Wharton's Jelly Mesenchymal Stem Cells-secreted IDO as Candidate of Anti-inflammation Therapy

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Abstract: Local inflammation in lung can induce by viral pneumonia which causes acute respiratory distress syndrome (ARDS). ARDS also caused by COVID-19 SARS-COV-2 infection. hWJMSCs will release anti-inflammatory signals such as indoleamine 2,3 dioxygenase (IDO) as tissue homeostasis between MSCs and resident macrophages as anti-inflammatory signals. This led to the idea of investigating potential of hWJMSCs-Secreted IDO as candidate of anti-inflammation therapy. The hWJMSCs have been isolated from the human umbilical cord using an explant method and characterized using a flow cytometer to detect the cell surface markers CD105, CD73, CD44, CD90, and negative lineage expression of hWJMSCs. The hWJMSCs secretome was characterized by measuring the level of indoleamine 2,3-dioxygenase (IDO) in various starving cells. The isolated umbilical cord hWJMSCs showed positive expression of CD105, CD73, CD44, CD90, and negative lineage expression from 5, 10, and 15 passage. The hWJMSCs IDO secretion level was 5.86 ng/mL for non-starving cells, 6.84 ng/mL for 24 h starving cells, 9.59 ng/mL for 48 h starving cells, and 13.32 ng/mL for 72 h starving cells. The early, medium, and old passage of hWJMSCs have the same characteristics. Longer starvation periods up to regulate the IDO level in hWJMSCs secretome which indicate as anti-inflammation therapy.

1 INTRODUCTION

Inflammation is a critical biological reaction to damage that is linked to a variety of disorder such as acute respiratory distress syndrome (ARDS). ARDS is caused by viral pneumonia as the virus infects the respiratory tract, it induces local inflammation, which results in the production of pro- inflammatory

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cytokines and chemokines. T cells and monocytes from the blood are drawn to the infectious site as a result (Tay et al., 2020; Canham et al., 2020). Excessive release of proteases and reactive oxygen species (ROS) is caused by the uncontrolled invasion of inflammatory cells into the lungs (Abraham & Krasnodembskaya; 2020). Cytokine Storm Syndrome (CSS) or Cytokine Release Syndrome (CRS) is a condition in which the body produces excessive amounts of cytokines (Ye et al., 2020; Azmi et al., 2020). SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of human cells for cell entry (Azmi et al., 2020), Increased serum cytokines such as Interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF)-, peripheral lymphopenia, elevated ferritin level, lactate dehydrogenase (LDH), d-dimer, C-reactive protein (CRP), and coagulation factors are all part of the host's immune response to COVID-19 (Zhou et al., 2020; Chen et al., 2019; Acosta, 2020). Infection with SARS-CoV-2 causes an increase in IL-1 Receptor Antagonist (IL- 1RA), IL-2, IL-6, IL-7, IL-9, IL-10, Interferon Gamma (IFN), Interferon Gamma Inducible Protein-10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP1), Granulocyte-Colony Stimulating Factor (GCSF), Basic Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF), and Inflammatory Protein 1- Alpha (MIP1-α), MIP-1β (Durand et al., 2020, Huang et al 2020; Canham et al., 2020). There are also high levels of chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9 and CXCL10) (Huang et al., 2019; Williams & Chambers, 2014; Cetin & Topcul; 2020), edema in the alveoli, reduced efficiency of gas exchange, ARDS, and acute cardiac injury (ACI), which can result in hypoxemia, secondary infection (Huang et al 2020; Canham et al., 2020), and death (Huang et al., 2020).ARDS caused by COVID-19 is due to respiratory failure (53%), respiratory failure coupled with cardiac failure (33%), myocardial damage and circulatory impairment (7%), or death by unknown cause (Gibson et al., 2020). CSS refers to a group of disorders that result in a violent immune system assault on the host body, such as systemic inflammation, multiorgan failure, and hyperferritinemia, and, if left untreated, death. (Behrens EM, Koretzky, 2017; Cetin & Topcul; 2020).

