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by Wahyu Widowati

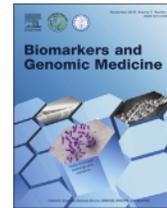
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ORIGINAL ARTICLE

Human platelet lysate enhances the proliferation of Wharton's jelly-derived mesenchymal stem cells



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Abstract This study was performed to elucidate the potential of human platelet lysate (huPL) as an alternative to xeno-free media for culturing mesenchymal stem cells (MSCs). In this work, Wharton's jelly-derived MSCs (WJ-MSCs; $n = 5$) were isolated, characterized, and cultured in huPL derived from type O platelets reconstituted with type AB platelet-poor plasma (huPL-ABO). WJ-MSCs from five donors were cultured under two different conditions [cell culture supplemented with 20% fetal bovine serum (FBS) and 0.250 mg/mL, 0.125 mg/mL, 0.063 mg/mL, and 0.031 mg/mL huPL]. Growth kinetics, cell surface markers, and *in vitro* differentiation potential toward the adipogenic, chondrogenic, and osteogenic lineages were evaluated. Our results indicate that WJ-MSCs cultured in the presence of huPL-ABO showed typical fibroblast-like morphology and had a significantly higher cell count ($p < 0.05$), compared with those cultured in FBS. Furthermore, results of immunophenotyping assay showed similar MSCs characteristics for both culture conditions. In addition, no significant differences were observed in the MSCs differentiation toward the adipogenic, chondrogenic, and osteogenic lineages. The optimal concentrations of huPL-ABO in the culture medium were 0.250 mg/mL and 0.125 mg/mL. Because of insignificant differences between the concentrations in terms of WJ-MSCs population doubling time, the concentration of 0.125 mg/mL was used in all comparisons between FBS and huPL-ABO. The average concentrations of insulin-like growth factor-1, platelet-derived growth factor-AB, vascular endothelial growth factor, and transforming growth factor- β 1 in huPL-ABO were 287.89 pg/mg, 47,096.63 pg/mg,

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150.93 pg/mg, and 74,817.76 pg/mg, respectively. huPL–ABO produced by this method was able to enhance the WJ-MSCs proliferation rate constantly and decrease the required time to reach confluence compared with the FBS culture condition.

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Introduction

Mesenchymal stem cells (MSCs) have been a popular tool for therapeutic application due to their multipotent differentiation capacity and immunomodulatory characteristics.¹ Although the bone marrow is an extensively studied source of MSCs, the amount of bone-marrow-derived MSCs is very limited and involves an invasive harvesting procedure.² Human umbilical cord contains an extra-embryonic membrane with a rich source of MSCs called “Wharton’s jelly.” Wharton’s jelly-derived MSCs (WJ-MSCs) are believed to be more primitive than MSCs derived from other tissue sources.³ WJ-MSCs have the unique properties of high proliferation rates, wide multipotency, and hypo-immunogenicity, as well as anticancer properties. Moreover, WJ-MSCs expressed low levels of pluripotent embryonic stem cell markers including POUF1, NANOG, SOX2, and LIN28, which explains why they do not induce teratomas.^{4–8} Most isolation and expansion protocols for clinical-scale production of MSCs use culture media supplemented with fetal bovine serum (FBS); however, MSCs cultured in FBS might pose an infection risk, which can cause immunological reactions in the host.⁹ Thus, there is a significant safety concern when administering MSCs cultured in FBS. It has also been reported that patients who have received cell transplantation with MSCs expanded in FBS exhibit antibodies against bovine antigens.¹⁰ Furthermore, different batches of FBS give variable efficiencies of MSCs expansion *in vitro*.¹¹

Human platelet lysate (huPL) is another culture medium supplement, which can be a potential substitute for FBS for culturing MSCs.¹² huPL contains various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), and epidermal growth factor that promote migration and proliferation of MSCs.^{13–18} Although several studies were carried out on huPL, conflicting results were obtained. For example, in one study an increase in proliferation rate with huPL was reported, whereas in some others huPL inhibited cell proliferation.¹³ huPL was reported to induce cell differentiation in some studies, whereas some studies reported that it did not exhibit any cell differentiation properties.¹⁴ These differences could be the result of different preparation methods of huPL between studies and variability of growth factors concentration between donors. Many extensive studies have been carried out in this regard, but creating a standardized huPL protocol for culturing MSCs remains a challenging task.^{19–21} Therefore, it is very important to establish a method to produce huPL with a reproducible concentration of growth factors and reliable effectiveness to expand MSCs *in vitro* for clinical application. It has been reported that a mixture

of type O platelets and type AB plasma (huPL–ABO) does not induce possible influences of ABH antigens and isoagglutinins.²²

In this study, we established a standardized method for producing huPL–ABO for WJ-MSCs expansion that is completely xeno free and comparable with culturing WJ-MSCs in FBS in terms of cell proliferation, phenotype characterization, and differentiation capacities. We also measured the concentration of growth factors in huPL from three batches.

