Heat Shock Preconditioning Mesenchymal Stem Cells Attenuate Acute Lung Injury via Reducing NLRP3 Inflammasome Activation in Macrophages

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Keywords: Heat shock, Umbilical cord-derived mesenchymal stem cells, Acute lung injury, Macrophage, NLRP3 inflammasome

Posted Date: January 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-139954/v1>

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Abstract

Objectives: Acute lung injury (ALI) remains one of the common causes of morbidity and mortality worldwide, so far, without any effective therapeutic approach. Previous researches have revealed that topical administration of umbilical cord-derived mesenchymal stem cells (UC-MSCs) can attenuate pathological changes in experimental acute lung injury. Heat shock (HS) pretreatment has been identified as a method to enhance survival and function of cells. The present study aimed to assess whether HS-pretreated mesenchymal stem cells (MSCs) could strengthen the immunomodulation and recovery from ALI.

Materials and Methods: HS pretreatment was defined 42°C for 1h, the changes of biological characteristics and the secreted functions were detected. In the mouse model of ALI, we intranasally dripped the pretreated UC-MSCs in vivo, confirmed their therapeutic effects and detected the phenotypes of macrophages in bronchoalveolar lavage fluid (BALF). To elucidate their mechanisms, we co-cultured the pretreated UC-MSCs with macrophages in vitro, and the expression levels of inflammasome-related proteins in macrophages were assessed. Finally, Apoptozole was used for further determine the role of HSP70 in HS-pretreated UC-MSCs-based therapy.

Results: The data showed that UC-MSCs did not represented significant changes in viability and biological characterizations after received HS pretreatment. Administration of HS-pretreated UC-MSCs into the ALI model, improved pathological changes and lung damage-related indexes, reduced of the levels of pro-inflammatory cytokines and modulated the balance of M1/M2. Mechanistically, both in vivo and in vitro studies demonstrated that HS pretreatment enhanced the protein level of HSP70 in UC-MSCs and subsequently upregulated the synthesis and secretion of PGE2, which negatively modulated the NLRP3 inflammasome activation of alveolar macrophages. And these effects was partially reversed by Apoptozole.

Conclusion: HS pretreatment can strengthen the beneficial effects of UC-MSCs on inhibiting NLRP3 inflammasome activation of macrophages in ALI. The mechanism may be contributed to the upregulated expression of HSP70 to further induce PGE2 synthesis and secretion.

Introduction

Acute lung injury (ALI) is a common inflammatory disease, which is pathologically characterized by diffuse inflammation and subsequently increased vascular permeability of the lung, leading to the reduce function of alveolar gas exchange and finally the development of acute respiratory distress syndrome (ARDS)[1, 2]. ARDS remains a common and life-threatening lung disease that its mortality rate ranges from 30–50% [3, 4]. Although health care advancements, including high-frequency oscillatory ventilation and carbon dioxide removal extracorporeally, have been improved, there is still no pharmacological approach exhibiting therapeutic effectiveness to ARDS[5]. Therefore, it is badly in need of the exploration of promising therapeutic strategies for targeting these pathophysiological features to alleviate ARDS.

As the imbalance between anti-inflammatory and pro-inflammatory factors is one of the critical characterizations of ARDS, cytokine modulation may become a potential treatment approach[6]. Of all, alveolar macrophages, which are main components of the lung immune system and locate at the air-tissue interface, display heterogeneous roles in lung injury as their various classification accorded to the phenotype and the cytokines they secrete[7, 8]. In a murine model of ALI, proinflammatory macrophages (M1-polarized) are predominance to reside in the lung to induce pulmonary injury, while these cells repolarize, and express surface markers correlated to reparative phenotype (M2-polarized) during the resolution of ALI [9]. Treatment with mesenchymal stem cells (MSCs) might be an ideal therapeutic approach because of their potentials to contribute to immunoregulation and tissue repair. Our previous researches provided reliable results on the beneficial effects of MSCs on improving liver ischemia/reperfusion injury (IRI) in vivo and in vitro[10, 11]. In addition, our and other several studies have also shown that MSCs could alleviate lipopolysaccharide (LPS)-induced ALI via their well-defined roles in immunomodulation and differentiation into multiple different cell types, including adipocytes, osteoblasts and lung-related cell types[12–17].

Indeed, recent researches focus on how to strengthen the therapeutic effects of MSCs for various diseases. In the past study, we demonstrated that preconditioning MSCs by rapamycin could increase hepatoprotective roles against liver IRI by enhancing immunosuppression and strengthening the migratory capacity of these cells [18]. Heat shock (HS) is also identified as an effective way to protect cell survival and enhance their function in vitro from various environmental cultures[19, 20]. Several studies reported that HS pretreatment of transfused MSCs enhanced their survival following administration into liver IRI and chemotherapy-induced granulosa cell apoptosis[21, 22]. Other findings have revealed that HS pretreatment of transplanted cells could strengthen their therapeutic functions[23]. However, whether HS pretreatment enhance MSCs function to protect against ALI is still unknown.

The aim of this study is to investigate the protective effects and the potential mechanisms of HS-pretreated UC-MSCs on alleviating ALI. We hypothesized that HS not only maintains viability of MSCs, but also strengthens the pulmonary protection of MSCs on ALI via modulating NLRP3 inflammasome of macrophages. In the further investigation, we targeted to HSP70, which was upregulated by heat shock and subsequently induced prostaglandin E2 (PGE2) synthesis.