Because of the COVID-19's serious respiratory effects as a result of CSS, infection prevention, surveillance, and supportive care, such as supplementary oxygen and mechanical ventilation, are now needed in the clinical management of critically ill patients (Baruah & Bose, 2020; Golchin et al., 2020). Since there is currently no specific cure for COVID-19 it is critical to develop new treatment approaches that are more innovative, safe, and promising in the treatment of ARDS. Cell-based therapy, especially stem cell therapy, is currently viewed as a promising therapy for curing incurable diseases (Golchin & Farahany, 2019; Golchin et al., 2020). Adult stem cells derived from mesenchymal stem cells (MSCs) is a promising source for cell therapy and tissue engineering (Widowati et al., 2015). MSCs are a more superior care than the others, and they've gotten a lot of coverage because of: i). source potential, easily accessible, and can be isolated from a variety of tissues including bone marrow (BM), adipose tissues (AT) (such as infrapatellar fat pad, abdominal fat, and buccal fat pad), neonatal birth-associated tissues such as placenta (PL), amniotic fluid (AF), Wharton jelly (WJ), umbilical cord (UC), and cord blood (CB), dental pulp, menstrual blood, buccal fat pad, fetal liver; ii). high proliferation rate; iii). multipotent stem cells with high proliferation rate; iv). simple culture and harvesting procedures; v). easy *ex vivo* expansion to clinical volume; vi). can be processed for repeated therapeutic use; vii). trophic paracrine secretion, producing a large amount of therapeutic growth factor and cytokines; and viii). autologous and allogenic clinical therapy (Golchin et al., 2018; Golchin et al., 2020). MSCs have low major histocompatibility complex (MHC) type 1 expression and no MHC type 2 expression, making them non-immunogenic and suitable for allogeneic therapy (Berglund et al., 2017; Canham et al., 2020).

MSCs have ability to restore the balancing immunological response at inflammation sites and in the surrounding environment by communicating with different immune system components. MSCs have the ability to interact with adaptive immune and innate immune systems by sensing the inflammatory state and detecting the presence of microbes through stimulation of Toll-like receptors (TLRs) on their surface. In the presence of an inflammatory microenvironment, such as high levels of Interferon-γ (INF-γ) and Tumor Necrosis Factor-α (TNF-α) or TLR3 receptor stimulation by viral RNA, MSCs will release anti-inflammatory signals such as indoleamine 2,3 dioxygenase (IDO), Tumor Growth Factor- β (TGF-β), and prostaglandin E-2 (PGE-2), as tissue homeostasis between MSCs and resident macrophages as anti-inflammatory signals that cause the emergence of both regulatory T and dendritic cells (Bernardo & Fibbe, 2013; Glenn & Whartenby, 2014; Canham et al., 2020). When MSCs interact directly with immune cells, they perform paracrine

modulation as a result of the immune response by releasing cytokines such as IDO, IL-10, TGF- β, and IL-1 receptor antagonist (IL- 1RA), and nitric oxide (NO) (van Buul et al., 2012). Several studies have shown that tryptophan catabolism occurs predominantly at areas of tissue inflammation and that IDO expression might well be involved in inhibiting the inflammatory reaction and therefore decreasing tissue injury (Wolf et al, 2004; Nikolaus, et al., 2017). IDO is a rate-limiting enzyme of tryptophan catabolism along the kynurenine (Kyn) pathway. The immunosuppressive mechanism of IDO is mediated by depletion of tryptophan, accumulation of kynurenines (Lee et al., 2016).

Several soluble factors either produced constitutively by MSCs or as a result of cross-talk with target immune cells have been attributed to immunomodulatory property of MSCs, including PGE2, IDO, NO, IL-10, and hepatocyte growth factor (HGF) (Meesuk et al., 2016). MSCs exhibit as anti- inflammatory several factors, including IDO and TNF- stimulated gene 6 (TSG-6). IDO controls the TSG-6 mediated anti-inflammatory therapeutic potent of MSCs (Wang et al., 2018). TSG-6-knockdown (TSG- 6-KD) MSCs have less therapeutic effect on lipopolysaccharide (LPS)-induced Acute Lung Injury (ALI) model mice compared to MSCs control. This data demonstrates that IDO expression by MSCs has capability to alleviates ALI by regulating the TSG-6. Expression (Wang et al., 2018).

