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Original Article

hWJMSCs inhibit inflammation and apoptosis in an ARDS cell model

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الملخص

أهداف البحث: متلازمة الضائقة التنفسية الحادة هي قصور رئوي ناتج عن السوائل ونقص الأكسجة في الدم. أظهرت الخلايا الجذعية الوسيطة القدرة على خفض مستويات الوسيط المؤيد للالتهابات والخلايا الالتهابية. لديهم أنشطة مضادة للالتهابات ومضادة للاستماتة ومضادة للميكروبات، فضلا عن القدرة على تجنب إصابات الرئة. قيم هذا البحث إمكانات هلام وارتون للخلايا الجذعية الوسيطة البشرية لتثبيط الالتهاب وموت الخلايا المبرمج في خلايا رئة الفئران التي يسببها عديد السكاريد الدهني.

طريقة البحث: استخدم علاج هلام وارتون للخلايا الجذعية الوسيطة البشرية في خلايا الرئة الناتجة عن عديد السكاريد الدهني نسبا مختلفة من خلايا هلام وارتون للخلايا الجذعية الوسيطة البشرية و "إل 2"- ، وهي 1:- 1- ، 1:- 5- ، 1:10 ، و 1:25. تم قياس التعبيرات الجينية للإنزيم المحول للأنجيوتنسين -2، ومستقبل المنتجات النهائية المتقدمة للجليكيشن، والعامل النووي كابا ب، و سي-اكس-سي عنصر كيموكين ليجيند - 9 باستخدام النسخ العكسي من تفاعل البوليميراز المتسلسل و تم قياس مستويات البروتين التفاعلي سي و انترلوكين-12 وعامل نخر الورم ألفا باستخدام طريقة تقنية الإليزا.

النتائج: زاد هلام وارتون للخلايا الجذعية الوسيطة البشرية من إنزيم تحويل الأنجيوتنسين -2 وخفضت سي-اكس-سي عنصر كيموكين ليجيند-9، والعامل النووي كابا ب، ومستقبلات المنتجات النهائية المتقدمة للجليكيشن. كما أدى العلاج أيضا إلى تثبيط البروتين التفاعلي سي، و انترلوكين-12 وعامل نخر الورم ألفا وزيادة نسبة الخلايا الحية، ولكنه قلل من نسبة الخلايا الميتة والخلايا المبرمجة في خلايا رئة الفئران الالتهابية كنموذج خلايا متلازمة الضائقة التنفسية الحادة.

الاستنتاجات: خففت الثقافة المشتركة لخلايا هلام وارتون للخلايا الجذعية الوسيطة البشرية و "إل 2" من الالتهاب من خلال زيادة التعبير الجيني للإنزيم المحول للأنجيوتنسين-2 مع تقليل تعبيرات الجين سي-اكس-سي عنصر كيموكين ليجيند-9 والعامل النووي كابا ب ومستقبلات المنتجات النهائية المتقدمة للجليكيشن؛ تقليل مستويات بروتين عامل نخر الورم ألفا والبروتين التفاعلي سي؛ وتقليل النخر والاستماتة المبكرة والمتأخرة بشكل أكثر فاعلية بنسبة 1: 1.

الكلمات المفتاحية: متلازمة الضائقة التنفسية الحادة؛ موت الخلايا المبرمج؛ الالتهاب؛ هلام وارتون للخلايا الجذعية الوسيطة البشرية؛ العامل النووي كابا ب

Abstract

Acute respiratory distress syndrome (ARDS) is a type of lung failure caused by fluids and hypoxemia. Mesenchymal stem cells (MSCs) have been shown to decrease levels of pro-inflammatory mediators and inflammatory cells. These cells have anti-inflammatory, anti-apoptotic, and anti-microbial activity, and protect against lung injury.

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Objective: This research evaluated the potential of human Wharton's jelly MSCs (hWJMSCs) to inhibit inflammation and apoptosis in lipopolysaccharide (LPS)-induced rat lung cells (L2).

Methods: hWJMSC treatment in LPS-induced rat lung cells was performed with 1:1, 1:5, 1:10, or 1:25 ratios of hWJMSCs to L2 cells. The gene expression of angiotensin-converting enzyme-2 (ACE-2), receptor for advanced glycation end products (RAGE), nuclear factor kappa B (NFκB), and C-X-C motif chemokine ligand-9 (CXCL-9) was quantified with RT-PCR, and the levels of C-reactive protein (CRP), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α) were measured with ELISA.

