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Human Wharton's jelly mesenchymal stem cells inhibit cytokine storm in acute respiratory distress syndrome in a rat model

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ABSTRACT

Objective: To evaluate the potential effect of human Wharton's jelly mesenchymal stem cells (hWJMSCs) on acute respiratory distress syndrome in lipopolysaccharide (LPS)-induced rats.

Methods: The hWJMSCs (5×10^4/mL, 5×10^5/mL, 5×10^6/mL) were administered to rats on day 1 and day 8 after being induced by LPS (5 mg/kg body weight). TNF-α levels in the lung and IL-18 and IL-1β levels in the serum were measured using ELISA. In addition, *caspase-1* expression in lung tissues was quantified using qRT-PCR, and NF-κB and IL-6 expressions were assessed using immunohistochemistry.

Results: The hWJMSCs decreased TNF-α levels in the lung and plasma IL-18 and IL-1β levels. Moreover, the hWJMSCs downregulated the expressions of *caspase-1*, IL-6, and NF-κB in lung tissues.

Conclusions: The hWJMSCs can decrease inflammatory markers of acute respiratory distress syndrome in a rat model and may be further investigated for the treatment of acute respiratory distress syndrome.

KEYWORDS: Human Wharton's jelly mesenchymal stem cells; Acute respiratory distress syndrome; TNF-α; IL-18; NF-κB; Inflammatory marker

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has elicited the pathogenesis of acute respiratory distress syndrome (ARDS). ARDS is a highly dangerous illness marked by insufficient oxygenation and rigid or non-compliant lungs. ARDS is associated with capillary endothelial injury and widespread alveolar destruction. Patients with ARDS frequently exhibit varying degrees of pulmonary artery vasoconstriction and may proceed to pulmonary hypertension. ARDS has a high mortality rate, and there are few effective treatment options for this deadly illness[1]. The major cause

Significance

Acute respiratory distress syndrome is correlated with capillary endothelial impairment along with extensive alveolar dysfunction. Human Wharton's jelly mesenchymal stem cells (hWJMSCs) have the potential to restore the balance of immunological response at inflammatory sites by interacting with various immune system mechanisms. The present study showed that hWJMSCs had anti-inflammatory activity, and supports further clinical development of hWJMSCs for the treatment of acute respiratory distress syndrome.

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of death from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the development of ARDS. Approximately one-third of hospitalized patients with SARS-CoV-2 fit the criteria for ARDS[2,3]. Cytokine storm or cytokine hyperproduction is crucial for the inflammatory response during coronavirus infections, producing a strong immunological reaction and invasion of certain other normal alveolar cells, resulting in ARDS and multi-organ damage, and causing severe illness and death[4,5]. The virus causes a cytokine storm in the lungs, releasing cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-18, granulocyte colony-stimulating factor, IFN-γ-induced protein 10 (IP-10), and monocyte chemoattractant protein-1 and resulting in edema, air exchange dysfunction, ARDS, acute cardiac injury, and secondary infection, thus causing death[6].

Controlling and inhibiting the cytokine storm is a strategy for preventing COVID-19 progression and saving the patient[7]. Cell therapy using mesenchymal stem cells (MSCs) is a safe and promising treatment for ARDS. MSCs-based treatment in patients with COVID-19 can act as an immunomodulator as well as repair and regenerate lung tissue[8]. MSCs have several advantages, such as being safe, shielding injured lungs and locations, and having preferred immune systems, immunomodulators, and antibacterial and regenerative properties[9].

MSCs have hematopoiesis-supporting and immunomodulatory capabilities. Growth factors, chemokines, adhesion molecules, and Toll-like receptors (TLRs) all play roles in MSCs migration[10]. MSCs produce antimicrobial peptides, which combat infections caused by viruses, bacteria, and fungi[11,12]. The antibacterial action of MSCs was mediated in part by the human cathelicidin antimicrobial peptides, human cathelicidin antimicrobial protein-18 (hCAP-18), and its C-terminal peptide LL-37 (hCAP-18/LL-37). MSCs therapy decreased bacterial load in the lungs while increasing LL-37 alveolar concentrations[9,12,13]. MSCs can prevent the epithelial-mesenchymal transition of alveolar epithelial cells, which is regulated by inhibiting tumor growth factor (TGF) gene transcripts such as zinc finger E-box binding homeobox-1, twist-related protein-1 and connective tissue growth factor and is mediated in part by fibroblast growth factor-7/keratinocyte growth factor (FGF-7/KGF)[14]. Claudin-4, which is involved in tight junction formation, is required for MSCs clearance of alveolar fluid[15]. MSCs can alter immune function. MSCs are both safe and efficacious for individuals with COVID-19-related pneumonia and acute illnesses[16].

