Potential of Human Wharton's Jelly

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Potential of Human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) Secretome for COVID-19 Adjuvant Therapy Candidate

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***Abstract*— SARS-CoV-2 is a virus that causes a serious life-threatening disease known as COVID-19. Patients with COVID-19 can develop acute respiratory distress syndrome (ARDS) and multiorgan failure requiring safe and effective therapy. The human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) can act as an anti-inflammatory and regenerative properties due to their secretion such as cytokine, growth factor, antimicrobial, and chemokine. A natural way of producing the secreted proteins of a cell (secretome) is by culturing mesenchymal stem cells (MSCs) under starvation or serum deprivation condition. This research was performed to measure the level of Fibroblast Growth Factor-7 (FGF-7), antibacterial protein LL-37 (LL-37), interleukin 1 Receptor Antagonist (IL-1ra), respectively, in the secretome of hWJMSCs. The secretome levels at: non-starvation, 24-, 48-, and 72-hours starvation periods were measured using ELISA method. The hWJMSCs secreted levels of FGF-7, LL-37, and IL-1ra were 46.74 – 72.13 pg/mL, 1.70 – 4.14 ng/mL, 1.35 – 8.32 pg/mL, respectively. The longer the starvation period of cells, the higher the protein levels produced. The hWJMSCs secrete FGF-7, LL-37, IL-1ra, longer starvation periods of cells will increase secretome levels. The hWJMSCs could potentially serve as adjuvant therapy for COVID-19.**

***Keywords*— hWJMSCs, Acute Respiratory Distress Syndrome, cytokine storm, SARS-CoV-2**

I. INTRODUCTION

Coronavirus disease 2019 (COVID-19) began in December 2019 in Wuhan, China and spread quickly and continuously [1], responsible for 116 million cases, with 2.5 million deaths [2,3]. The COVID-19 has caused significant morbidity and death in the respiratory system [4]. The spike glycoprotein (S protein) receptor-binding domain (RBD) is a type I viral fusion protein involved target cell receptors and requires the membrane-linked angiotensin-converting enzyme 2 (ACE2) for cell entrance to mediate the early phases of infection [5][6]. The ACE2 is found in the upper and lower respiratory tracts, but also inside the intestines, the heart, and blood vessel endothelial cells [6-8].

The development of viral pneumonia-induced acute respiratory distress syndrome (ARDS) is the primary cause of death from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [9][10]. Approximately one-third of SARS-CoV-2 patients hospitalized fulfill the criteria for ARDS [3,11]. Cytokine storm or cytokine hyperproduction is important for the inflammatory response during coronavirus infections, provoke a severe immunological reaction and an invasion on some other normal alveolar cells, resulting in ARDS and multi-organ damage and thereby causing severe illness and death [12-14].

The cytokine storm is characterized by the release of large amounts of pro-inflammatory cytokines and chemokines into the bloodstreams of COVID-19 patients, particularly Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Interferon-γ (INF-γ), chemokine (C-X-C motif) ligand-9 (CXCL-9), and (CXCL-10) [6][15][16]. Elevated circulating chemokines and cytokines such as IL-6, IL-1ra, C-C Motif Chemokine Ligand-2 (CCL-2), CCL-8, CXCL-2, CXCL-8, CXCL-9, and CXCL-16 show an increase of

generalized inflammation in COVID-19 patients [6,7]. The levels of IL-2 (IL-2R), IL-1, and IL-6 receptors were shown to be positively associated with disease severity of COVID-19 patients [12,18]. Virus stimulates cytokine storm in the lungs and releases cytokines such as TNF-α, IL-2, IL-6, IL-7, Granulocyte Colony-Stimulating Factor (GSCF), interferon-induced protein (IP10), monocyte chemoattractant protein-1 (MCP-1), followed by the edema, air exchange dysfunction, ARDS, Acute Cardiac Injury (ACI) and secondary infection that may cause death [19].