Results: hWJMSCs increased ACE-2 gene expression, and decreased CXCL-9, NFκB, and RAGE gene expression. The treatment also suppressed CRP, TNF-α, and IL-12 levels, and increased the percentage of live cells, but decreased the percentages of necrotic cells and apoptotic cells in inflammatory rat lung cells, which served as an ARDS cell model.

Conclusion: Co-culture of hWJMSCs and L2 cells mitigated inflammation through increasing ACE-2 gene expression, and decreasing CXCL-9, NFκB, and RAGE gene expression; decreasing TNF-α and CRP protein levels; and decreasing necrosis, and early and late apoptosis. A co-culture ratio of 1:1 was most effective.

Keywords: Apoptosis; ARDS; hWJMSCs; Inflammation; NFκB

Introduction

Acute respiratory distress syndrome (ARDS) is a type of lung failure characterized by the presence of fluid and hypoxemia.[1] It is the main symptom of coronavirus disease 2019 (COVID-19), which was recently responsible for a highly fatal pandemic.[2] To date, the World Health Organization has confirmed 763,740,140 global cases and 6,908,554 deaths,[3] including 6,759,513 cases and 161,140 deaths in Indonesia.[4] Edema in the lungs promotes lung inflammation and epithelial cell destruction.[5,6] Cytokines and chemokines are abundantly released in the initial phase of infection. The pathophysiology becomes complicated, owing to inflammatory mediators including tumor necrosis factor-α (TNF-α); interleukin-1β (IL-1β), IL-4, IL-6, IL-8, IL-19, IL-12, and IL-13; and chemokines (C-X-C motif ligand (CXCL-8, CXCL-9, and CXCL-10) and C−C motif ligand (CCL-2, CCL-3, and CCL-5)).[7]

ARDS is also characterized by angiotensin-converting enzyme-2 (ACE-2) downregulation, which results in multiple organ injuries in patients with COVID-19.[8−10] In homeostasis, ACE-2 neutralizes severe effects on the renin-angiotensin system and decreases inflammatory mediators, such as IL-12 and TNF-α.[10] Furthermore, ARDS pathology appears to be consistent with changes in C-reactive protein (CRP) levels. CRP levels in systemic inflammation are frequently used for some diagnoses, although the link between CRP and ARDS has been poorly explained.[11]

One critical mechanism in ARDS pathology is the nuclear factor kappa B (NFκB) signaling pathway.[12,13] NFκB hyperactivity exacerbates ARDS symptoms.[12,13] The activity of NFκB is influenced by the receptor for advanced glycation end products (RAGE).[14]

Mesenchymal stem cells (MSCs) have many benefits in mitigating inflammation through regulating inflammatory mediators.[15,16] In ARDS models, MSCs therapy has been reported to suppress pro-inflammatory cytokines and decrease inflammatory cell numbers. MSCs also have activity against apoptosis, microbial infections, cellular damage, and other lung injuries, as indicated by a growing number of in vivo and pre-clinical investigations.[17] In contrast, studying ARDS requires a model of cytokine storms. Inflamed lung cells are a feasible ARDS model.[18] Therefore, this study evaluated the effects of human Wharton's jelly MSCs (hWJMSCs) in suppressing inflammation in lipopolysaccharide (LPS)-induced rat lung cells, as an ARDS cell model. ACE-2, CXCL-9, NFκB, and RAGE gene expression, as well as CRP, TNF-α, and IL-12 levels, and apoptosis percentages were measured.

Materials and Methods

L2 and hWJMSC cultures

Rat lung cells (L2 cells) (ATCC®CCL-149) and primary cells of characterized hWJMSCs were obtained from the Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. L2 cell culture was performed in Dulbecco's modified Eagle's medium, high glucose (Biowest, L0103-500), whereas hWJMSC culture was performed in minimum essential medium-α (Biowest, L-475-500). Each basal medium was supplemented with 10% fetal bovine serum (Biowest, S181B-500), 1% antibiotic-antimycotic agent (ABAM) (Biowest, L0010-100), 1% amphotericin B (Biowest, L0009-050), 1% minimum essential medium vitamins, 100 × without L-glutamine (Biowest, X0556-100), 1% glutamine, stable, 100×, 200 mM (Biowest, X0551-100), and 0.1% gentamicin (Biowest, L0012-100).[19]

LPS induction and co-culture treatment

L2 cells that had reached 80−90% confluence were harvested and seeded at 1×10^6 cells per T25 flask. After reaching 80% confluence, the cells were treated with 1 μg/mL LPS for 18 h to establish the ARDS cell model[20] and were then co-cultured with hWJMSCs for 24 h at 37 °C in a 5% CO_2 incubator. The treatment was performed with co-culture ratios of 1:1, 1:5, 1:10, and 1:25 hWJMSCs:L2 cells. A transwell plate with 3 μm pores was used with ARDS model cells (LPS-induced L2 cells) in the lower chamber and hWJMSCs in the upper chamber. The cell supernatants were sampled for ELISA, and the pellets were sampled for gene expression analysis.[21]