Lipopolysaccharide (LPS) is an essential component of Gram-negative bacteria's cell walls. LPS is found in nearly all Gram-negative bacteria and is a potent inflammatory inducer[17,18]. The ARDS rat model was created to imitate the neutrophilic inflammatory response that was observed in patients with ARDS[19]. This research aimed to examine the anti-inflammatory effects of human Wharton's jelly MSCs (hWJMSCs) on inflammatory cytokines in LPS-induced ARDS rats.

2. Materials and methods

2.1. hWJMSC preparation

Experiments were performed on hWJMSCs passage 5 obtained from human umbilical cord of normal delivery women aged 25-40 years who have signed an informed consent form[20]. The isolated hWJMSCs had a fibroblastic spindle-like appearance with plastic adherence. The hWJMSCs showed high expression of CD105, CD90, CD73, and CD44 and low expression of CD45, CD34, CD19, CD11b, and HLADR; the hWJMSCs could differentiate into chondrocyte, osteocyte, and adipocyte[20–22]. The cells with a density of 8×10^3 cells/cm^2 were seeded in Minimum Essential Medium Alpha (MEM-α) (L0475-500; Biowest) supplemented with 10% fetal bovine serum (FBS, S1810-500; Biowest), 1% antibiotic-antimycotic solution (15240062; Gibco), 0.5% gentamicin (15750060; Gibco), 1% amphotericin B (L0009-100; Biowest), and 1% nanomycopulitin (L-X16-100; Biowest). The cells were cultured in a starved condition (FBS-free media) for 24 h after they achieved 80%-90% confluence[22].

2.2. Animal and experimental design

Twenty-five of 6-week-old white male Sprague-Dawley rats (weighing 115-135 g) were provided by iRATco Veterinary Laboratory Services (Bogor, West Java, Indonesia). Individually ventilated cages were utilized to house the rats that were kept in an air-conditioned room with a temperature of 20-24 ℃ and a 12-hour light/dark cycle (lights on 06:00-18:00). The humidity in the atmosphere was kept constant during the procedure[23]. Throughout the experiments, the animals were fed *ad libitum* meals containing 14% protein and 5% fat (PT Indoofeed). The rats were acclimatized for 7 d. Then, LPS dissolved in normal saline was induced once intratracheally at a dose of 5 mg/kg body weight (BW). After 8 h, LPS-induced rats showed positive ARDS (rats discharged in the nostrils, rats sneezed) and rats were treated with hWJMSCs. The rats were divided into five groups according to the number of treatments that were used (Ⅰ: negative control, Aquadest; Ⅱ: positive control, LPS 5 mg/kg BW + Aquadest; Ⅲ: LPS+5×10^4 cells/kg BW in 1 mL FBS; Ⅳ: LPS+5×10^5 cells/kg BW in 1 mL FBS; Ⅴ: LPS+5×10^6 cells/kg BW in 1 mL FBS). Each treatment was repeated for five replications; hWJMSCs were administered twice on the first and eighth days after LPS induction and maintained for 21 d[24]. The BW of rats in all groups was monitored and recorded weekly. After 21 d, rats suffered ARDS. Furthermore, rats were anesthetized with

ketamine (Ikapharmindo Putramas) 100 mg/kg BW and xylazine (361453, Interchemie) 15 mg/kg BW *via* intraperitoneal injection. At the end of the experiment, rats were sacrificed after being deeply anesthetized. The abdominal cavities and chest were opened and the blood was collected and centrifuged at 3 500 rpm for 10 min to obtain serum. Briefly, the lung was removed, a part of the lung was frozen in liquid nitrogen and stored at −80 ℃ until it was used for the ELISA assay, and the remaining part of the lung was fixed in 10% formalin for further experiment[19,23–25].