Material and methods

Isolation, activation, and quantification of platelets

Seven samples of type O blood and two samples of type AB blood were collected in a 250-mL blood bag with citrate phosphate dextrose as anticoagulant (Terumo, Terumo Medical Corporation, Shibuya, Japan). The freshly collected blood samples were centrifuged (1000 g, 5 min) to separate red blood cells from the rest of blood components. The upper fraction was collected and centrifuged to separate platelets from plasma. The collected type O platelets were then reconstituted with type AB platelet-poor plasma. Platelet-rich plasma (PRP) was activated through repeated freeze–thaw cycles, and the platelet lysate produced by these methods was centrifuged to separate it from other debris. The supernatant was collected for the quantification of protein using NanoDrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA) and for the quantification of insulin-like growth factor-1 (IGF-1), PDGF-AB, vascular endothelial growth factor (VEGF), and TGF- β 1 concentration using Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer’s protocols. The remaining platelet lysates from donors were stored at -20°C until further use for cell culture medium supplementation.²²

Isolation and culture of WJ-MSCs

Fresh human umbilical cords ($n = 5$) were obtained after full-term births (caesarian section) with informed consent, according to the guidelines approved by the Ethics Committee at the Stem Cell and Cancer Institute (Jakarta, Indonesia) and by the Ethics Committee collaboration between Maranatha Christian University (Bandung, Indonesia) and Immanuel Hospital (Bandung, Indonesia). After rinsing in normal saline (0.9% w/v sodium chloride), Wharton’s jelly tissue was cut into small pieces (approximately 1–2 mm), and these were then placed in a tissue culture

dish. The explants were cultured in minimum essential medium (MEM) alpha with 2mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 20% FBS (Invitrogen) or 0.03–0.250 mg/mL huPL–ABO, and 100 U/mL penicillin–100 µg/mL streptomycin/amphotericin B (Invitrogen). Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Approximately 3 weeks after cultivation, the adherent cells and tissue fragments were detached using trypsin–EDTA solution (TrypLE Express, Invitrogen) followed by inactivation with a basal medium. The cells were harvested and replanted at 8×10^3 cells/cm².^{23,24}

Growth factors assay of huPL

Assays of IGF-1, PDGF-AB, VEGF, and TGF-β1 were performed to measure the concentration of these growth factors in huPL from three batches of seven samples of type O blood and two samples of type AB blood. The concentration of growth factors was measured using the ELISA kit. The IGF-1 assay was performed using Quantikine ELISA human IGF-I immunoassay kit (R&D Systems). The PDGF-AB assay was performed using Quantikine human PDGF-AB immunoassay kit (R&D Systems). The VEGF assay was carried out using Quantikine human VEGF immunoassay kit (R&D Systems). The TGF-β1 assay was performed using human TGF-1 immunoassay kit (R&D Systems).

Proliferation kinetics

WJ-MSCs were plated at 8×10^3 cells/cm² in tissue culture dishes and expanded using two different supplements, namely, (1) 20% FBS and (2) 0.031 mg/mL, 0.063 mg/mL, 0.125 mg/mL, and 0.250 mg/mL of huPL–ABO. Cells were counted and passaged at a confluence of 80%. At each passage, the population doubling (PD) was determined using the following formula:

$$PD = [\log_{10}[NH] - \log_{10}[NI]] / \log_{10} 2$$

where NI is the cell number of the inoculum and NH is the cell harvest number.

The PD for each passage was calculated and added to the PD of the previous passages to generate cumulative PD data. The PD time (PDT) was obtained using the following formula:

$$PDT = t / PD \text{ (hours)}$$

where *t* is the culture time (hours).