Quantification of gene expression

The gene expression of ACE-2, NFκB, RAGE, and CXCL-9 was quantified with RT-PCR. RNAs were isolated with a Direct-zol RNA Miniprep Plus Kit (Zymo, R2073), then processed for cDNA synthesis with iScript Reverse Transcription Supermix (Bio-Rad, 170-8841). The obtained cDNA was subsequently mixed with primers (Macrogen), nuclease free water (Zymo, R2073), and SsoFast Evagreen Supermix (Bio-Rad, 172-5200). Reactions were run on an AriaMx RT-PCR system (Agilent). Primer designs, RNA purity and concentration are shown in Tables 1 and 2 respectively.[21–23]

Measurement of TNF-α, IL-12, and CRP levels

TNF-α, IL-12, and CRP were measured with rat TNF-α, rat IL-12, and rat CRP ELISA kits (Elabscience, E-EL-R2856, E-EL-R0064, and E-EL-R0506, respectively). The results were read at 450 nm in a microplate reader (Multiskan Go, Thermo Scientific, 1510-00778C).[24]

Quantification of live and dead cells

Apoptotic, live, and necrotic cells were quantified with flow cytometry. The treated cells were sampled and then washed twice with 500 µL FACS buffer. Cell pellets were prepared with an Annexin V-FITC/PI Apoptosis Detection Kit (Elabscience, E-CK-A211). Subsequently, the cells were measured with MACSQuant Analyser 10 (Miltenyi Biotec).[20]

Statistical analysis

The data were processed in IBM SPSS 20. After a normality test was performed, normal data were analyzed with analysis of variance followed by Tukey's HSD post hoc test, with a significance threshold of $p \leq 0.05$.

Results

ACE-2, CXCL-9, NFκB, and RAGE gene expression

The expression of the ACE-2, CXCL-9, NFκB, and RAGE genes was determined (Figure 1). LPS induction significantly decreased ACE-2 gene expression, and increased CXCL-9, NFκB, and RAGE gene expression. hWJMSC co-cultures with LPS-induced L2 cells showed upregulated ACE-2 gene expression, and downregulated CXCL-9, NFκB, and RAGE

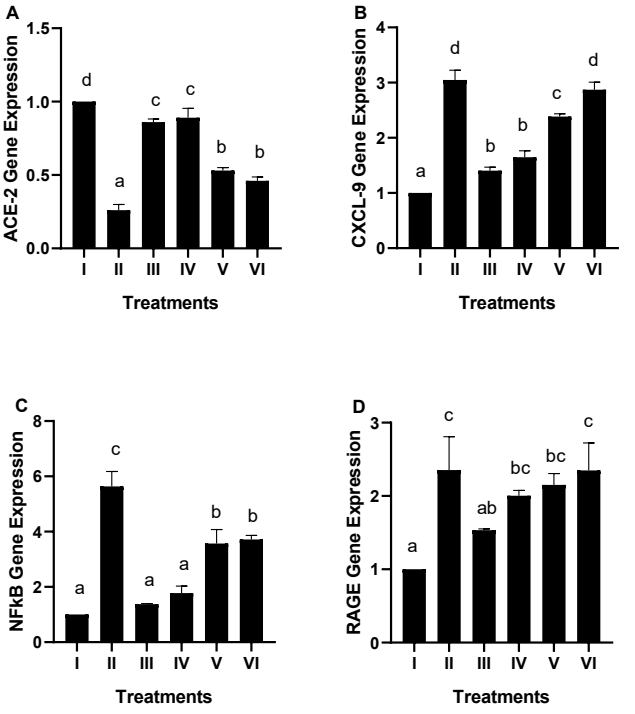

Figure 1: Effects of hWJMSCs treatment on ACE-2, CXCL-9, NFκB, and RAGE gene expression in LPS-induced L2 cells, as an ARDS cell model. Data are shown as averages with standard deviations. Treatment I: negative control; II: positive control (LPS-induced rat lung cells), as an ARDS cell model; III: hWJMSCs with ARDS cell co-culture (1:1); IV: hWJMSCs with ARDS cell co-culture (1:5); V: hWJMSCs with ARDS cell co-culture (1:10); VI: hWJMSCs with ARDS cell co-culture (1:25). Different letters (a, b, c, and d in 1A-B; a, b, and c in 1C; and a, ab, bc, and c in 1D) indicate statistical differences, according to Tukey's HSD post hoc test at $p \leq 0.05$.