2.3. Quantification of lung TNF–α and serum IL–18 and IL–1β levels

The chilled lung of rats was extracted and then assessed using ELISA Kit TNF-α (E-EL-R0019; Elabsci) and for serum using IL-1β (E-EL-R0012; Elabsci), and IL-18 (E-EL-R0567; Elabsci), in line with the manufacturer's instructions. Microplate readers were used to measure sample absorbances at 450 nm (Multiskan GO, Thermo Scientific). TNF-α, IL-1β, and IL-18 levels were determined using a standard curve[18,21,22].

2.4. Total protein assay

The stock of bovine serum albumin (BSA, A9576; Sigma Aldrich) in 1 000 µL ddH_2O, 20 µL of standard solutions, samples, and 200 µL of QuickStart Dye Reagent 1× (5000205; Bio-Rad) was each added to the well plate and then incubated for 5 min at room temperature. After incubation, absorbance was measured using a microplate reader at 595 nm[18].

2.5. Immunohistochemical analysis

On day 21 after rats suffered ARDS, animals were sacrificed by anesthesia. Lung samples were fixed in 10% formalin. The samples were embedded in paraffin and cut into 5 µm sections using a microtome (Leica, Leica Biosystem Nussloch GmbH). Then, the lung slices were deparaffinized in xylene and rehydrated in a graded alcohol series. Immunohistochemistry (IHC) staining and antigen retrieval (ab208572; Abcam) were accomplished in citrate buffer with a pH of 6.0 for 10 min at 121 ℃. Endogenous peroxidase was blocked for 15 min at room temperature in 3% H_2O_2 (107209; Merck) and methanol (106009; Merck). The IL-6 Polyclonal Antibody (E-AB-40073; Elabsci), and NF-κB-p65 Polyclonal Antibody (E-AB-32232, Elabsci) were incubated at room temperature overnight. The Rabbit-Specific HRP/DAB (ABC) Detection IHC Kit (ab64261; Abcam) was then used to examine the target proteins (ab64261; Abcam). Hematoxylin was used as a counterstaining agent. The dyed tissues were then examined by a PrimoStar (Zeiss) microscope equipped with a Lumenera Infinity 1-3c camera. The quantification method of scoring used ImageJ software to assess indices of positive cells on IHC slides[23,25,26].

2.6. Caspase–1 gene expression

Total RNA was extracted and purified from the lung of a rat using the Direct-zol RNA MiniPrep Plus Kit (R2073; Zymo) according to the manufacturer's instructions. In a two-step RT-PCR method, the iScript Reverse Transcription Supermix for RT-PCR (170-8841; Bio-Rad) was used to reverse-transcribe RNA into single-stranded complementary DNA. To measure gene expression, the AriaMx 3000 Real-Time PCR System (G8830A; Agilent) was utilized. The primer sequences are shown in Supplementary Table 1. Supplementary Table 2 shows the concentration and purity of RNA in each sample measured at 260/280 nm[21,22].

2.7. Statistical analysis

The SPSS 22.0 program (IBM Corp., Armonk, NY, USA) was used to analyze the statistical data. The experimental results are shown as mean±SD. The data were analyzed using the one-way analysis of variance (ANOVA) and followed by Tukey's HSD *post hoc* test. The level of statistical significance was determined at $P<0.05$.

2.8. Ethical statement

This study was conducted according to the Institutional Ethics Committee of Maranatha Christian University in Bandung, Indonesia (No. 097/KEP/VII/2020)[20].

3. Results

3.1. Effect of hWJMSCs on body weight

In the present study, BW was measured every week after rats were induced by LPS. In the week 1, there was no significant difference among treatments; the data showed that the rats had homogeneous BW and after LPS was induced in the LPS group, the BW was lower compared with the negative control group. Treatment with hWJMSCs at three doses elevated the BW compared with the LPS group (Supplementary Figure 1).