Methods And Materials

Isolation and culture of umbilical cord-derived MSCs (UC-MSCs)

The procedures of isolated UC-MSCs was conducted abiding by the Declaration of Helsinki and was approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). All these procedures were performed under aseptic conditions accorded to the previous described[24]. After received the informed consent from the patients, fresh umbilical cords (UCs) were obtained and emerged in phosphate-buffered saline (PBS) at 4°C. After removed remnant blood

using PBS, the UCs were cut into 10 mm³/piece and placed in type I collagenase (Sigma, USA) with 0.1% hyaluronidase containing 3 mM CaCl₂ and digested at 37°C for 4 h. The sample were incubated in low-sugar Dulbecco's modified Eagle's medium (DMEM, 1 g/L, Gibco, Life, Australia) with 10% fetal bovine serum (FBS, PAN-Biotech, Germany) at 37°C with 5% CO₂ in a humidified atmosphere. The medium was rejuvenated every three days to discard nonadherent cells, while the rest of adherent cells were cultured and passaged.

Preparation of HS-pretreated UC-MSCs

The procedure of preparing HS-pretreated UC-MSCs was performed according the previously described [25]. In brief, the 3rd passage of UC-MSCs was collected, and the culture medium was refreshed to remove non-adherent cells. Then, the cells were exposed to HS condition for 1 h in a 42°C water bath and subsequently cultured at 37°C with 5% CO₂ in a humidified atmosphere for 24 h. The control group was that UC-MSCs were incubated under common condition without HS pretreatment (Fig. 2A).

Cytotoxicity assay

CCK-8 assay: UC-MSCs were planked in 96-well plates with a density of 2000 cells/well. A Cell Counting Kit-8 (CCK-8, Kaiji Bio-Technology Co. Ltd., Jiangsu, China) was used after 12, 24 and 48 h treatment accorded to the manufacturer's protocol. The optical density (OD) was measured at 450 nm using an automatic microplate reader (Biotek Vermont, USA). PI/Annexin V: UC-MSCs were stained with Annexin V/propidium iodide to quantify cell apoptosis in each group following to the manufacture's instruction. In brief, UC-MSCs were cultured in 6-well plates, and the cells were treated according to the group design for 24 h and, then, harvested and stained with Annexin V/PI (Kaiji Bio-Technology) for 15 min. The apoptosis rate was detected by flow cytometry. Colony formation assay: UC-MSCs were seeded in 6-well plates, treated according to the group deign and then cultured for another 14 days. Finally, the cells were fixed with paraformaldehyde (4% PFA) for 30 min and stained with crystal violet (0.5%) for 30 min. Images were taken using a light inverted microscope (Leica, Germany).

Multipotential differentiation of UC-MSCs

To detect the potentials of adipogenic and osteogenic differentiation, UC-MSCs were incubated with specific adipogenesis medium and specific osteogenesis medium (Gibco, Life Technologies, NY, USA), respectively. The medium of each specimens was refreshed every three days. The capability of UC-MSCs to differentiate into adipocytes and osteocytes was evaluated by Oil Red O and Alizarin Red S, respectively, after 14 or 21 days incubation.

Flow cytometry analysis

The cell surface antigens of UC-MSCs were detected using flow cytometry analysis. After reached 70%-80% confluence, the cultured cells were trypsinized and respectively incubated with monoclonal antibodies, including PE-Cy7-CD34, PE-CD29, PE-Cy7-CD45, APC-CD44, APC-CD90, PE-CD166, FITC-CD105 and FITC-CD73, for 30 min at 4°C in the dark. The flow cytometry analysis was performed using an eight-color FACS Calibur™ flow cytometer (BD Biosciences, NJ, USA).

We also used flow cytometry analysis to detect the changes of macrophages polarization in vivo and in vitro study. For surface markers staining, the cells, harvested from both bronchoalveolar lavage fluid (BALF) and in vitro experiments, were incubated with primary antibody against APC-CD206 and PE-CD11b at 4°C for 30 min in the dark. The fluorescence of the cells was evaluated using an eight-color FACS Calibur™ flow cytometer.

All data were analyzed using FlowJo 7.6 software (Becton Dickinson, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA extraction and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) were conducted following to the previous report[18]. Total RNA was extracted from UC-MSCs and macrophages using TRIzol (Invitrogen) accorded to the manufacturer's protocol. Biophotometer plus (Eppendorf, Germany) was used to detect the amount and purity of total RNA at 260 nm and 280 nm of the absorbance wavelengths. Then, cDNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Applied Science, USA) and amplified through heat for 10 min at 65°C, incubation at 55°C for 30 min, deactivation at 85°C for 5 min and finally storage at 4°C for 5 min by a PCR Thermal Cycler (Bio-Rad, USA). RT-qPCR was conducted by SYBR Master Mix (Roche Applied Science) using reverse transcription system (LC-480, Roche, USA). β -actin was used as a housekeeping gene. The primer sequences of target genes used in this study are shown in Table 1.

Enzyme-linked immunosorbent assay

The levels of IL-10, PEG2 and TGF- β in the MSC conditioned medium (MSC-CM) and the concentrations of TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18 in supernatant and BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits following to the manufacturer's instructions (EIAAB Science Company, Wuhan, China). An automatic microplate reader (Biotek Vermont, USA) was used to detect the optical density (OD) at 450 nm.