Controlling and suppressing the cytokine storm in COVID-19 is one of strategy to prevent the progression of COVID-19 and save the patient [12]. Mesenchymal Stem Cells (MSCs)-based cell therapy is a safe and promising treatment for ARDS. In patients with COVID-19, MSCs-based therapy can act as an immunomodulator as well as heal and renew lung tissue [20]. The MSCs are beneficials and safe in protecting the lungs and damaged sites, privileged immune systems, immunomodulators, antimicrobial and regenerative [21]. The MSCs support hematopoiesis and immuno-modulatory properties. The migration of MSCs is mediated by growth factors, chemokines, adhesion molecules, and toll-like receptors [22]. The MSCs secrete low molecular weight proteins in the form of Antimicrobial Peptides (AMPs) to fight infections like viruses, bacteria, and fungus [21,23]. The MSCs' antibacterial activity is partly mediated by human cathelicidin antimicrobial peptide, hCAP-18/LL-37, an AMP. The treatment with MSCs was able to reduce the lung bacterial load while increasing LL-37 alveolar concentrations [21,24].

The MSCs can inhibit the epithelial-mesenchymal transition of alveolar epithelial cells, which is controlled by inhibiting TGF- gene transcripts such as Zinc Finger E-box Binding homeobox-1 (ZEB-1), Twist-Related Protein-1 (TWIST-1), and Connective Tissue Growth Factor (CTGF), and is partially mediated by KGF/FGF-7 [25]. The clearance of alveolar fluid by MSCs is dependent on claudin-4 that is involved in tight junction formation [26]. MSCs can change immune function levels [1][27] and are also safe and effective for patients with COVID-19-related pneumonia or patients with acute diseases [27]. This research was conducted to measure the level of FGF-7, LL-37, IL-1ra in the secretome of hWJMSCs.

II. Materials and Methods

A. hWJMSCs isolation and markers detection

Human Umbilical Cord (UC) were obtained from normal delivery women aged 25 to 40 years who have signed an informed consent form the Institutional Ethics Committee of Maranatha Christian University in Bandung, Indonesia, and Immanuel Hospital Bandung, Bandung, Indonesia [28]. The WJMSCs were isolated from the WJ of the umbilical cord. The hWJMSCs at 80-90% confluence were harvested and analyzed for the surface marker using flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotec). The cells were stained with specific antibodies (CD90 FITC, CD73 APC, CD105 PerCP-Cy5, CD44 PE, negative lineage: CD34/CD45/CD11b/CD19/HLA-DR PE) according to manufacturer's protocol (BD stem flowTM, 562245) [28][29][30][31][32].

Negative lineage: CD34/CD45/CD11b/CD19/HLA-DR PE) according to manufacturer's protocol (BD stem flow™, 562245) [28][29][30][31][32].

B. Preparation of conditioned medium from hWJMSCs

The hWJMSCs passage 5 (P5) were used for experiments. The 8×103 cells/cm2 cells were seeded in Minimum Essential Medium (MEM-α) (Biowest, L0475-500) added with 10% fetal bovine serum (FBS) (Biowest, S1810-500), 1% ABAM, 0.1% Gentamicin (Gibco, 15750060), 1% Amphotericin B (Amp B) (Biowest, L0009-100), 1% Nanomycopulitin (Biowest, LX16-100) incubated in a humidified atmosphere with 5% CO2. After the cells reached 80-90% confluence, the cells were grown in a starving medium (FBS free medium) for 24, 48, and 72 hours. The medium was collected and centrifuged at 3,000 g for 4 minutes at 37 °C, and the supernatant was purified using a 0.22-mm MillexeGV Filter Unit with Durapore (Millipore Corporation, SLGV 033 RS) and used as hWJMSCs secretome [28, 29].

C. FGF-7, LL-37, IL-1RA level of hWJMSCs secretome

The level of FGF-7, LL-37, and IL-1ra in the cell-free supernatant of hWJMSCs was measured using Human FGF-7/KGF (Fibroblast Growth Factor 7) ELISA Kit (Elabsci, E-EL-H0092), Human LL-37 (Antibacterial Protein LL-37) ELISA Kit (Elabsci, E-EL-H2438), and Human IL-1ra/IL-1F3 (Interleukin 1 Receptor Antagonist) ELISA Kit (Elabsci, E-EL-H0089), respectively. Regarding the manual, 50 µL of stop solution was applied to each well, and the absorbance was read at 450 nm microplate reader (Multiskan Go, Thermos Fisher Scientific) [28].