3.2. Effect of hWJMSCs on lung TNF–α and serum IL–18 and IL–1β levels

TNF-α level in the lung of the LPS group was significantly increased compared with the negative control group. The hWJMSCs at three doses reduced TNF-α levels significantly in LPS-induced

rats (P<0.05) (Figure 1).

Treatment with hWJMSCs also resulted in a relatively lower IL-18 level than the ARDS rat model. IL-18 level was reduced more significantly in the serum of the ARDS rat model treated with hWJMSCs at 5×10^5 and 5×10^6 cells/kg BW as shown in Figure 2. Moreover, LPS markedly increased serum IL-1β level in ARDS rats, while hWJMSCs at all doses reversed increased IL-1β level in LPS-induced rats (Figure 3).

3.3. Effect of hWJMSCs on caspase-1 gene expression

The effect of hWJMSCs on *caspase-1* gene expression is shown in Figure 4. According to the results, LPS markedly enhanced *caspase-1* gene expression compared with the negative control, which could be reduced by hWJMSCs treatments at 5×10^5 and 5×10^6 cells/kg BW.

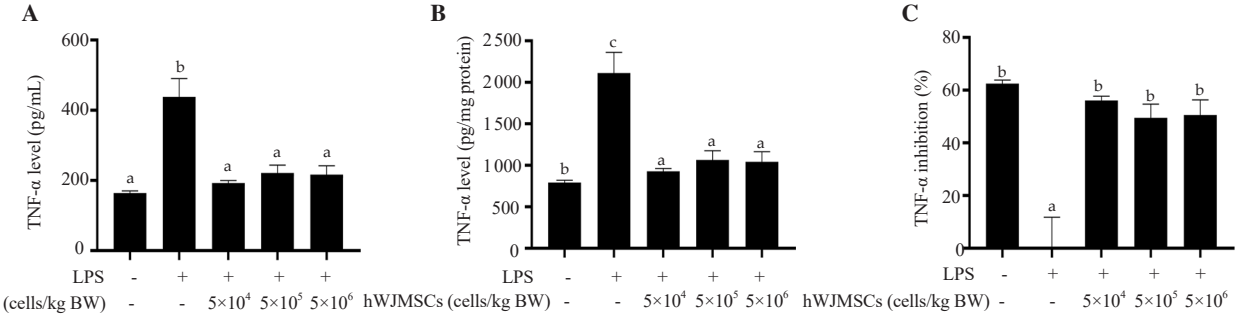

Figure 1. Effect of various doses of hWJMSCs on the level and inhibitory activity of lung TNF-α in the ARDS rat model. (A-B) TNF-α level was determined by ELISA and total protein assay, respectively. (C) The inhibitory rate of TNF-α was calculated. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, P<0.05.

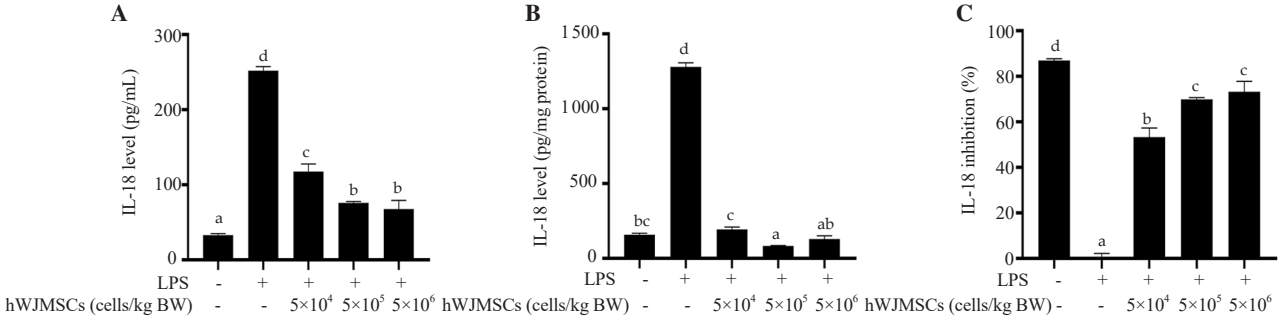

Figure 2. Effect of various doses of hWJMSCs on the level and inhibitory activity of serum IL-18 in the ARDS rat model. (A-B) IL-18 level was determined by ELISA and total protein assay, respectively. (C) The inhibitory rate of IL-18 was calculated. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, P<0.05.