Table 1: Annealing of GAPDH: 57.

Gene Symbol	Forward (5′–3′)	Reverse (5′–3′)	Product Size (bp)	Annealing (°C)	Reference
ACE-2	AACAAGCACAGACTACAATCGT	ACGGTTTGATCTCTTTGAAGGT	248	58	NM_001012006.2
RAGE	CGAGTCTACCAGATTCCTG	CTTTGCCATCAGGAATCAG	163	56	NM_053336.2
NFκB	GGACTATGACTTGAATGCGG	ACACCTCAATGTCTTCTTTCTG	230	57	NM_199267.2
CXCL-9	ACTGAAATCATCGCTACACTG	GTGTATTAAAGGGAAGGCGT	278	54	NM_145672.4
GAPDH	TCA AGA TGG TGA AGCAG	ATGTAGGCCATGAGGTCCAC	217		NM_001289726.1

Table 2: RNA purity and concentration.

Group	Treatment	Concentration (ng/mL)	Purity (λ260/λ280 nm)
I	Negative Control (untreated L2 cells, normal L2 cells)	168.04	2.162
II	Positive Control (LPS-induced L2 cells, ARDS cells model)	315.80	2.217
III	WJMSCs 1:1 (hWJMSCs:ARDS cells)	312.00	2.188
IV	WJMSCs 1:5 (hWJMSCs:ARDS cells)	564.72	2.329
V	WJMSCs 1:10 (hWJMSCs:ARDS cells)	370.76	2.177
VI	WJMSCs 1:25 (hWJMSCs:ARDS cells)	435.80	2.163