D. Protein Total assay

The standard solution of Bovine Standard Albumin (BSA) was produced from a dilution sequence of BSA stock. In each well plate, 2 mg of BSA (Sigma Aldrich, A9576) was dissolved in 1,000 L ddH2O, followed by 20 L of standard solutions and 200 L Quick Start Dye Reagen 1X (Biorad, 5000205). After 5 minutes of incubation at room temperature, the absorbance at 595 nm was measured using a microplate reader [32]. The protein total of samples was used to convert the level of FGF-7, LL-37, and IL-1ra in unit/mg protein.

E. Statistical Analysis

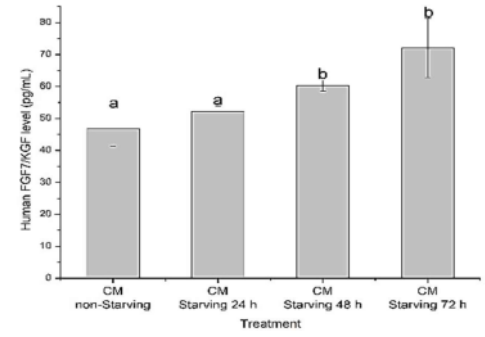
The mean and standard deviation are used to represent all of the data. The data were analyzed using the One Way ANOVA test, which was followed by the Dunnett T3 post hoc test (0.05 was considered significant).

III. Results and Discussion

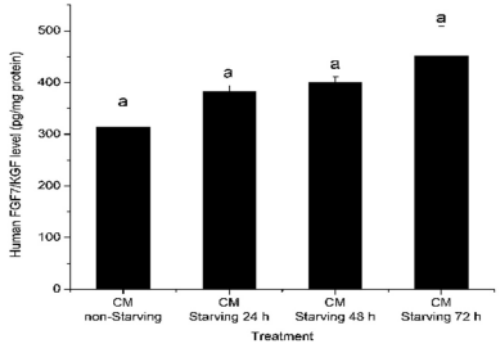
A. FGF-7 level of hWJMSCs-secretome

The human Wharton's Jelly-derived MSCs (hWJMSCs) are more useful and easier to isolate, have expansion and banking capabilities, a higher self-renewal and proliferation rate, immunosuppressive potential than adult tissue-derived MSCs, and can also be utilized in both autologous and allogeneic treatments [29, 30]. The hWJMSCs are more primitive and do not express the major histocompatibility complex (MHC) of class II protein, which contributes to their hypo-immunogenicity in the context of transplantation, than the MSCs which is isolated from other tissue sources [30].

The P5 of hWJMSCs which starving (free FBS) for 24, 48, 72 hours and the secretome (conditioned medium) were harvested and the the FGF-7 level hWJMSCs-secretome was measured. The FGF-7 level of hWJMSCs-secretome is shown in Fig. 1. Fig. 1A shows that FGF-7 levels increase for longer periods of starvation although Fig. 1B does not show significant differences between different starvation periods, but long-term starvation tends to show increases in FGF-7 levels.



(A) FGF-7 level (ng/mL)



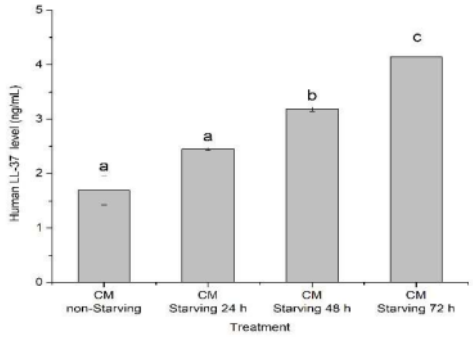
(B) FGF-7 level (ng/mg protein)

Fig. 1. Effect of starving period treatment to FGF-7 level of hWJMSCs-secretomes.
*The data was presented as mean + standard deviation. A different letter (a, b) shows a significant difference between the various starving time for FGF-7 level in pg/mL (Fig. 1A). This data shows an insignificant difference between the various starving times for the level of FGF-7 in pg/mg protein (Fig. 1B) based on the Dunnett T3 post hoc test ($p<0.05$).