The microenvironment of cells, such as food deprivation and low oxygen stress, has an effect on their characteristics (Ferro et al., 2019). Many studies show that starving MSCs *in vitro* to mimic *in vivo* post- transplantation improves MSC survival and therapeutic efficacy (Ferro et al., 2019). Human MSCs are protected from a rapid transition from *in vitro* culture to a harsh environment *in vivo* by using fetal bovine serum (FBS) and glucose deprivation before transplantation (Moya et al., 2015; Ferro et al., 2019). COVID-19 therapy using stem cells, especially WJMSCs, has several advantages, including a high capability for regeneration and differentiation, as well as the ability to rapidly expand (Garzon et al., 2020). WJMSCs are a non-controversial stem cell source (Yang et al., 2012; Bongso et al., 2013). WJMSCs are more useful and straightforward in terms of donor entry, expansion, proliferative ability, and banking; they can also be used in clinical and experimental therapy (Tamura et al., 2011). Between the amniotic epithelium and the umbilical vessels is the WJ, which is embryonic mucous connective tissue. Adult tissue- derived MSCs have a lower proliferation rate and self-renewal capability than WJ derived MSCs or WJMSCs (Marino et al., 2019; Widowati et al., 2014). This study looked at the immunophenotyping of human WJMSCs (hWJMSCs) at different passages, including positive CD105, CD73, CD44, CD90, and negative lineage expression, as well as the IDO secretion of hWJMSCs.

2 MATERIAL AND METHOD

2.1 hWJMSCs Isolation

Human umbilical cords (UC) were collected from normal delivery women aged 25 to 40 who signed an informed consent document that was accepted by the Institutional Ethics Committee of Maranatha Christian University, Bandung, Indonesia, and Immanuel Hospital Bandung, Bandung, Indonesia (Widowati et al., 2017). Phosphate Buffer Saline (PBS) (Biowest, X0515500) was used to wash UC's blood, which was then supplemented with antibiotics and transported to the laboratory using transport medium (Widowati et al., 2019a; Widowati et al., 2019b).

The vessel was extracted from the UC after being transferred and washed in PBS (1x). Wharton jelly tissue explants were dissected into 1-2 mm^3 pieces and plated on 6 well plates in Minimum Essential Medium (MEM- α) (Biowest, L0475-500) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810-500), 1% ABAM, 0.1% Gentamicin (Gibco, 15750060), 1% Amphotericin B (Amp B) (Biowest, L0009-100), and 1% Nanomycopulitin (Biowest, LX16-100). After 3 weeks of incubation at 37ºC in a humidified atmosphere with 5% CO2, adherent cells and tissue fragments were detached using Trypsin-EDTA solution (Biowest, L0931-500) and washed with basal medium. The cells were harvested and re-plated at a density 8 x 10^3 cells/cm^2 when cells reached 80-90% confluence (Widowati et al., 2014; Widowati et al., 2017; Widowati et al., 2019a; Widowati et al., 2019b).

2.2 Markers Detection of hWJMSCs using Fluorescence Activated Cell Sorting

The hWJMSCs surface marker was observed in Passage 5 (P5), 10, and 15 cell cultures. The cells culture that had reached 80-90% confluence were

harvested and analyzed for 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 flow™ kit, 562245). The surface marker of hWJMSCs were conducted in triplicate for each passage (Widowati et al., 2014; Widowati et al., 2019a; Widowati et al., 2019b).

2.3 Preparation of Conditioned Medium From hWJMSCs

The medium was collected and centrifuged at 3000 rpm for 4 minutes at room temperature, and the supernatant was filtered by a 0.22-mm MillexeGV Filter Unit with Durapore (Millipore Corporation, SLGV 033 RS) and used as hWJMSCs secretome (Widowati et al., 2015).

2.4 IDO level of hWJMSCs Secretome

The hWJMSCs P5 cell culture was used for experiments. The cells were seeded $8x10^3$ cells/cm^2 in complete medium. After the cells reached 80-90% confluence, the cells were grown in starving medium (MEM-α no- phenol red, 1% ABAM, 0.1% Gentamicin, 1% Amphotericin B and 1% Nanomycopulitin) for 24, 48 and 72 hours.

The level of IDO in the cell-free supernatant of hWJMSCs was measured using human IDO (Indoleamine-2,3- Dioxygenase) ELISA Kit (Elabsci, E-EL-H2162). 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) (Widowati et al., 2017).

3 RESULTS AND DISCUSSION

3.1 The Effect of Different Passages on hWJMSC Markers

The result of evaluating the effect of different passages (P5, P10, P15) on hWJMSC surface markers. The hWJMSCs were positive for CD90, CD44, CD105, CD73 and negative for CD11b, CD19, CD34, CD45, and HLA-II. The effect of different passages on the surface marker of hWJMSCs are given in Table 1. Positive and

Table 1: Effects of different passages on the percentage of hWJMSCs with positive and negative surface marker lineages.