Western blotting assay

Lung tissues and macrophages were lysed by cold radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 10% SDS, 10% sodium deoxycholate, 2 mM EDTA and protease cocktail inhibitor (Roche, Basel, Switzerland). Proteins were equaled, subjected to 12% SDS polyacrylamide gel electrophoresis (PAGE) and subsequently transferred onto polyvinylidene difluoride membranes (PVDF) (Millipore, Billerica, MA, USA). After incubated in 5% non-fat milk for 1 h to block non-specific antigen, the membranes were treated with the primary antibodies, including NLRP3, IL-1 β , pro-Caspase 1 and cleaved Caspase 1, at 4°C overnight. After washed three times with tri-buffered saline tween (TBST), the membranes were respectively incubated with the secondary antibody (1:5000, Sigma-Aldrich) for 1 h at room temperature. Then the membranes were treated with an enhanced chemiluminescence (ECL) substrate, and the blots were detected using a

FluorChem System imager (ProteinSimple, CA, USA). An image analyzer (ImageJ software, USA) was used to analyze the intensities of the blots.

Animals

C57BL/6 mice (B6 mice, male, 8–10 weeks, 22–25 g) were purchased from Biomedical Research Institute of Nanjing University (Jiangsu, China). All mice were housed in the Laboratory Animal Center of South China Agricultural University under specific pathogen free (SPF) conditions and were taken care abiding by the Guideline of South China Agricultural University for Animal Experimentation. Animals were maintained at constant environment, 20°C temperature, 50% humidity and 12 h dark and light cycle and were received free access standard laboratory diet and water.

Model of LPS-induced ALI and BALF collected

All procedures of animal experiments were abided by the guidelines of the Ethics Committee on Animal Welfare, South China Agricultural University. The standard procedures of establishment of a mouse LPS-induced ALI model were performed as previously described [26]. In brief, the mice were anesthetized by intraperitoneally injecting pentobarbital sodium (0.1%, 100 µl/10 g), and, then, were divided into four groups, including PBS + PBS, lipopolysaccharide (LPS) + PBS, LPS + UC-MSCs, LPS + HS-UC-MSCs. The mice model of ALI was developed by dripping intranasally of LPS (5 mg/kg, Sigma, USA). And the sham group was replaced with the same volume of PBS. After 4 h treatment, the mice were intranasally dripped of UC-MSCs ($1 \times 10^7/30 \mu\text{l}$), PBS (30 µl) and HS-UC-MSCs ($1 \times 10^7/30 \mu\text{l}$), respectively, according to the group design mentioned above. The mice in these four groups were euthanized after 24 h of the model establishment.

To collect BALF, an intravenous infusion needle (BD, USA) was used to flush the lung 3 times with 0.5 ml 4°C PBS through the trachea. After 500 g centrifugation for 10 min at 4°C, the supernatant of BALF was removed, and the cells were resuspended by ice-cold PBS. The total number of the cells was counted using a hemocytometer, and the number of neutrophils was counted after Wright-Giemsa staining.

Lung edema evaluation

Lung wet/dry weight ratio was calculated to evaluate the severity of lung edema. In brief, lung tissues were obtained immediately after satisfaction and the value of wet weight was documented. Then, the samples were transferred in a 65°C incubator for 72 h and the value of dry weight was also recorded.

Lung histopathology

4 µm-thick paraffin-embedded lung tissue sections was prepared and stained with hematoxylin and eosin (H&E). The histological score indicating the severity of lung injury was determined according to the previous study, including hyaline membrane formation, alveolar and interstitial inflammation and hemorrhage, necrosis, atelectasis and edema [27]. The slides were evaluated under a light microscope by an observer who did not know the group design. Five selected fields of each section were selected to calculate the injury score.

Fluorescent TUNEL

To investigate cell apoptosis in lung, we performed fluorescent TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay) using a commercial in situ apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA) on 16 μm thick cryosections. Briefly, after washed with equilibration buffer, the cryosections were incubated with the TUNEL reaction mixture, including the enzyme and label solution, for 60 min in the dark at 37°C. Then, the sections were treated with DAPI for 2 min in the dark and mounted using Prolong Diamond Antifade Mounting Agent. The slides were investigated under a fluorescent inverted microscope (Leica, Germany).

Immunohistochemical staining

The 4 μm thick paraffin sections were received dewaxing, rehydrating, repairing by ethylene diamine tetraacetic acid (EDTA, pH 8.0) and treating with primary antibodies at 4°C overnight. Subsequently, the sections were incubated with secondary antibody for 30 min at 37°C (DAKO, USA) after washed three times with PBS. The sections were observed under a light microscope (Leica, Germany) after treated with diaminobenzidine.

Cell co-culture experiments

UC-MSCs or HS- pretreated UC-MSCs were respectively seeded on a Transwell insert (0.4 μm , Corning) at a density of $4 \times 10^5/\text{ml}$. In addition, the macrophage cell line (THP1 cells) were planted in the lower chamber of a 6-well plate at a concentration of $5 \times 10^5/\text{ml}$ and stimulated by PMA (50 ng/ml) for 24 h. Then, the upper chamber of culturing UC-MSCs, HS-UC-MSCs or HS-UC-MSCs + Apoptozole (a HSP70 inhibitor, Selleck, USA) was placed to the 6-well plate to co-culture with THP1 cells containing LPS (1 mg/ml) and IFN- γ (50 U/ml) for 24 h (30446660). After all, the THP1 cells were harvested using EDTA (0.5 mM).