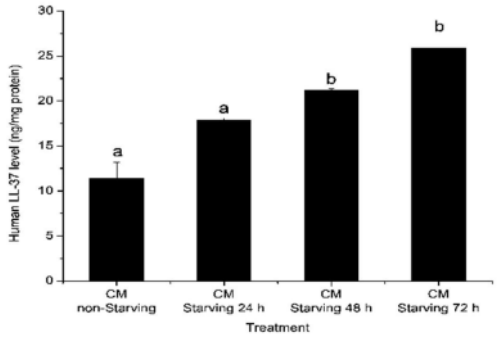
Based on this research finding results, the hWJMSCs was able to secrete FGF-7 at various level of 46.76 pg/mL – 72.13 pg/mL or 313.87 pg/mg protein – 450.94 pg/mg protein (Fig. 1). This finding result was validated with the previous study for which MSCs can inhibit the epithelial-mesenchymal transition of alveolar epithelial cells, which is controlled by suppressing TGF- gene transcripts such as Zinc-Finger E-Box-Binding Homeobox 1 (ZEB-1), Twist-Related Protein-1 (TWIST-1), and Connective Tissue Growth Factor (CTGF), which is mediated in part by FGF-7 [25]. In vivo ARDS model and endotoxin-induced human lung show that MSCs administration increased the alveolar fluid clearance [33, 34]. These effects might be linked to FGF-7 release and extracellular microvesicles. The MSCs' therapeutic impact in ARDS can heal alveolar epithelial cells by increasing secretion of HGF, KGF/FGF-7, PGE-2, ANG-1, IL-IRN, EVs, MMP-8, and clearing the alveolar fluid by increasing secretion of FGF-7, HGF, ANG-1, as well as inhibiting apoptosis by increasing secretion of HGF, KGF, IGF, IL-10, EVs [34].

B. LL-37 level of hWJMSCs-secretomes

The level of LL-37 of hWJMSCs-secretome for 24, 48, 72 starving hours can be seen in Fig. 2. Fig. 2A shows that the level of LL-37 was increased for a longer starving period. The level of LL-37 after 24-hour starvation was not significantly different from non-starving (Fig. 2A, 2B).



(A) LL-37 level (ng/mL)



(B) LL-37 level (ng/mg protein)

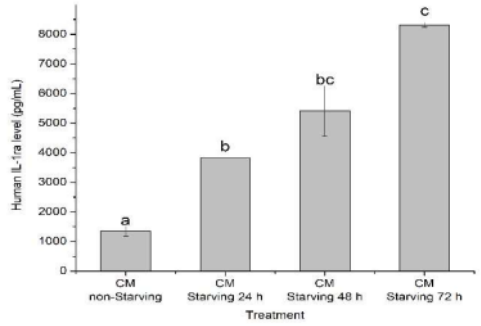
Fig. 2. Effect of starving period treatment to LL-37 level of hWJMSCs-secretomes.
*The data was presented as mean + standard deviation. A different letter (a, b, c) shows a significant difference between the various starving times for LL-37 level in ng/mL (Fig. 2A). A different letter (a, b) shows a significant difference between the various starving times for LL-37 level in ng/mg protein (Fig. 2B) based on the Dunnett T3 post hoc test ($p<0.05$).

The LL-37 secretion of hWJMSCs was 1.70 ng/mL – 4.14 ng/mL or 11.39 – 25.89 ng/mg protein (Fig. 2.). This data was in line with a previous study on the therapeutic effect of MSCs on the activity of bacteria or phagocytosis by secreting LL-37, Lipocalin-2, B-Defensive-2, Hepcidin, KGF [34]. Secret antimicrobial factors in MSCs, such as peptides LL-37 and lipocalin-2 promote macrophage phagocytosis in the lungs and increase bacteria clearance [24]. The MSC-induced bacterial clearance triggered by LL-37 has also been observed in cystic fibrosis animal models [21, 35]. In a mouse model of E. coli-induced ARDS, the

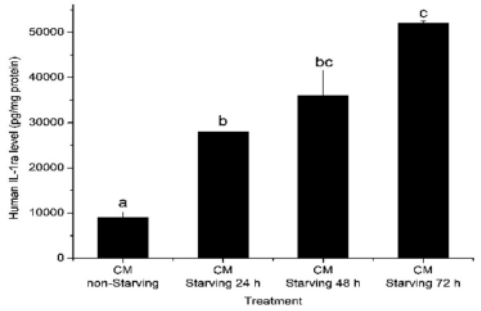
MSC treatment decreased the development of E. coli colony-forming units in bronchoalveolar lavage (BAL) [21, 36, 37].