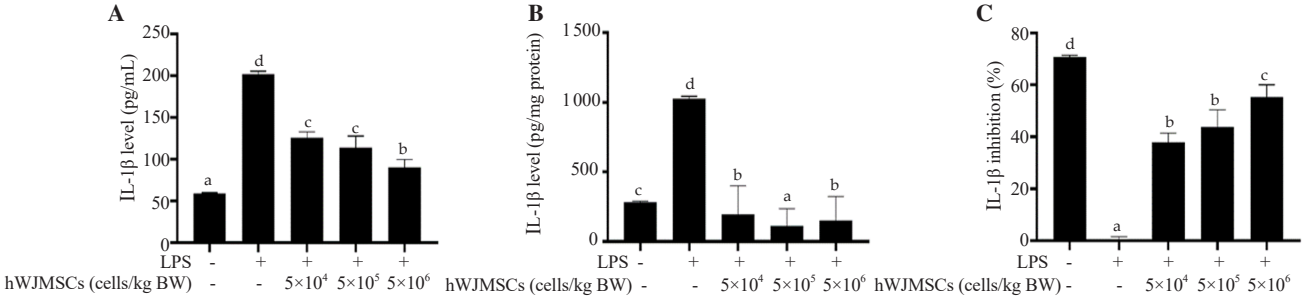

Figure 3. Effect of various doses of hWJMSCs on the level and inhibitory activity of serum IL-1β in the ARDS rat model. (A-B) IL-1β level was determined by ELISA and total protein assay, respectively. (C) The inhibitory rate of IL-1β was calculated. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, P<0.05.

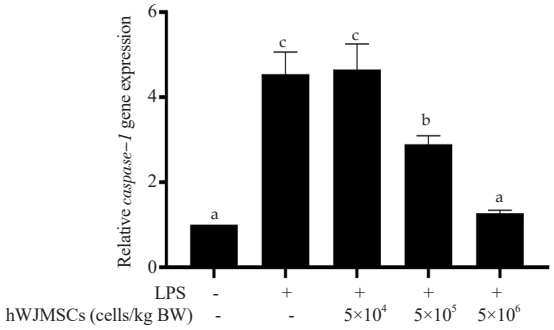

Figure 4. Effect of hWJMSCs treatment on *caspase-1* gene expression in the ARDS rat model. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, $P<0.05$.

3.4. Effect of hWJMSCs on IL-6 and NF-κB expression

IHC was performed to determine the IL-6 expression. The LPS-induced rats showed significantly higher IL-6 expression compared with the negative control ($P<0.05$). The hWJMSCs at all doses markedly decreased IL-6 expression compared with the positive control ($P<0.05$) (Figure 5).

The same trend was also observed in NF-κB expression. LPS could elevate NF-κB expression, which was reduced by hWJMSC treatment at all doses (Figure 6).

4. Discussion

Based on the current results, LPS can induce ARDS by activating inflammatory markers including TNF-α, IL-1β, IL-6, IL-18, and NF-κB.

In previous studies, male Wistar rats were induced by LPS to suffer ARDS[19,24,27,28]. ARDS was detected in all LPS-induced rats, and LPS induction was associated with significant acute lung injury and high mortality[28]. LPS induces uncontrolled cytokine production, leading to hemodynamic instability, cardiovascular failure, and lung damage. The cause of depression is related to inflammatory cytokine imbalance induced by LPS. Sepsis and multiple organ failure are caused by LPS-induced systemic inflammatory response syndrome,

