factors in MSC-conditioned media

	IGF-BP-7	VEGF	MCP-1	HGF	TIMP-1	MIG	G-CSF
DMEM	ND	ND	ND	ND	ND	ND	ND
CM	6245	671	668	766	800	977	619

ND = non-detectable, **CM** = MSC-conditioned media.

MSC-CM improve the H9c2 cell viability after exposure to H/SD

Fig. 2 shows the decrease in cell survival ($p < 0.01$) in H/SD environment, and this decrease was significantly improved with MSC-CM, while LY294002 in CM inhibited a positive effect. Furthermore, we determined the cell survival by using additional Hoechst 33342 staining. The results showed that H9c2 cells exposed to H/SD showed increased floating ones and chromatin condensation compared to the control group. However, cells treated with MSC-CM had regular nuclei, with relatively few showing the characteristic of condensed chromatin, suggesting that MSC-CM improves the H9c2 cell viability under H/SD conditions (Figs. 3A–3C).

MSC-CM displays a paracrine protective effect against the apoptotic damage of H9c2 cells in vitro

In the flow cytometry analyses, 24 h of H/SD increased the apoptotic rate of H9c2 cells in comparison with the control group (21.73 ± 2.58% *vs.* 5.20 ± 1.43%; $p < 0.01$). However, MSC-CM, when added to H9c2 cells during hypoxia, significantly decreased apoptosis from 24.67% to 12.58% ($p < 0.05$). More importantly, the Akt inhibitor LY294002 suppresses MSC-CM protection of H9c2 cells ($p < 0.05$) (Figs. 4A and 4B). In line with this result, chromosomal condensation, a hallmark of apoptosis, was reduced in MSC-CM cultured H9c2 cells.

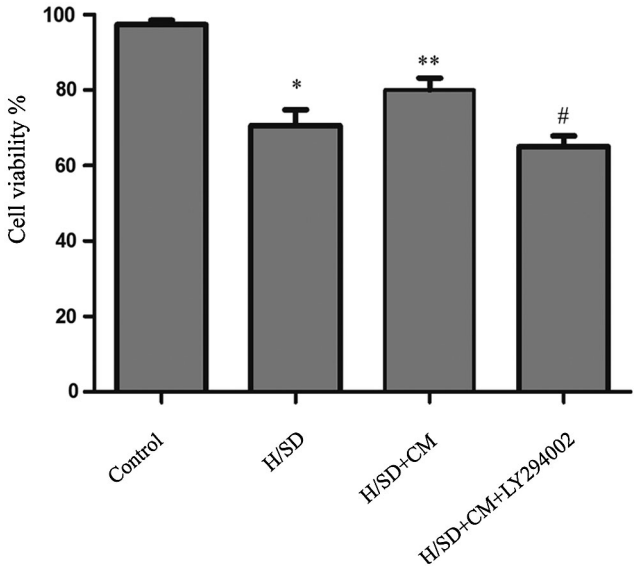

FIGURE 2. Effect of CM on H9c2 cell viability. Cell viability was remarkably reduced in the H/SD group. CM increased cell viability, but the effect was abrogated by LY294002. N = 3, *$p < 0.01$ *vs.* control group; **$p < 0.05$ *vs.* H/SD group; #$p < 0.05$ *vs.* H/SD + CM group.

Moreover, to test the hypothesis that paracrine factors secreted by MSCs inhibit apoptosis, we also determined the expressions of caspase-3, a classical marker of apoptosis. H9c2 cells were cultured in the presence or absence of MSC-CM while under hypoxic conditions for 24 h to induce apoptosis. As shown in Fig. 5, MSC-CM was able to significantly ($p < 0.05$) reduce caspase-3 activity.

MSC-CM activates Akt in H9c2 cells

Protein levels of Akt and the activated form, p-Akt, were detected in cell homogenates. Hypoxia significantly decreased p-Akt in h9c2 cells cultured without MSC-CM in comparison with h9c2 cells in normoxia ($p < 0.01$), while its expression was increased after MSC-CM treatment, the ratio of p-Akt/Akt was obviously increased in the CM group compared with the H/SD group (Fig. 5).