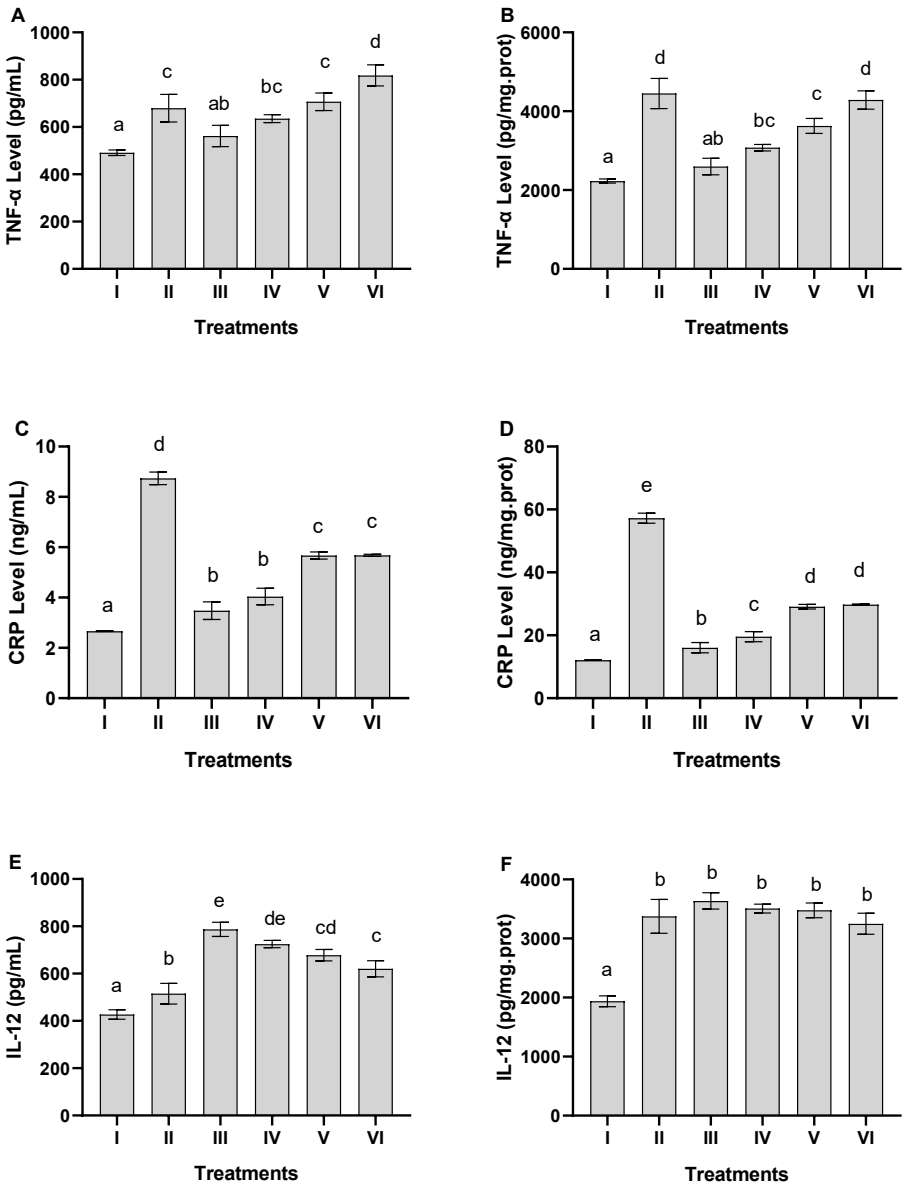

Figure 2: Effects of hWJMSCs on TNF-α, CRP, and IL-12 protein levels in LPS-induced rat lung cells, as an ARDS cell model. Data are shown as averages with standard deviations. Treatment I: negative control, II: positive control (LPS-induced rat lung cells), as an ARDS cell model, III: hWJMSCs with ARDS cell co-culture (1:1), IV: hWJMSCs with ARDS cell co-culture (1:5), V: hWJMSCs with ARDS cell co-culture (1:10), VI: hWJMSCs with ARDS cell co-culture (1:25). Different letters (a, ab, bc, c, and d in A-B; a, b, c, and d in 2C; a, b, c, d, and e in Figure 1D; a, b, c, cd, de, and e in 2E; and a and b in 2F) indicate statistical differences, according to Tukey's HSD post hoc test at $p \leq 0.05$.

gene expression. The highest ACE-2 gene upregulation was induced by hWJMSC-L2 co-culture with L2 cells at a ratio of 1:5, whereas the highest CXCL-9, NFκB, and RAGE gene downregulation was induced at a co-culture ratio of 1:1. In general, hWJMSC and L2 co-culture at a ratio of 1:1 showed the greatest therapeutic effects.

Protein levels

The protein levels of TNF-α, CRP, and IL-12 were determined (Figure 2). LPS induction increased the release of those proteins in L2 cells. hWJMSC-L2 co-culture decreased the levels of these proteins, except IL-12. Co-culture at a ratio of 1:1 resulted in the greatest decreases in TNF-α and CRP protein.

Live, necrotic, and apoptotic cells

Apoptotic cell assays indicated the percentages of live, necrotic, early apoptotic, and late apoptotic cells (Figures 3 and 4). The co-culture of hWJMSCs with LPS-susceptible L2 cells did not influence the percentage of live cells but did influence the percentage of dead cells. The co-culture of hWJMSCs and L2 cells resulted in significantly less necrosis, early apoptosis, and late apoptosis than observed in the positive control. Co-culture at a ratio of 1:5 hWJMSCs to L2