Statistical analysis

The data are presented as mean $\pm$ standard error of the mean (SEM) or as values directly. As appropriate, differences between the groups was compared using unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA). A probability value (p-value) < 0.05 was considered as statistical significance. The statistical analyses were conducting by GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

Results

Culture and identification of UC-MSCs

As shown in Fig. 1A, UC-MSCs were identified as a population of fibroblast-like cells. And their surface positively expressed CD73, CD90, CD29, CD106 and CD44, and negatively expressed CD34, CD45 and

HLA-DR (Fig. 1B). After specific incubation for 14 days or 21 days, respectively, the cells could differentiate into adipocytes or osteocytes by staining with Oil red O or crystal violet solution (Fig. 1C).

The Biological Features And Viability Of Hs Pretreated Ucmscs

As shown in Fig. 2B and 2C, pretreating HS 1 h and subsequently culturing in common condition for 24 h did not affect the surface characterizations and the potentials of multi-differentiation of UC-MSCs. CCK-8 assay was conducted to determine the proliferative ability of UC-MSCs from these two groups according to the OD value. The results of the cell growth curve showed that there was no significant difference of the cell proliferation rate between UC-MSCs culturing in common condition and HS-pretreated UC-MSCs (Fig. 2D). And the results from flow cytometry analysis revealed that the apoptotic rate of HS pretreated UC-MSCs did not statistical difference from that in the control group (Fig. 2E).

Administration Of Hs-pretreated Ucmscs Attenuates Lps-induced Ali

We assessed the severity of lung injury in each group to result whether HS-pretreated UC-MSCs can improve pulmonary performance after LPS administration. A mouse model of ALI was established following to intratracheally injected with LPS. After 24 h, the lung injury was pathologically confirmed which characterized by lung edema and widespread septal thickening as well as filled with neutrophil/mononuclear infiltration in alveolar spaces, while the changes were significantly improved after administration of UC-MSCs, and treatment with HS-pretreated UC-MSCs showed the further recovery of histopathological characterizations (Fig. 3A). The lung injury score which was evaluated from the staining of lung sections with H&E showed the consistent results (Fig. 3B). Meanwhile, the results found that LPS induced an increased lung wet/dry weight ratio of mice, while UC-MSCs could significantly improve the pulmonary edema (Fig. 3C). Interestingly, the better therapeutic effect was observed in the HS-pretreated UC-MSCs, compared with that in the UC-MSCs group (Fig. 3C). Finally, the changes in BALF are important indicator for assessing lung function. The results from the cell smear showed that HS pretreatment could further improve UC-MSCs-caused the decreased BALF inflammatory cell count (Fig. 3D). In addition, a TUNEL assay was also performed to evaluate endothelial apoptosis from each group. As shown in Fig. 3E, the apoptotic index of the lung endothelium obviously increased after exposed to LPS, whereas the increased index was remarkably decreased when treated with UC-MSCs. Moreover, in UC-MSCs with HS pretreatment, the apoptotic value of the lung endothelium was significantly lower than that in the UC-MSCs group. These results exhibited that HS pretreatment play a critical role in strengthening lung-protective roles of UC-MSCs.

HS-pretreated UC-MSCs modulate the functions of alveolar macrophages in vivo and in vitro

Growing evidence demonstrated that the polarization of alveolar macrophages is a critical factor to affect the pathological process of ALI[28]. Thus, we next collected BALF and detected the change of alveolar macrophage phenotype from each group. As shown in Fig. 4A, the results of flow cytometry analysis showed that UC-MSCs reversed the increased expression of a pro-inflammatory marker, TNF- α , in alveolar macrophages (F4/80+) stimulating by LPS, and meanwhile, promoted the anti-inflammatory marker, CD206, expression on these cells. HS pretreatment could further strengthen the immunoregulative effect of UC-MSCs to induce M2 macrophage transformation compared to UC-MSCs cultured in common condition (Fig. 4A). Furthermore, we detected the concentrations of several cytokines to evaluate the secreted function of alveolar macrophages indirectly, and found that the roles of HS-pretreated UC-MSCs in reducing the increased pro-inflammatory cytokines, including TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18, in BALF by LPS stimulation was significant better than that in the UC-MSC group (Fig. 4B).

Moreover, we used the LPS-stimulated THP1 cells as an in vitro model. The level of M1 marker was significantly increased after treated with LPS compared with that in the control group, while co-cultured with non-pretreated or HS-pretreated UC-MSCs significantly reversed this phenomenon (Fig. 4C). Furthermore, we also detected the level of M2 marker in each group. After stimulation, the expression of CD206 was significantly increased in macrophages when co-cultured with non-pretreated UC-MSCs compared to that in the control group, and this change was further obviously upregulated on macrophages after co-cultured with HS-pretreated UC-MSCs (Fig. 4C). To evaluate the secretive function of macrophages from each group, we also measured the concentrations of pro-inflammatory cytokines in co-cultured supernatants using ELISA. As shown in Fig. 4D, LPS promoted macrophages to secrete various cytokines, including TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18, while UC-MSCs could reversed these changes of macrophages. As expect, HS pretreatment could further strengthen these immunoregulative roles of UC-MSCs. These results suggested that HS pretreatment act as an approach to enhance effects of UC-MSCs on modulating polarization and secretive functions of alveolar macrophages.