Moreover, the difference in the signal pathway between STEMI patients and healthy controls were detected by protein Chip. The results showed that the PI3K-Akt signaling pathway is significantly different between the two groups of patients (Fig. 6).

Akt inhibition decreases MSC-CM protection in H9c2 cells

We performed Akt inhibition studies with LY294002, a specific Akt inhibitor. Consistent with our initial studies, 24 h of hypoxia significantly decreased Akt phosphorylation in h9c2 cells, this decrease was significantly prevented by MSC-CM. In contrast, LY294002 significantly abrogated the elevated p-Akt and attenuated the protective effects of CM on the H/SD-induced cell apoptosis rate (22.54 ± 3.89% *vs.* 12.34 ± 2.00%; $p < 0.05$) (Figs. 4A and 4B). Meantime, the effect of LY294002 on caspase-3 expression was determined; importantly, it was able to reverse the effect of CM on caspase-3, and the caspase-3 activity was increased significantly (Fig. 5).

Discussion

Our study clearly identifies specific umbilical cord blood-derived MSCs secreted paracrine factors that are able to inhibit hypoxia-induced apoptosis in H9c2 cells; the protection of MSC-CM was mediated through the activation of Akt pathway, activated Akt significantly limits the activity of the apoptotic protein caspase-3. These suggested that the paracrine factors of MSCs exerted cytoprotective effects on H9c2 cells exposed to hypoxia.

MSCs have been reported to transdifferentiation to functional Cardiomyocyte-like Cells *in vitro*, and suggest that *in vitro* pre-differentiation could be a potential strategy to increase their regenerative efficacy *in vivo* (Szaraz *et al.*,