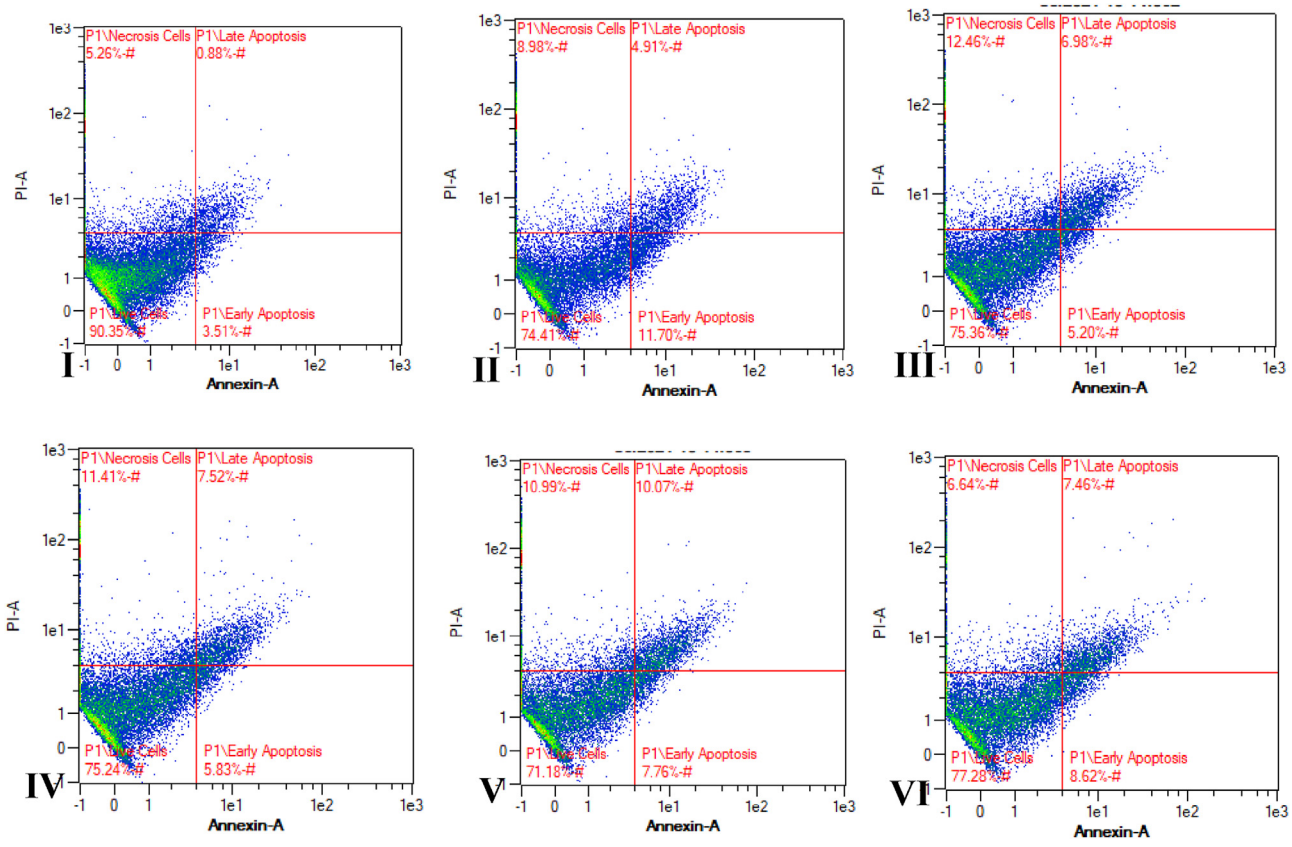

Figure 3: Plots of apoptosis assays, determined by flow cytometry. Treatment I: negative control; II: positive control (LPS-induced rat lung cells), as an ARDS cell model; III: hWJMSCs with ARDS cell co-culture (1:1); IV: hWJMSCs with ARDS cell co-culture (1:5); V: hWJMSCs with ARDS cell co-culture (1:10); VI: hWJMSCs with ARDS cell co-culture (1:25).