HS-pretreated UC-MSCs reduce the activation of NLRP3 inflammasome in macrophages

It is accepted that the levels of NLRP3 inflammasome is positively associated with the severity of ALI, and MSCs could significantly reduce NLRP3 inflammasome. Therefore, we further investigated whether HS pretreatment enhanced therapeutic potential of UC-MSCs on NLRP3 inflammasome activation. As shown in Fig. 5A and 5B, the elevation of the protein expressions of NLRP3, ASC, IL-1 β , pro-Caspase 1, cleaved-Caspase 1 and IL-18 in the lung by LPS exposure were remarkably decreased after treatment with UC-MSCs, whereas the expressions of these proteins in the HS pretreating UC-MSCs group were significant lower than that in the UC-MSCs group. And these results were consistent with the decreased concentrations of IL-1 β and IL-18.

In the previous studies, NLRP3 inflammasome is demonstrated to mainly express in the activated macrophages. Thus, we also detected the effects of HS pretreated UC-MSCs on NLRP3 inflammasome activation of macrophage cell line in vitro. The results from Western blot showed that the upregulated NLRP3 activation of macrophages, as evidenced by the increased expressions of NLRP3, IL-1 β , pro-

Caspase 1 and cleaved-Caspase 1, was obviously suppressed by UC-MSCs treatment. And pretreatment with HP strengthened the role of reducing NLRP3 inflammasome activation in macrophages of UC-MSCs. Again, the levels of IL-1 β and IL-18 secreted by macrophages were markedly decreased by both UC-MSCs and HS pretreated UC-MSCs. Together, these results indicate that HS pretreatment enhances the lung protective capacity of UC-MSCs in ALI model via inhibiting the activation of NLRP3 inflammasome in macrophages.

HS pretreatment enhanced the secretive abilities of UC-MSCs via increasing HSP70 expression

We further explore the potential mechanism that how HS-pretreated UC-MSCs improved ALI and reduced the NLRP3 inflammasome activation of macrophages. In the previous studies, both IL-10 and PGE2 could negatively regulate inflammasome activation [29, 30]. Therefore, in this setting, we detected the levels of IL-10, TGF- β and PGE2 in cultured supernatant to evaluate the secreted abilities of UC-MSCs that presented or absent HS. ELISA results shown that compared with the control group, HS led UC-MSCs to synthesize and secrete IL-10 and PGE2 remarkably, but not TGF- β (Fig. 6A). In addition, as the evidence from Fan et al. demonstrated that HSP70 promoted PGE2 production via regulating COX-2 [31, 32], we, herein, targeted the expression of HSP70 in UC-MSCs in each group. As expect, the results from Western blot assay shown that the level of HSP70 was significantly higher in the HS-pretreated UC-MSC group than that in the control group (Fig. 6B). To further confirm this correlation, we added Apoptozole, an antagonist of HSP70, to treat HS-pretreated UC-MSCs, and the results showed that inhibiting HSP70 expression obviously reversed the increased PGE2 secretion of UC-MSCs induced by HS. Meanwhile, we used these cells to co-culture with macrophage and found from the results of ELISA and flow cytometry analysis that administration of Apoptozole significantly blocked the effect of HS on strengthening immunoregulative roles of UC-MSCs to modulate macrophage functions (Fig. 6C and 6D). Finally, we also performed Western blot assay to detect the inflammasome activation of macrophages in each group. The results indicated that Apoptozole might reversed the role of HS-pretreated UC-MSCs in reducing NLRP3 inflammasome activation of macrophages through downregulated PGE2 secretion (Fig. 6E).

Inhibition of HSP70 weakened the protective effect of HS-pretreated UC-MSCs on attenuating LPS-induced ALI

Furthermore, we also evaluated the degree of lung injury in each group to explore whether pretreatment of UC-MSCs with HS attenuate LPS-induced ALI model via regulating HSP70. The results showed that addition of Apoptozole to inhibit HSP70 abolished the protective effect of HS-pretreated UC-MSCs, as evidenced by the reversely aggravating pathological changes in lung architecture (H&E and injury score) and the increased lung wet/dry weight ratio (Fig. 7A, 7B and 7C). In addition, combined application with HS-pretreated UC-MSCs and Apoptozole showed that Apoptozole could reversely increase the reduced BALF inflammatory cell count treating by HS-pretreated UC-MSCs (Fig. 7D). And the results for investigating the TUNEL-positive cells indicated that the role of HS-pretreated UC-MSCs in alleviating alveolar epithelial cells was weakened by utilization of Apoptozole (Fig. 7E). In addition, we also performed ELISA assay to evaluate the inflammatory response, and the results showed that the

significantly decreased pro-inflammatory cytokines, including TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18, by HS-pretreated UC-MSCs in this ALI model were remarkably reversed after union used with Apoptozole (Fig. 7F).

Discussion

Till now, ALI remains one of the most serious respiratory inflammatory syndromes that is recognized to be a major factor of mortality. Current medical therapeutic approaches have not been available to halt the progression of ALI[5]. Therefore, exploring optimal therapeutic strategies has been the subject of urgent investigation. With the capacities of immunomodulation and differentiation into a variety of cell types, MSCs have been considered as a novel and effective therapeutic option for ALI. In the previous study, Lee et al. found that MSCs product various soluble factors, including angiopoietin-1 (Ang-1), interleukin-10 (IL-10), keratinocyte growth factor (KGF) and prostaglandin E2 (PGE2), to improve lung epithelial and endothelial permeability, reduce inflammatory response, enhance alveolar fluid clearance and facilitate endothelial repair [33]. And Khubutiya et al. demonstrated that these beneficial effects of MSCs for tissue repair mainly relied on their paracrine ability rather than differentiation [34]. A large-sample clinical trials relating an evaluation of the effect of MSCs on ALI are underway.