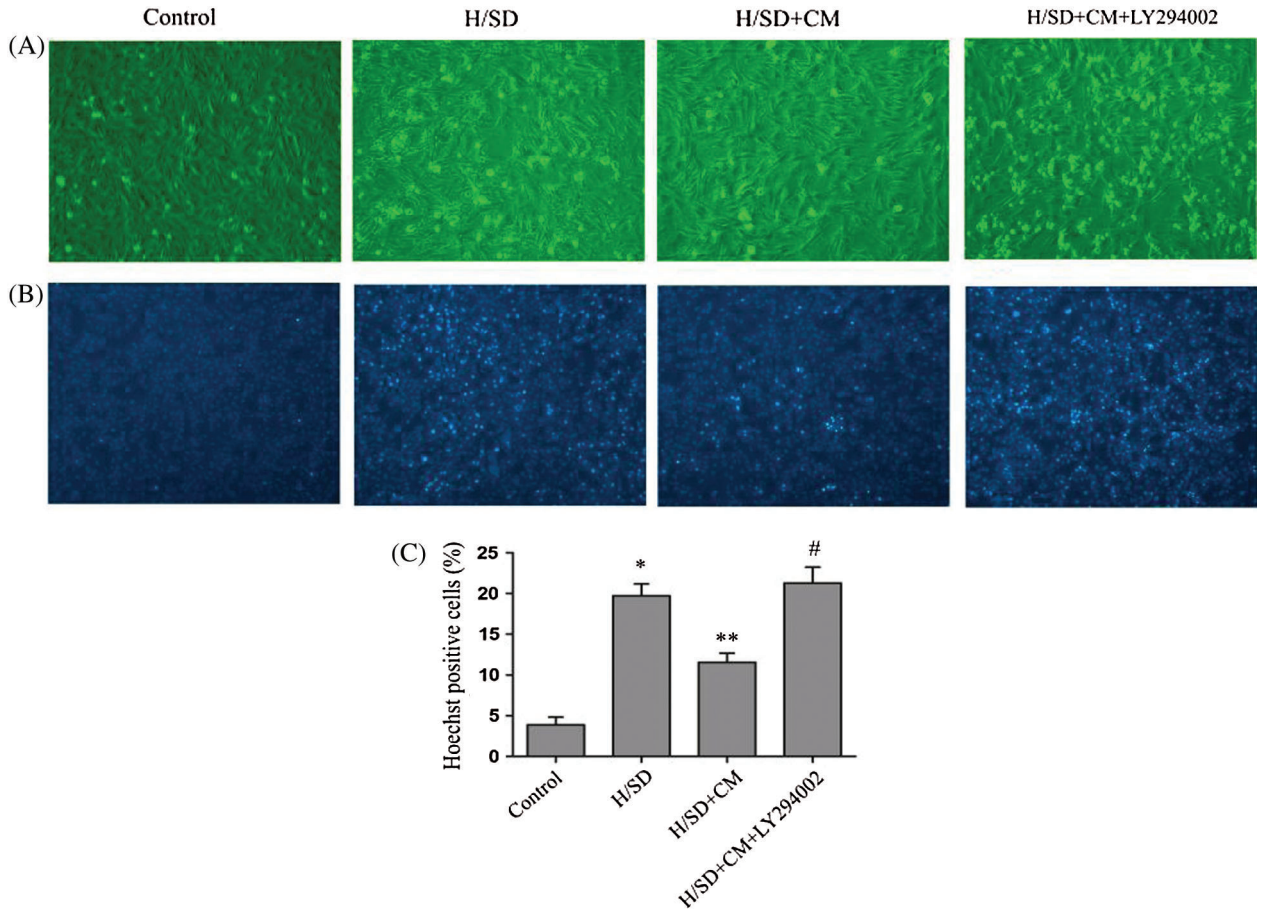

FIGURE 3. Effect of CM on apoptosis induced by H/SD in H9c2 cells. (A) Morphological alterations were detected by a phase-contrast microscope (100X). (B) Representative pictures of apoptotic nuclear inspected by fluorescence microscopy upon Hoechst 33342 staining (100X). (C) Quantifications of the apoptotic H9c2 cells were presented as the percentage of Hoechst positive cells. The data represent the results of three independent experiments. *$p < 0.01$ *vs.* the control group; **$p < 0.05$ *vs.* the H/D group, #$p < 0.05$ *vs.* H/SD + CM group.

2017). However, no permanent engraftment of transplanted MSCs (Muller-Ehmsen *et al.*, 2006) and significant levels of stem cell differentiation into cardiomyocytes (Yasin, 2013) were verified *in vivo*; many investigators believe that the evidence of cardiomyocyte differentiation was insufficient. More importantly, the differentiation is not likely to occur acutely and does not readily explain the prompt beneficial effects of MSCs in the treatment of acute myocardial infarctions (Henning *et al.*, 2008). Hence, the beneficial effect of MSCs treatment may be attributed to paracrine factors, a mechanism supported by more and more investigators (Haider and Aramini, 2020, Linero and Chaparro, 2014).

UCB-MSCs paracrine factors

The present study assessed the effects of conditioned medium from UCB-MSCs *in vitro* on H9c2 cells subjected to hypoxia. First, the protein chip assay demonstrates that UCB-MSCs do secrete more paracrine factors, including Ang-1, VEGF, HGF, MCP-1, TIMP-1, MIG, IGF-1, IGF-BP-7, and G-CSF, that can have anti-apoptosis and cellular protective effects. For example, MCP-1 was able to play a constructive role in cell survival, which has been shown to be both pro-and anti-apoptotic in cardiomyocytes (Zhou *et al.*, 2006). Bader *et al.* also found that MSC-CM contained more HGF than fibroblast medium, which protected cardiomyocyte-like HL-1 cells from simulated ischemia (Bader *et al.*, 2014). Therefore, CM derived from MSCs cultures may be the next generation for regenerative medicine (Gunawardena *et al.*, 2019).