Passage	CD44 (%)	CD73 (%)	CD90 (%)	CD10 5 (%)	negative-lineage (%)
P5	99.44±0.01	96.97±0.24	99.44±0.03	99.56±0.03	0.23±0.02
P10	98.77±0.26	97.68±0.09	98.79±0.13	98.86±0.27	0.71±0.05
P15	99.75±0.06	99.36±0.20	99.71±0.03	99.78±0.05	0.43±0.04

*Data are expressed as mean ± standard deviation from 3 replications; CD90, CD44, CD105, CD73 are positive lineage- markers; CD14, CD19, CD34, CD45, HLA-II are negative lineage- markers.

negative surface marker expression of hWJMSCs P5, P10, and P15 were not significantly different ($p>0.05$). MSCs should have CD44, CD73, CD90, and CD105 positive lineage markers and CD11b, CD19, CD34, CD45, and HLA-DR negative lineage markers (Figure 1). Propyl etidium (PE), fluorescein isothiocyanate (FITC), and peridinin chlorophyll protein-5 (PerCP-Cy5) staining were used to detect the surface markers of hWJMMSCs. The MSC characteristics were visible in different passages, including early (P5), medium (P10), and old passage (P15) (Table 1).

This finding is also in line with another previous study that human adipose tissue-derived MSCs (hATMSCs) exhibited positive lineage markers (CD44, CD73, CD90, CD105) and negative lineage markers (CD11b, CD19, CD34, CD45, HLA-DR) from passage 4 to 15 (Widowati et al., 2014; Widowati et al., 2019a; Widowati et al., 2019b). The passaging from P4-P15 affects the cells proliferation but not affects the cells morphology and cells characteristic (Widowati et al., 2019b). Our previous research that passage 3 and passage 8 of hWJMSCs isolated by explant and enzymatic method exhibited un-significantly differences between P3 and P8, between explant and enzymatic isolation (Widowati et al., 2019a). Our previous research exhibited that passage P4 and P8 of hWJMSCs cultured in normoxic and hypoxic condition showed hWJMSCs un-significantly differences between P4 and P8, between normoxic and hypoxic condition (Widowati et al., 2014). The flow cytometric analysis showed that oxygen level, isolation method and passage did not affect the MSC's character. The hWJMSCs from P5, P10, P15 showed a very little expression (0.23-0.71%) of negative lineage (CD11b, CD19, CD34, CD45, HLA-DR).

3.2 Effect Starvation on hWJMSCs-secretome IDO Levels

The secretome (conditioned medium) and P5 of hWJMSCs that had been starved (fee FBS) for 24, 48, and 72 hours were harvested, and the IDO levels of the hWJMSCs-secretome was calculated. The IDO level was measured using enzyme-linked immunosorbent assay (ELISA) kit assay. The IDO levels of hWJMSCs- secretome are shown Figure 1.

The results indicate that hWJMSCs secrete IDO at concentrations ranging from 5.86 ng/mL to 13.12 ng/mL or 39.39 ng/mg protein to 82.05 ng/mg (Figure 1). This data was supported with previous study that MSCs release TGF-β, IL-10, IL-1RA, NO, IDO (van Buul et al., 2012). MSCs change inflammation from releasing pro- inflammatory cytokines including IL-1, IL-6, IL-12, IL- 17, MCP-1, MIP-2, CXCL-1, CXCL-2, TNF-α, IFN-γ, proteases like MMP-2, MMP-9 and MMP-12 to an anti- inflammatory status with releasing anti-inflammatory TGF-β, CCL18, IL-4, IL-10, PGE2, IDO, NO, inflammation resolving lipoxin A4 (LXA4) which enable reduce inflammation and improve tissue repair (Zheng et al., 2015; Mao et al., 2015). MSCs control excessive inflammation, improve the microenvironment for tissue repairing LPS-induced ALI model mice but MSCs of IDO knockdown (IDO- KD) didn't increase the inflammation compared to control group, indicating that IDO is important in mediating the inflammation therapeutic role of MSCs (Wang et al., 2018).