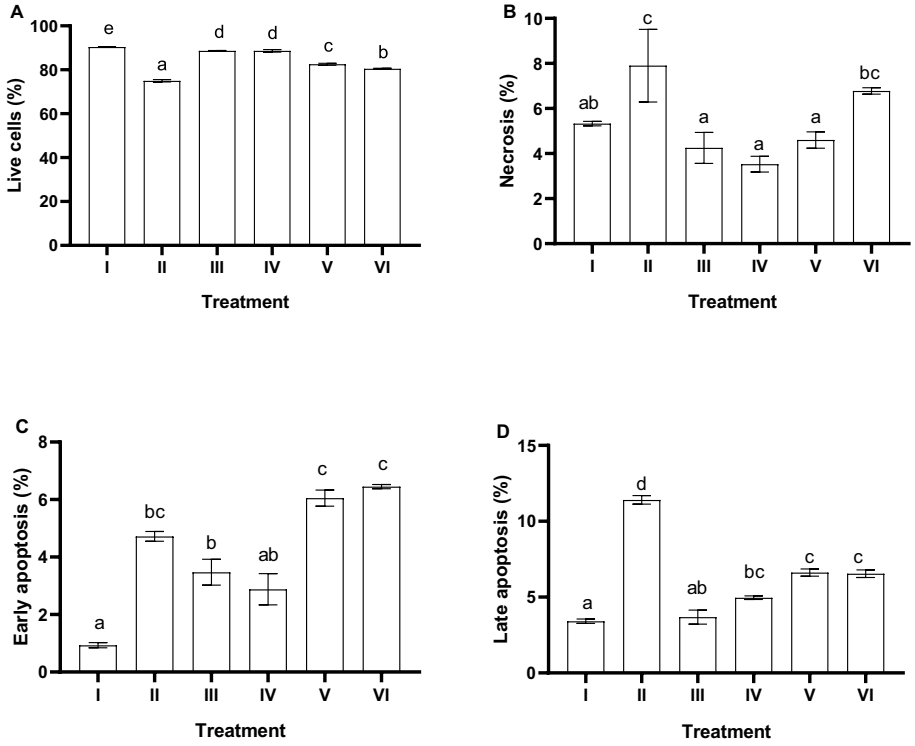

Figure 4: Effects of hWJMSCs on live cells, necrosis, early apoptosis, and late apoptosis in LPS-induced rat lung cells, as an ARDS cell model. Data are shown as averages with standard deviations. Treatment I: negative control; II: positive control (LPS-induced rat lung cells); as an ARDS cell model; III: hWJMSCs with ARDS cell co-culture (1:1); IV: hWJMSCs with ARDS cell co-culture (1:5); V: hWJMSCs with ARDS cell co-culture (1:10); VI: hWJMSCs with ARDS cell co-culture (1:25). Different letters (a, b, c, d, and e in 4A; a, ab, bc, and c in 4B; a, ab, b, bc, and c, in 4C; and a, ab, bc, c and d in 4D) indicate statistical differences, according to Tukey's HSD post hoc test at $p \leq 0.05$.

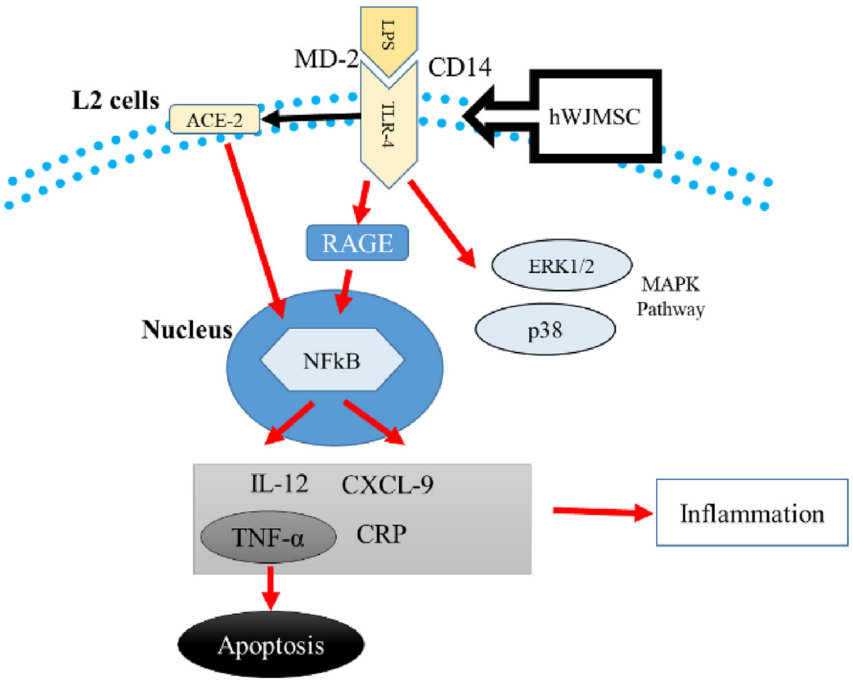

Figure 5: Proposed anti-inflammation and anti-apoptotic pathways of hWJMSCs in lung cell inflammation. LPS: lipopolysaccharide; hWJMSCs: human Wharton's jelly mesenchymal stem cells; TLR-4: Toll-like receptor-4; ACE-2: angiotensin converting enzyme-2; RAGE: receptor for advanced glycation end products; ERK1/2: extracellular signal-regulated kinase; NFκB: nuclear factor kappa B; CXCL-9: C-X-C motif chemokine ligand-9; CRP: C-reactive protein; IL-8: interleukin-8; TNF-α: tumor necrosis factor-alpha. Red arrow: decrease; black arrow: increase. The inflammatory effect of LPS induction is inhibited by hWJMSCs treatment. This inhibition upregulates ACE-2 production, thereby decreasing TNF-α; downregulates RAGE, thereby decreasing NFκB cascade activity; and decreases CRP and CXCL-9 production. The anti-inflammatory effects of hWJMSCs inhibit apoptosis in lung cells.

cells generated the highest decrease in necrosis and early apoptosis, and a ratio of 1:1 generated the highest decrease in late apoptosis among the treatments.

Discussion

This research investigated the effects of hWJMSCs-L2 co-culture on L2 cells that had been exposed to LPS. LPS enters cells via CD14 and Myeloid Differentiation factor-2 (MD-2) binding to Toll-like receptor-4 (TLR)-4. This binding inhibits ACE-2[25,26] and stimulates the activation of mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK1/2), and p38. This activation causes NFκB to stimulate the release pro-inflammatory cytokines, such as TNF-α, thereby resulting in inflammation (Figure 5).[27–29]

In this study, LPS induction successfully induced ARDS in L2 cells, as evidenced by increased NFκB, CXCL-9, and RAGE gene expression, and decreased ACE-2 gene expression (Figure 1), as well as increased TNF-α and CRP protein expression (Figure 2A–B) in all positive controls. These findings were consistent with those from previous research showing that LPS-induced L2 cells express inflammatory genes, such as CXCL-9, IL-12, and CCL-2.[29]