Except for the treatment of myocardial infarction, paracrine factors from MSCs have been reported to have the potential for treating many diseases such as renal failure (Van Koppen *et al.*, 2012), musculoskeletal tissue damage (Veronesi and Borsari, 2018), and ischemic stroke (Lee *et al.*, 2010). Therefore, all these elucidated how multiple factors contained in MSC-CM promote cell survival, our cell viability assay test also proved that MSC-CM increased the survival rate of H9c2 cells in H/SD environment.

Anti-apoptosis of MSC-CM in H9c2 cells

We were able to demonstrate significant anti-apoptosis activity for the factors secreted by MSCs. In h9c2 cells, hypoxia increased the apoptosis, and CM can reduce the apoptosis by AV/PI staining. Moreover, the caspase-3, a core protein of apoptosis, was activated by hypoxia; however, MSC-CM reversed the effect also. Consistent with the previous experiment, Farahmand *et al.* (2018) reported that anti-apoptotic and proliferative genes were up-regulated in co-cultured breast tumor cells with MSCs-CM that correlate with tumor progression and poor prognosis. Hardiany *et al.* (2019) also found the presence of these and other anti-apoptotic molecules in umbilical cord stem cell-derived CM. Taken together, these data suggest that

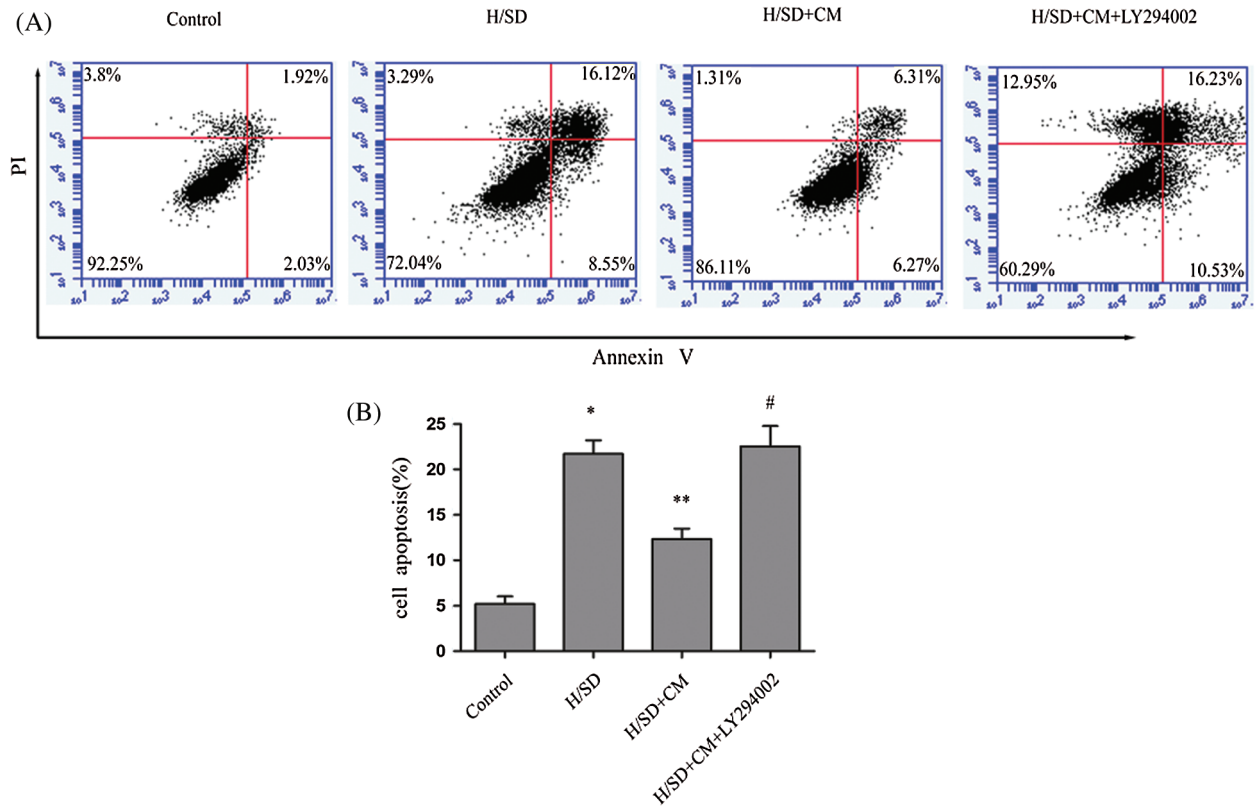

FIGURE 4. Anti-apoptotic effect of CM on H9c2 cells determined by FACS analysis. (A) Representative FACS pictures from each group, apoptosis was quantified after staining with Annexin V and propidium iodide (PI). (B) Quantifications of the apoptotic H9c2 cells were presented as the percentage of early and late apoptotic cells. The data represent the results of three independent experiments. *$p < 0.01$ *vs.* the control group; **$p < 0.05$ *vs.* the H/D group, #$p < 0.05$ *vs.* H/SD + CM group.

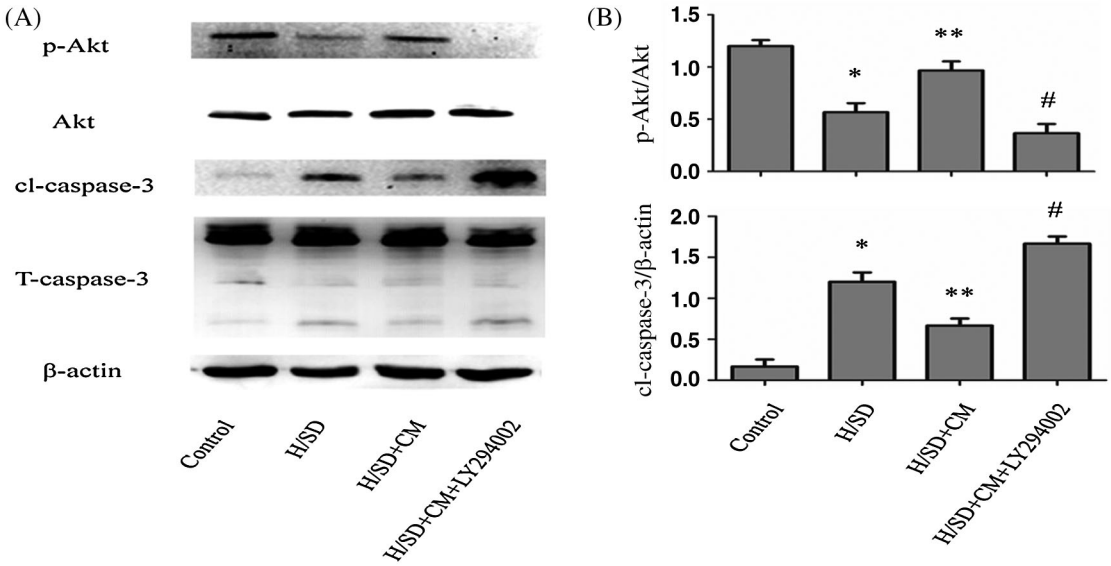

FIGURE 5. Akt pathway mediated the anti-apoptotic effect of CM on H9c2 cells. (A) CM remarkably increased the phosphorylation of Akt expression, whereas LY294002 attenuated the effect. Moreover, CM reduced the H/SD induced expression of caspase-3; LY294002 increased the caspase-3 activation. (B) Semiquantitative analysis of the western blots, the data represent the results of three independent experiments. *$p < 0.01$ *vs.* the control group, **$p < 0.05$ *vs.* the H/SD group, #$p < 0.01$ *vs.* H/SD + CM group.

multiple paracrine factors in MSC-CM can significantly inhibit cell apoptosis under the harsh environment.