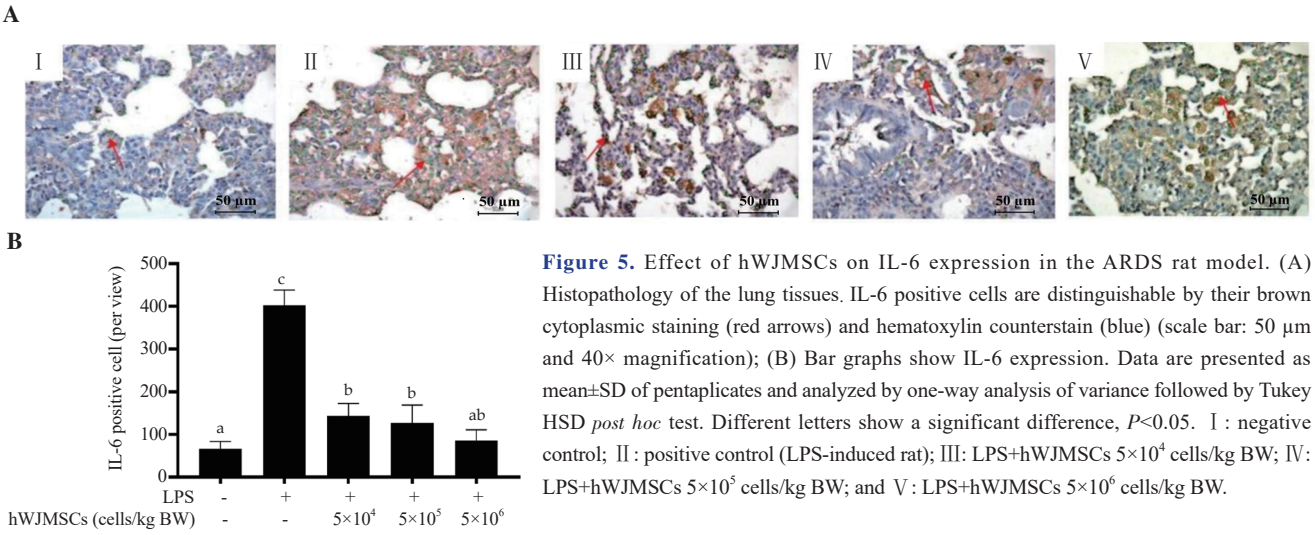

Figure 5. Effect of hWJMSCs on IL-6 expression in the ARDS rat model. (A) Histopathology of the lung tissues. IL-6 positive cells are distinguishable by their brown cytoplasmic staining (red arrows) and hematoxylin counterstain (blue) (scale bar: 50 μm and 40× magnification); (B) Bar graphs show IL-6 expression. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, $P<0.05$. Ⅰ: negative control; Ⅱ: positive control (LPS-induced rat); Ⅲ: LPS+hWJMSCs 5×10^4 cells/kg BW; Ⅳ: LPS+hWJMSCs 5×10^5 cells/kg BW; and Ⅴ: LPS+hWJMSCs 5×10^6 cells/kg BW.