Some studies have confirmed that ACE-2 is a counter-regulatory protein in ARDS.[13] Recent research has suggested that ACE-2 might regulate the release of cytokines, such as TNF-α and IL-12.[9] Evidence has also indicated that ACE-2 functions as an anti-inflammatory protein.[30,31] In a recent study, ACE-2 levels have been found to be lower in LPS-induced ARDS in mice.[32]

hWJMSC therapy has been widely studied in the treatment of lung inflammation due to COVID-19. Immunoregulatory, angiogenic, antiapoptotic, and cell migration factors are all paracrine factors secreted by MSCs. MSCs migration and targeting to injured areas for healing are facilitated by these cytokines. MSCs promote macrophage M2 phenotype differentiation, increase release of anti-inflammatory cytokines, and decrease levels of pro-inflammatory molecules TNF-α, IL-6, and IL-1, thus facilitating healing and protecting tissue against cytokine storms.[33]

Our results indicated that hWJMSCs ameliorated ARDS in lung cells, as indicated by lower expression of the NFκB, RAGE, and CXCL-9 genes (Figure 1) and higher expression of ACE-2 than observed in the positive control. Likewise, the ELISA data indicated that hWJMSCs decreased the levels of CRP and TNF-α, but increased the levels of IL-12 proteins (Figure 2). The most effective hWJMSCs-L2 co-culture ratio was 1:1 (treatment III). hWJMSCs have been shown to alleviate cytokine storms.[33–35] Our previous study has reported that starved hWJMSCs secrete anti-inflammatory proteins, such as Interleukin 1 receptor antagonist (IL-1ra), Fibroblast Growth Factor-7 (FGF-7), and antibacterial protein LL-37.[27] We also reported that hWJMSCs secrete indolamine 2,3 dioxygenase (IDO), an anti-inflammatory protein that regulates TNF-stimulated gene-6 (TSG-6) expression and consequently ameliorates inflammation.[34] However an absence of IL-12 suppression suggested that IL-12 was not the ultimate inflammatory mediator. In our previous study, hWJMSCs were found to decrease lung inflammation

in ARDS rats through decreasing IL-18 and IL-1β, and consequently suppressing the NFκB cascade.[18]

Flow cytometry analysis demonstrated that LPS induction caused cell death (Figure 4), which was associated with an increase in TNF-α levels. The binding of TNF-α to its receptors (e.g., p75, CD120B, or TNFRSF1B) results in recruitment of TNF receptor associated death domain (TNFRADD) and Fas-associated death domain (FADD). The resultant complex then activates caspase-8 (CASP-8), followed by CASP-3, and promotes apoptosis.[36] hWJMSCs treatment decreased cell death. On the basis of our results, treatment III (1:1) elicited the least late apoptosis, whereas treatment IV (hWJMSCs-L2 co-culture at a ratio of 1:5) resulted in more live cells, less necrosis, and less early apoptosis than the other treatments. These data demonstrated hWJMSCs' anti-apoptotic effects in lung cells, owing to less TNF-α release after treatment. Moreover, a previous study has indicated that hWJMSCs treatment in animals lungs upregulates FGF-7, thereby inhibiting apoptosis.[1,18,33]

In summary, this study elucidated how hWJMSCs mitigate ARDS through decreasing inflammation and apoptosis in LPS-induced L2 cells. At a co-culture ratio of 1:1 with L2 cells, hWJMSCs generated the best amelioration of ARDS. Inflammatory mediators, such as CXCL, NFκB, RAGE, CRP, and TNF-α, were downregulated, and apoptosis was inhibited. The proposed pathway of ARDS mitigation by hWJMSCs is shown in Figure 5.

Conclusions

hWJMSCs treatment in LPS-induced lung cells mitigated inflammation by increasing ACE-2 gene expression, while decreasing CXCL-9, NFκB, and RAGE gene expression; decreasing TNF-α and CRP levels; and decreasing necrosis, early, and late apoptosis. A ratio of 1:1 of hWJMSCs to lung cells was the most effective.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The donors of hWJMSCs provided signed informed consent issued by Maranatha Christian University and Immanuel Hospital, Bandung, Indonesia, with certification number 097/KEP/VII/2020 dated on July 25, 2020.

Authors' contribution

WW, TLW, FR, RFG, MEG: Conceptualization, writing/reviewing, and editing. WW, DP, and RR: Supervision, data curation, and validation. WW, AY and AN: Methodology, investigation, and original draft preparation. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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