IFN-γ, IL-12, and IL-18 are powerful inducers of IDO expression. However, IDO acts as a negative feedback loop that can inhibit pro-inflammatory activation (IFN-γ, IL-12, and IL-18). Thus Wolf et al (2004) hypothesized that IDO has an anti-inflammatory role characterized by Th1 overexpression (Wolf et al., 2004; Nikolaus et al., 2017). This study was supported by previous research, it has shown that indoleamine 2,3-dioxygenase (IDO) plays a critical role in the immunomodulatory ability of human MSCs. This enzyme catalyzes the first and rate-limiting step of tryptophan catabolism along the kynurenine pathway, and IDO and several of its downstream

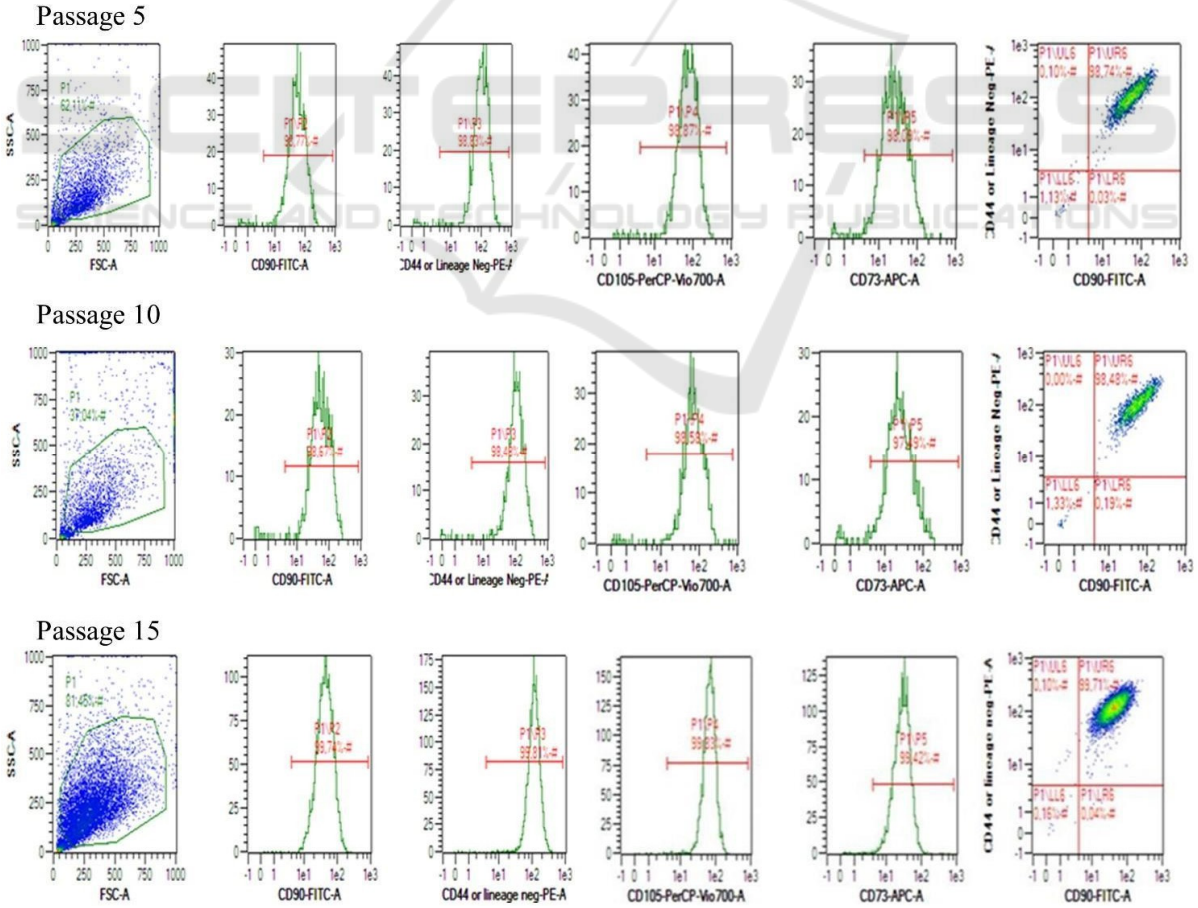

Figure 1: Dot plot of immunophenotype representative hWJ-MSCs from P5, P10, P15.