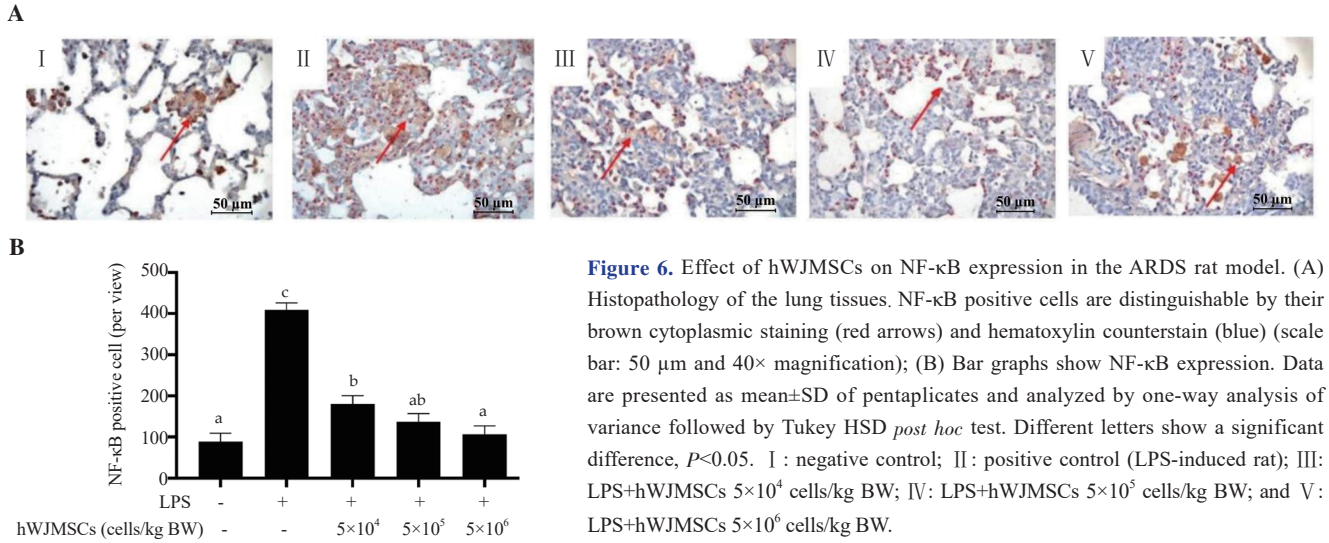

Figure 6. Effect of hWJMSCs on NF-κB expression in the ARDS rat model. (A) Histopathology of the lung tissues. NF-κB positive cells are distinguishable by their brown cytoplasmic staining (red arrows) and hematoxylin counterstain (blue) (scale bar: 50 μm and 40× magnification); (B) Bar graphs show NF-κB expression. Data are presented as mean±SD of pentaplicates and analyzed by one-way analysis of variance followed by Tukey HSD *post hoc* test. Different letters show a significant difference, $P<0.05$. Ⅰ: negative control; Ⅱ: positive control (LPS-induced rat); Ⅲ: LPS+hWJMSCs 5×10^4 cells/kg BW; Ⅳ: LPS+hWJMSCs 5×10^5 cells/kg BW; and Ⅴ: LPS+hWJMSCs 5×10^6 cells/kg BW.

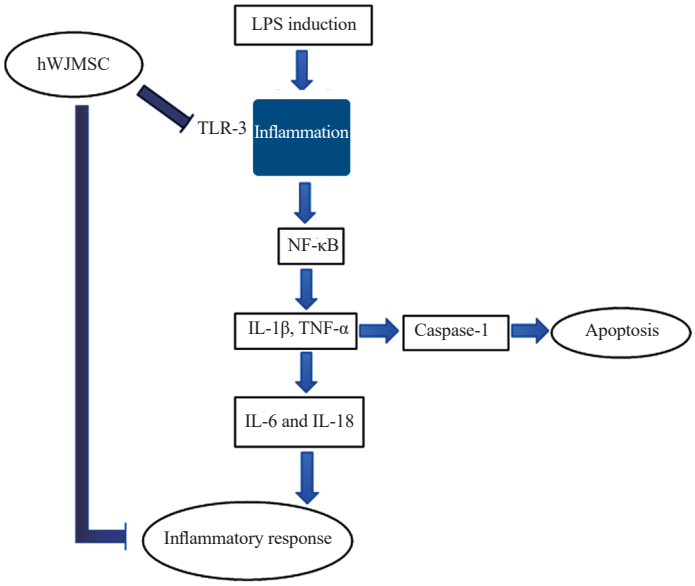

Figure 7. The proposed mechanism of hWJMSCs treatment for acute respiratory distress syndrome. The hWJMSCs have a therapeutic effect by inhibiting the production of NF-κB and proinflammatory cytokines such as TNF-α, IL-18, IL-6, IL-1β and decreasing the expression of *caspase-1*.

which is associated with high morbidity and mortality[29,30]. This result is also supported by previous studies, which found that serum inflammatory cytokines such as IL-6, IL-1β, IL-12, IL-17, IL-18, and TNF-α levels were significantly increased in patients with COVID-19 cytokine storm syndrome[6,29].

The hWJMSCs are more beneficial and convenient to isolate than adult tissue-derived MSCs with a greater expansion and banking capacity, a higher self-renewal and proliferation rate, and immunosuppressive capability, and can be used in both autologous and allogeneic treatments[20,30]. A variety of immune systems interact, and MSCs can restore a balanced immune response in the inflammatory site and the surrounding environment. MSCs can detect inflammation through TLRs on its surface, thereby interacting with the adaptive and innate immune systems. MSCs will release anti-inflammatory signals such as indoleamine 2,3-dioxygenase (IDO) and TGF as tissue homeostasis and anti-inflammatory signals in an inflammatory microenvironment, TNF-α, IL-1β, and TLR3 are inflammation markers that have viral RNA[31,32]. This previous research supported the recent study that determined hWJMSCs' ability to decrease inflammatory cytokines such as IL-18, IL-1β, and TNF-α (Figure 7)[33].