The IL-1ra secretion of hWJMSCs was 1347.44 pg/mL – 8317.47 pg/mL or 9049.03 pg/mg protein – 51997.12 pg/mg protein. This finding result is supported by a previous study that the release of IL-1ra by MSCs decreases IL-/ activity by producing tumor necrosis factor-stimulated gene-6 (TSG-6), which results in the suppression of NF-B signaling and the decrease of inflammatory cytokine production [38]. The MSCs inhibited IL-1 production in an IL-1ra-dependent way; MSC-derived IL-1ra in the treatment of acute lung damage [39, 40]. Among MSCs-sourced factors, IDO, IL-6, ,TGF-β, NO, IL-10, PGE-2, leukocyte inhibitory factor (LIF), IL-1ra have beneficial MSCs in the attenuation of acute and chronic inflammation [41].

C. IL-1ra level of hWJMSCs-secretome



(A) IL-1ra level (pg/mL);



(B) IL-1ra level (pg/mg protein)

Fig. 3. Effect of starving period treatment to IL-1ra level hWJMSCs-secretomes.
*The data was presented as mean + standard deviation. A different letter (a, b, bc, c) shows a significant difference between various starving time for IL-1RA level in pg/mL (Fig. 3A). A different letter (a,b,bc,c) shows a significant difference between the various starving times for IL-1ra level in pg/mg protein (Fig. 3B) based on the Dunnett T3 post hoc test ($p<0.05$).

The starving hWJMSCs-secretome (conditioned medium) were harvested and the hWJMSCs-secretome was measured for the IL-1ra level. The IL-1ra level of hWJMSCs-secretome is shown in Fig. 3. Based on the obtained results, the level of IL-1ra was increased for a longer starving period (Figure 1A), with the highest level of IL-1ra was about 72 hours of starvation.

Based on Figs. 1-3, longer starvation (24, 48, 72 h) increased FGF-7, LL-37, IL-1ra level, where the longest starvation resulted in the highest levels of FGF-7, LL-37, and IL-1ra, respectively. These findings were consistent with the previous study that Starvation did not cause an evident apoptotic response in immortalized human MSCs (ihMSCs) till 120 hours of starvation [42].

Longer starvation (72 hours) increased factors and triggered the secretion of FGF-7, LL-37, IL-1ra. This finding was consistent with previous studies that cellular starvation is a result of inflammatory tissue injury, that cells starvation activates adaptive responses, and that cells starvation upregulates numerous cytokines and chemokines including IL-6 and IL-8 in response to starvation cells [43]. To increase the factors number released by MSCs can be done by modifying them to over express beneficial genes or pre-treating with a series of preconditioning methods that can enhance their therapeutic benefits. An increase in MSC engraftment and survival in the lung, a reduction in oxidative damage, and enhanced anti-inflammation, anti-apoptosis, and angiogenesis effects all contribute to improved therapeutic benefits [15]. The MSCs cultured under hypoxia release bioactive substances such as HGF, IGF-1, VEGF, and BAL-2, which have pro-angiogenic and anti-apoptotic properties [15][45].

Pre-activation with serum from ARDS patients improves MSC anti-inflammatory ability, as shown by increases in IL-10 and IL-1RN production [15][45]. These preconditioning methods may hold promise for improving MSC potency in the treatment of ARDS [15]. Exosomes produced from serum-deprived MSCs are up to 22-fold more efficient than exosomes derived from control cells at delivering small interfering RNAs (siRNAs) to cells of interest (injury cells) [46]. Cellular stress increases the EVs activity by altering their protein and RNA composition. Serum deprivation may induce cellular stress and is widely used in extracellular vesicle production, to alter EVs level, activity, and composition [46].

IV. Conclusion

The hWJMSCs was able to secrete the FGF-7, LL-37, and IL-1ra, respectively, in both non-starvation and starvation-serum condition. Longer periods of starvation was able to increase FGF-7, LL-37, and IL-1ra levels of secretion in hWJMSCs; 72 hours of starvation was the inflammatory model to induce FGF-7, LL-37, and IL-1ra secretion in hWJMSCs. In conclusion, the hWJMSCs is highly potential to be used as an adjuvant therapy for COVID-19 patients.

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