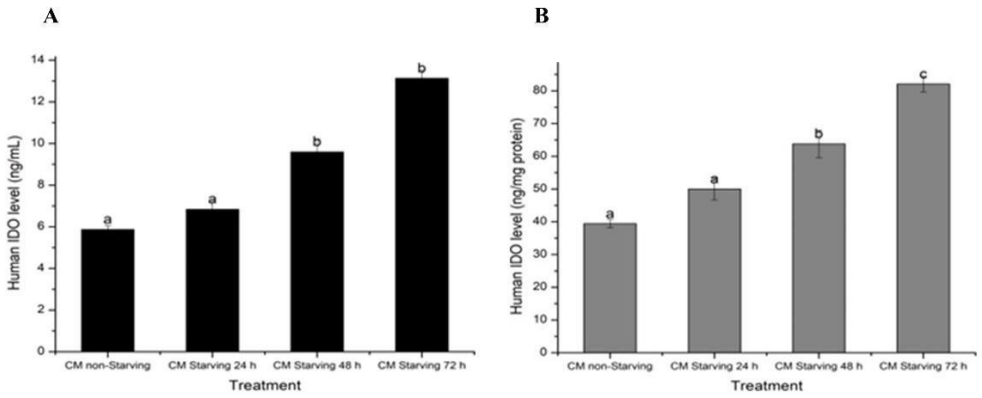

Figure 2: Effect starving time on hWJMSCs IDO levels (A) IDO level (ng/mL); (B) IDO level (ng/mg protein). The data was presented as mean + standard deviation. Different letters (a,b) show a significant different between different starving time for IDO level in ng/mL (Figure A). Different letters (a,b,c) show a significant different between different starving time for IDO level in ng/mg protein (Figure B) based on Dunnett T3 post hoc test ($p<0.05$).

metabolites, such as kynurenine (KYN) and 3-hydroxyanthranilic acid, not only inhibit effector T-cell proliferation but also induce regulatory T-cell differentiation (Treg). Notably, IDO has been found to influence inflammation-associated gene expression, either directly as a signaling factor or indirectly through the production of bioactive intermediates such as kynurenic acid via the kynurenine pathway. MSC has a metabolite of IDO that controls the TSG-6-mediated anti-inflammatory therapeutic effects (Wang et al., 2018). The other study reported that MSCs can improve inflammation and repair tissue from chronic inflammation (Rubtsov et al., 2017).

Figure 1 shows that longer deprivation increased IDO levels, with the longest deprivation resulting in the highest level of IDO. This data was confirmed by a previous study, which found that deprivation did not cause an obvious apoptotic response in immortalized human MSC (ihMSCs) until~120 h of deprivation (Nuschke et al., 2016). In response to starvation stress, cells cause adaptive responses such as angiogenesis, which promote tissue reorganization and repair, as well as up-regulation of multiple cytokines and chemokines, including IL-6 and IL-8 (Püschel et al., 2020).

Starvation for 3 days (see Figure 2) on umbilical cord MSCs (UCMSCs) increase L-Kynurenine (correlated IDO activity) 6 µM compared to untreated UCMSCs 2 µM. IFN-γ, IFN-β, TGF- β increase IDO activity 27 µM, 10 µM, 3 µM (de Witte et al., 2017). IDO level increase in human adipose stem cells (hASCs) in the presence of activated peripheral blood mononuclear cells (PBMCs) (Rubtsov et al., 2017). The amnion-derived MSCs (AM- MSCs) and BM-MSCs induced by Phytohemagglutinin (PHA) and IFN-γ exhibit that the IDO gene expression increase compared negative control (AMMSCs, BMMSCs) (Meesuk et al., 2016).

The AD-MSCs secrete IDO 52.82 IU/mL, IFN-γ induction on AD-MSCs increase IDO level 81.25-94.79 IU/mL, higher IFN-γ increase IDO level (Laksmitawati et al., 2011). hWJMSCs secretes IDO as an anti- inflammatory (Zheng et al., 2015; Mao et al., 2015), indicating that hWJMSCs secretion is a promising therapy candidate for enhancing cytokine storm in COVID-19.

4 CONCLUSIONS

The hWJMSCs have distinct MSCs until passage 15 that differ in a non-significant way in both positive and negative lineage surface markers. The IDO is secreted by hWJMSCs, and longer deprivation increases IDO levels. However, the longer starvation periods up to regulate the IDO level in hWJMSCs secretome are one of method as an alternative Covid- 19 therapy.

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