Recently, increasing researches focused on the strategies that how to enhance the therapeutic effectiveness of MSCs so that widely promoted the clinical administration of cellular therapy. Genetic modification is one of the particularly valued approaches due to it available to directly integrate exogenous genes into cells to induce MSCs producing desirable proteins. To solve the problem of low expression of homing receptors of MSCs, Yang et al. genetically overexpressed CXCR4 on MSCs using a lentiviral vector transfection to enhance their homing efficiency and therapeutic roles for ALI [35]. Overexpressing IL-1 receptor -like-1 (ST2) could increase the anti-inflammatory effect of MSCs for ALI via interleukin-33 (IL-33)/ST2 axis [36]. In addition, other studies focused on the culture of MSCs with several specific cytokines or small molecule compounds to enhance their therapeutic capacities. Witte showed that pre-stimulation with inflammatory cytokines could strengthen the immunomodulatory role of UC-MSCs [37, 38]. Our previous study demonstrated that preconditioning of UC-MSCs by rapamycin could increase cell migration and attenuate liver IRI via CXCR4/CXCL12 axis [18]. In this study, we identified heat shock (HS) pretreatment also strengthened the therapeutic potentials of UC-MSCs for ALI. HS is a stress source that facilitates organisms and cells damage. However, recent studies showed that adaptive heat shock pretreatment could improve the viability and anti-apoptosis property of MSCs. Peng found that HS pretreatment effectively induce autophagy to reduce apoptosis rate and enhance hepatoprotective roles of MSCs in liver IRI [21]. And other studies also reported that compared with MSCs, HS-pretreated MSCs were better applied in repairing chemotherapy-induced premature ovarian failure and preventing cisplatin-induced granulosa cell apoptosis [22, 25]. Inconsistent with previous studies mentioned above, we, herein, directly selected the condition of HS at 42°C for 1 h according the Qing et al. reported [22]. And the results of the biological characterizations of MSCs suggested that HS pretreatment did not affect the viability and anti-apoptosis ability of UC-MSCs. Furthermore, we

established a mouse LPS-induced ALI model, and the data indicated that the therapeutic effects of HS-pretreated UC-MSCs in ALI was better than UC-MSCs.

In this setting, we further detected the effect of HS-pretreated UC-MSCs on macrophages, as macrophages are a critical component of immune cells that reside in the lung to mediate adaptive and innate immunity [39, 40]. In addition, macrophages are also identified as a heterogeneous population of immune cells, which are mainly divided into two subgroups accorded to their functions and biomarkers. M1 macrophages are a pro-inflammatory phenotype that secrete various kinds of pro-inflammatory cytokines and chemokines as well as play an important role in antigen presentation, whereas M2 macrophages are immunoregulative cells that secreted IL-10 and TGF- β . During ALI, the activation of macrophages by LPS disrupts the balance of immune status, which is closely associated with a superabundant inflammatory response and subsequently lung injury [9]. Thus, recent studies have demonstrated that affecting macrophage polarization, which inhibited abnormal activated macrophages and promoted regulative phenotype, could be performed as therapeutic approaches for ALI [41, 42]. Through collected BALF and cultured supernatant, our both in vivo and in vitro studies not only demonstrated the effect of UC-MSCs on regulating the phenotypic transformation of macrophages, but also revealed that HS pretreatment strengthened immunosuppressive roles of UC-MSCs that further obviously reduced the proportion of activated macrophages and up-regulated the immunoregulative polarization.

Mechanistically, activating macrophages by LPS up-regulated TLR4 expression, subsequently stimulated its downstream signaling pathway, including NF- κ B, c-jun N-terminal kinase and p38 and finally triggered NLRP3 inflammasome to synthesize various kinds of pro-inflammatory cytokines, IL-1 β and IL-18 [43]. The imbalance of anti-inflammatory and pro-inflammatory responses leads to the facilitation of the progression of ALI. Past research found that melatonin alleviated ALI via inhibiting the NLRP3 inflammasome [44]. Yin et al. performed in vivo and in vitro experiments to demonstrate that isoflurane acted as a therapeutic approach to improve LPS-induced ALI by inhibiting NLRP3 inflammasome activation of alveolar macrophages [45]. MSCs also played an important role in modulating inflammasome activation. In vitro study, Joo et al. found that MSCs inhibited Caspase-1 activation and IL-1 β secretion of activated macrophages by reducing the level of mitochondrial reactive oxygen species (ROS) [46]. And in the murine models of rheumatoid arthritis and Crohn's disease, Won and Serena et al. respectively showed that MSCs could block inflammasome activation to regulate the immune properties of macrophages [47, 48]. In addition, Liu found that MSC-derived exosomes also reduce inflammasome activation in macrophages to alleviate LPS/galactosamine-induced acute liver failure via their inner microRNAs [49]. In the present study, our results from in vivo studies suggested that UC-MSCs could obviously inhibiting NLRP3 inflammasome activation in lung tissues, and the further in vitro studies revealed that MSCs could successfully blocked the expression of NLRP3, IL-1 β , IL-18 and cleaved-Caspase 1 in macrophage cell line. Combinedly, we concluded that MSCs may be utilized as a therapeutic approach to attenuate ALI via inhibiting NLRP3 inflammasome activation. Interesting, we also found at the first time that HS pretreatment not only promoted the roles of MSCs in lung protection, but also enhanced the potential of MSCs to modulate NLRP3 inflammasome of macrophages.