Akt pathway

It has been shown that CM derived from umbilical cord MSC regenerates atrophied muscles, the CM exerted its actions by stimulating the Akt signaling cascade (Kim *et al.*, 2016). Moreover, many studies suggested that paracrine action accounts for marked protection of ischemic heart by Akt-modified MSCs (Chen *et al.*, 2013), and Akt pathway are commonly involved in the stress-induced apoptosis in *in vitro* and *in vivo* models (Li *et al.*, 2019; Mao *et al.*, 2019).

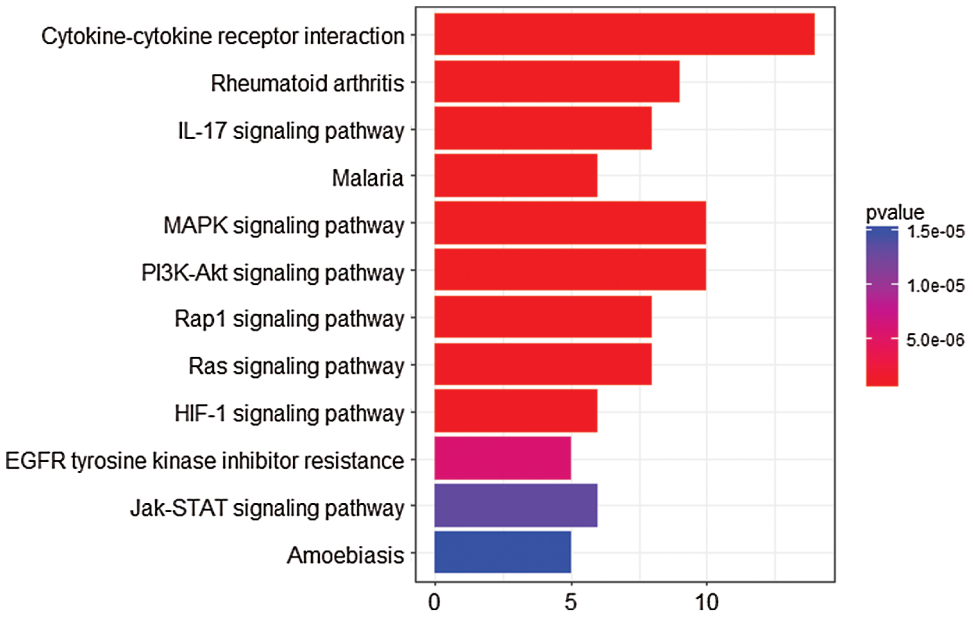

FIGURE 6. The differences in signaling pathways in STEMI patient and healthy controls. The results showed that the importance of the Akt signal pathway in the clinic.

Therefore, as to the anti-apoptosis mechanisms of umbilical cord blood MSC-CM, we further investigated the Akt pathway.

In our study, H/SD decreased the expression of p-Akt protein; however, MSC-CM treatment could reverse the suppression of p-Akt caused by H/SD. More importantly, when pretreated H9c2 cells with the specific Akt inhibitor LY294002, the result showed that the expression of p-Akt was decreased and the MSC-CM-mediated anti-apoptotic effects were significantly attenuated confirmed with AV/PI staining and caspase-3 activities, suggesting that Akt activation is important for the protection function of MSC-CM on H9c2 cells under H/SD conditions. Furthermore, the role of the Akt pathway was also evidenced in embryonic stem cells-CM of inhibiting H_2O_2-induced apoptosis in H9c2 cells (Singla *et al.*, 2008). Moreover, the change of downstream genes of the PI3K-Akt pathway will be the focus of our next research.

More importantly, in the clinical real world, we also found that the PI3K-Akt signaling pathway is significantly different between STEMI patients and healthy controls. This proves the importance of this signal pathway in the clinic.

Conclusions

In conclusion, the present experiments demonstrate that UCB-MSCs can significantly inhibit H9c2 cells apoptosis under H/SD environment by secreting growth factors and anti-apoptosis cytokines that activate the cell survival proteins Akt. These findings would suggest that the isolation of the paracrine factors may have a better therapeutic application for ischemic tissue damage.

Availability of Data and Materials: The datasets used or analyzed during the current study are available from the corresponding authors on reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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