Compared with the positive control, the expression of *caspase-1* gene was reduced in the rat ARDS model after treatment with hWJMSCs. Caspase-1 is a unique caspase because it can activate the pro-inflammatory cytokines IL-1β and IL-18 (Figure 7)[34]. According to an *in vitro* study, the activation of *caspase-1* and the subsequent release of IL-1β in macrophages are reduced by direct or trans-cocultivation with hMSCs[35].

Using IHC assay to evaluate IL-6 and NF-κB expression, it was discovered that hWJMSCs treatment led to decreased IL-6 and NF-κB expression in the ARDS rats. The transcription factor NF-κB, a key regulator of inflammatory response, regulates a variety of innate and adaptive immune activities. NF-κB also promotes the expression of several pro-inflammatory genes, including those that encode cytokines and chemokines, and also helps control inflammasomes[36]. Inhibition of pro-inflammatory mediators, such as NF-κB, requires the discovery of more anti-inflammatory drugs.

The hWJMSCs have anti-inflammatory activity due to their ability to reduce inflammatory cytokines[31,32]. Our results show that hWJMSCs could lower NF-κB expression of lung cells and serum IL-1β levels, which was validated by our previous research that hWJMSCs secreted interleukin 1 receptor antagonist (IL-1ra) from 9 049.03 pg/mg protein to 51 997.12 pg/mg protein[37]. The secretion of IL-1ra inhibits IL-α/β production by producing tumor necrosis factor-stimulated gene-6 (TSG-6), which is followed by suppression of NF-κB signaling and decreased inflammatory cytokine production[34,38-40]. Our previous research also exhibited that hWJMSCs secreted FGF-7 ranging from 313.87 pg/mg protein to 450.94 pg/mg protein[34]. In the present study, hWJMSCs inhibited *caspase-1* expression. The result is in agreement with the previous study in which the secretion of FGF-7, human growth hormone, and angiopoietin-1 by the MSCs could inhibit apoptosis[38,40]. The FGF-7 secretion by hWJMSCs administration on an ARDS *in-vivo* model showed the healing of alveolar epithelial cells and the alveolar fluid clearance[38-40]. MSCs administration increased the alveolar fluid clearance by increasing FGF-7 secretion[40]. Another previous research exhibited that hWJMSCs released IDO (5.86-13.12 ng/mL)[13], IDO acts as a negative feedback loop that can inhibit proinflammatory activation namely IL-12, and IL-18[37,41]. Consistent with the results of previous study, the present study revealed that IL-18 level was reduced by hWJMSCs treatment. The MSCs inhibit inflammation by suppressing the release of

proinflammatory markers such as IL-18, TNF-α, IL-1β, IL-6, and NF-κB and stimulating anti-inflammatory markers such as IDO, IL-4, and IL-10[42].

In conclusion, ARDS is a highly dangerous illness marked by insufficient oxygenation and rigid or non-compliant lungs. Based on the current results, LPS can induce ARDS by activating inflammatory markers including TNF-α, IL-1β, IL-6, IL-18, and NF-κB. The IL-1β and IL-18 levels in serum, as well as TNF-α level in the lung of an ARDS rat model, were inhibited by hWJMSCs. The hWJMSCs can also reduce the expression of *caspase−1*, IL-6, and NF-κB. Therefore, hWJMSCs may be used as a therapeutic agent in inflammatory diseases. But its efficacy needs to be further verifed in clinical trials.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

WW, TLW, FR, and RFG conceived and designed the study and also drafted the manuscript. DP, MEG, EA, and RR participated in the design of the study, carried out the experiment, and collected the data. WW and CRW contributed in analysis and interpretation of data, drafted and revised the manuscript. All authors read and approved the final manuscript.

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