PGE2 is one of endogenous lipid mediator that is synthesized by liberating free fatty acids from membrane phospholipids and converting arachidonic acid to prostaglandin H2 by two different cyclooxygenases (COXs), namely COX-1 and COX-2. PGE2 have various essential effects for the normal physiological functions. Recent evidence revealed that PGE2 also act as an immunosuppressive mediator that contribute to alleviate acute inflammation and facilitate tissue regeneration. The correlation between PGE2 and inflammasome is still controversy. Wu showed that PGE2 could activate inflammasome of macrophages through TLR2 and TLR4, whereas Milena reported that through EP4 receptor and subsequent increase of intracellular cAMP, PGE2 reversely reduced NLRP3 inflammasome activation [50, 51]. In addition, Hong found that MSC-derived PGE2 could alleviate colitis by suppressing inflammasome formation and limiting M1-macrophage population [30]. Therefore, we in this study measuring the levels of cytokines MSCs secreting to detect how HS pretreatment strengthened the therapeutic potentials of MSCs. Our findings suggested that HS pretreatment resulted in the increase production of PGE2 of MSCs that led to a decrease in NLRP3 inflammasome formation and IL-1 β secretion of macrophages. As the previous evidence from Fan et al. showed that HSP70 could upregulate COX-2 expression to promote PGE2 production, we finally detected the expression levels of a family of heat shock proteins, which are substantially upregulated during stress condition, including infection, oxidative stress, inflammation and HS [31, 32]. As expect, HS pretreatment could induce HSP70 expression of MSCs. And our data of pharmacological inhibition with a highly specific HSP70 inhibitor in conjunction with the investigations of the effects of HS-pretreated MSCs-secreted PGE2 on NLRP3 inflammasome activation of macrophages further confirmed the roles of HS pretreatment in strengthening the immunoregulative effects of MSCs.

In conclusion, the present study revealed that HS pretreatment can be used to strengthen the immunoregulative capacity of MSCs and to improve ALI. It is well known that the expressions of heat shock proteins were high induced after HS pretreatment. Our findings of increased HSP70 expression in UC-MSCs indicates that high level of HSP70 enhance UC-MSCs to secrete PGE2 that inhibits NLRP3 inflammasome activation of macrophages. Therefore, HS pretreatment may be a promising strategy to strengthen the therapeutic potential of MSCs n the treatment of ALI in the clinical setting.

Abbreviations

LPS
lipopolysaccharide
ALI
Acute lung injury
MSCs
mesenchymal stem cells
UC-MSCs
umbilical cord-derived mesenchymal stem cells
HS
Heat shock
HSP70

heat shock protein 70
BALF
bronchoalveolar lavage fluid
ARDS
acute respiratory distress syndrome
LPS
lipopolysaccharide
IRI
ischemia/ reperfusion injury
Ang-1
angiopoietin-1
IL-10
interleukin-10
KGF
keratinocyte growth factor
PGE2
prostaglandin E2
PBS
phosphate-buffered saline
PFA
paraformaldehyde
qRT-PCR
quantitative real-time reverse transcriptase-polymerase chain reaction
MSC-CM
MSC conditioned medium
ELISA
enzyme-linked immunosorbent assay
RIPA
radioimmunoprecipitation assay
EDTA
ethylene diamine tetraacetic acid
SEM
standard error of the mean
COXs
cyclooxygenases

Declarations

Ethics approval and consent to participate

The procedures of isolated UC-MSCs was conducted abiding by the Declaration of Helsinki and was approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the grants from the National key research and development program (2017YFA0104304), National Natural Science Foundation of China (81570593, 81670601, 81770648, 81802402, 81870449, 81901943, 81900597, 31701116), Guangdong Natural Science Foundation (2015A030312013, 2018A030310323, 2018A030310275), Sci-tech Research Development Program of Guangdong province (2017A020215023), Medical Scientific Research Foundation of Guangdong Province (A2018130), Sci-tech Research Development Program of Guangzhou city (158100076) and Young Teacher Development Program of Sun Yat-Sen University (17ykpy57, 20ykpy33), the China Postdoctoral Science Foundation (2019M653199), and the Fundamental Research Funds for the Sun Yat-sen Universities (18zxxt08).

Author's Contributions

H. Lv, X. Yuan, and J. Zhang: collection and/or assembly of data, data analysis, and interpretation, manuscript writing. T. Lu, J. Yao, J. Zheng, and J. Cai: provision of study material. J. Xiao, H. Chen, S. Xie and Y. Ruan: collection and/or assembly of data. H. Yi, X. Sui, and Y. An: conception and design, final approval of manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

Characterization of UC-MSCs. (A) The fibroblast-like morphology of cultured UC-MSCs. Scale bar: 50 μm (B) Flow cytometry analysis of the surface markers of UC-MSCs. (C) Oil red O staining of UC-MSCs cultured in specific adipogenesis differentiation medium for 14 days. Scale bar: 50 μm (D) Crystal violet solution staining of UC-MSCs cultured in specific osteogenesis differentiation medium for 21 days. Scale bar: 50 μm .

Figure 2

The optimal condition of HS pretreatment did not affect the viability and biological characterizations of UC-MSCs. (A) The methodological procedure for obtaining HS-pretreated UC-MSCs. (B) Flow cytometry analysis of the surface markers of HS-pretreated UC-MSCs. (C) Representative images of HS-pretreated UC-MSCs, which differentiate into adipogenesis and osteogenesis. Scale bar: 50 μ m (D) UC-MSCs and HS-pretreated UC-MSCs were subjected a CCK-8 assay for 0, 1, 2 days culture to assess cellular viability.

(E) Apoptosis rate of each group were detected using Annexin V/propidium iodide staining. Dara are presented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001 (all p values were obtained by unpaired two-tailed Student's t-test).

Figure 3

HS-pretreated UC-MSCs attenuate LPS-induced ALI. (A) Hematoxylin and eosin staining of lung tissues in each group to assess the amount of lung damage after ALI. Scale bar: 200 μm. (B) The lung injury score

for each group calculated by selecting five fields in each section. Results are presented as the mean $\pm$ SEM (n=5 mice/group). (C) The value of wet/dry ratio of lung tissue for each group were obtained to evaluate lung edema. Results are presented as mean $\pm$ SEM (n=5 mice/group). (D) Total cell count and neutrophil count in the BALF from each group were examined at 24 h after LPS treatment to assess inflammatory response. Results are presented as mean $\pm$ SEM (n=5 mice/group). (E) Representative lung sections of TUNEL staining from each group were collected. Scale bar: 200 μ m. The data are presented as mean $\pm$ SEM (n=5 mice/group). *p<0.05, **p<0.01, ***p<0.001 (all p values were obtained by one-way ANOVA).

Figure 4

HS-pretreated UC-MSCs modulate phenotype transition and secretive function of alveolar macrophages. (A) The surface markers, CD11b and CD206, of alveolar macrophages from BALF were detected by flow cytometry analysis. Data are presented as mean $\pm$ SEM (n=5 mice/group). (B) The levels of TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18 in BALF in each group were measured by ELISA assays. Data are presented as mean $\pm$ SEM (n=5 mice/group). (C) Flow cytometry analyses of TNF- α and CD206 expressions of

macrophages in each group. Data are presented as mean±SEM (n=3/group). (D) The levels of TNF-α, IFN-γ, IL-1β, IL-6 and IL-18 in co-cultured supernatant in each group were measured by ELISA assays. Data are presented as mean±SEM (n=3/group). *, p<0.05, **, p<0.01, ***, p<0.001 (all p values were obtained by one-way ANOVA).

Figure 5

HS-pretreated UC-MSCs reduce the NLRP3 inflammasome activation of alveolar macrophages. (A) Protein levels of NLRP3, ASC, pro-Caspase 1, cleaved-Caspase 1, IL-1β and IL-18 in murine lung samples were detected by Western blot analysis. (B) The results from Western blot analysis were quantified by gray value assay. Data are expressed as the mean±SEM (n=5 mice/group). (C) Protein levels of NLRP3, ASC, pro-Caspase 1, cleaved-Caspase 1, IL-1β and IL-18 in macrophages were detected by Western blot analysis. (B) The results from Western blot analysis were quantified by gray value assay. Data are expressed as the mean±SEM (n=3/group). *p<0.05, **p<0.01, ***p<0.001 (all p values were obtained by one-way ANOVA).

Figure 6

HS pretreatment enhanced the secretive abilities of UC-MSCs via increasing HSP70 expression. (A) The levels of IL-10, TGF- β and PGE2 in UC-MSCs cultured supernatant in each group were measured by ELISA assays. Data are presented as mean $\pm$ SEM (n=3/group). (B) Protein levels of HSP70 in UC-MSCs were detected by Western blot analysis. The results from Western blot analysis were quantified by gray value assay. Data are expressed as mean $\pm$ SEM (n=3/group). (C) Flow cytometry analyses of TNF- α and CD206

expressions of macrophages in each group. Data are presented as mean±SEM (n=3/group). (D) Protein levels of NLRP3, ASC, pro-Caspase 1, cleaved-Caspase 1, IL-1β and IL-18 in macrophages were detected by Western blot analysis. The results from Western blot analysis were quantified by gray value assay. Data are expressed as mean±SEM (n=3/group). *, p<0.05, **, p<0.01, ***, p<0.001 (all p values were obtained by one-way ANOVA).

Figure 7

The protective effect of HS-pretreated UC-MSCs on attenuating LPS-induced ALI via regulating HSP70. (A) Hematoxylin and eosin staining of lung tissues in each group to assess the amount of lung damage after ALI. Scale bar: 200 μ m. (B) The lung injury score for each group calculated by selecting five fields in each section. Results are presented as the mean $\pm$ SEM (n=5 mice/group). (C) The value of wet/dry ratio of lung tissue for each group were obtained to evaluate lung edema. Results are presented as mean $\pm$ SEM (n=5 mice/group). (D) Total cell count and neutrophil count in the BALF from each group were examined at 24 h after LPS treatment to assess inflammatory response. Results are presented as mean $\pm$ SEM (n=5 mice/group). (E) Representative lung sections of TUNEL staining from each group were collected. Scale bar: 200 μ m. The data are presented as mean $\pm$ SEM (n=5 mice/group). (F) The levels of TNF- α , IFN- γ , IL-1 β , IL-6 and IL-18 in BALF in each group were measured by ELISA assays. Data are presented as mean $\pm$ SEM (n=5 mice/group). Data are expressed as mean $\pm$ SEM (n=3/group). *, p<0.05, **, p<0.01, ***, p<0.001 (all p values were obtained by one-way ANOVA).

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