At the same time, a growth curve of WJ-MSCs from the two different conditions was plotted against time. A total of 200 cells/cm² were seeded in six-well plates. On Day 3, Day 5, Day 7, and Day 9, cells from one well were harvested and counted.²³

Immunophenotyping

For P4 and P8, WJ-MSCs cultured in 0.125 mg/mL huPL–ABO and 20% FBS were stained with antihuman antibodies, which mark the following cell surface markers:

CD105-PE, CD19-PE, HLADR-PE, CD14-FITC (Abcam, Cambridge, UK), CD73-PE, CD34-FITC, and CD45-FITC (BD Pharmingen, Franklin Lakes, NJ, USA). Cells incubated with identical concentrations of fluorescein isothiocyanate- or phycoerythrin-conjugated mouse immunoglobulin G isotype antibodies (BD Pharmingen) served as negative controls. The cells were analyzed by flow cytometry with a FACS-Calibur 3 argon laser 488 nm (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest Pro Acquisition on the BD FACStation™ Software (BD Biosciences). The experiments and measurement of surface marker were performed in triplicate.^{23,24}

Differentiation of WJ-MSCs

Multipotency was confirmed by the ability of WJ-MSCs to differentiate into adipocytes, chondrocytes, and osteocytes. WJ-MSCs (P4 and P8) were plated in six-well plates at 1×10^4 cells/cm² and incubated in the MSCs growth medium in a humidified atmosphere with 5% CO₂ at 37°C for 4 days.

For osteogenic differentiation, WJMSCs (P4 and P8) were seeded at a density of 1×10^4 cells/cm² in culture dishes using the StemPro osteogenesis differentiation kit (Gibco A10072-01, Invitrogen) for 3 weeks. Calcium deposits were visualized using alizarin red S (Amresco 9436; AMRESCO LLC, Solon, OH, USA). For chondrogenic differentiation, WJMSCs were seeded at a density 1×10^4 cells/cm² in culture dishes using StemPro chondrogenesis differentiation kit (Gibco A10071-01, Invitrogen) for 2 weeks. Chondrocytes were visualized using alcian blue (Amresco 0298). Adipogenic differentiation of WJMSCs was performed using the StemPro adipogenesis differentiation kit (Gibco A10070-01, Invitrogen) for 2 weeks. We used oil red O (Sigma-Aldrich-U0625) to confirm lipid droplets.^{24–27}

Statistical analysis

Data were presented as means ± standard deviation. Statistical comparisons were performed using one-way analysis of variance. All *p* values < 0.05 were considered statistically significant.

Results

Growth factors concentration of huPL–ABO

The concentration of growth factor (pg/mg) was calculated by dividing the concentration of growth factors in huPL–ABO (pg/mL) with the total protein concentration (mg/mL; Tables 1 and 2).

Immunophenotypic characterization of WJ-MSCs

WJ-MSCs at the early and late passages (P4 and P8) were characterized by flow cytometry. A panel of seven markers including CD105, CD73, CD14, CD19, CD34, CD45, and HLA-II was tested. All WJ-MSCs cultured in MEM alpha and supplemented with 0.25 mg/mL huPL–ABO and 20% FBS were negative for lymphocyte marker CD19, hematopoietic

Table 1 Growth factors concentration (pg/mL) of huPL-ABO from three batches.

Growth factors	Concentration (pg/mL)			
	Batch I	Batch II	Batch III	Average
IGF-1	1247.53	1435.78	1115.98	1266.43 ± 160.73
PDGF-AB	235,140.79	193,220.56	196,408.45	208,256 ± 23,336.89
VEGF	726.54	533,46	735.38	665.13 ± 114.11
TGF-β1	319,500	286,000	378,300	327,933.33 ± 46,724.33

huPL = human platelet lysate; IGF-1 = insulin-like growth factor-1; PDGF = platelet-derived growth factor; TGF-β1 = transforming growth factor-β1; VEGF = vascular endothelial growth factor.

Table 2 Growth factors concentration (pg/mg protein) of huPL-ABO from three batches.

Growth factors	Concentration (pg/mg protein)			
	Batch I	Batch II	Batch III	Average
Protein (mg/mL)	4.85	4.27	4.18	4.42 ± 0.38
IGF-1	257.22	336.25	270.21	287.89 ± 42.38
PDGF-AB	48,482.64	45,250.72	47,556.52	47,096.63 ± 1664.32
VEGF	149.80	124.93	178.06	150.93 ± 26.58
TGF-β1	65,876.29	66,978.92	91,598.06	74,817.76 ± 14,542.62

huPL = human platelet lysate; IGF-1 = insulin-like growth factor-1; PDGF = platelet-derived growth factor; TGF-β1 = transforming growth factor-β1; VEGF = vascular endothelial growth factor.

markers CD34 and CD45, and HLA-DR. The cells in both culture conditions were also consistently positive for CD73 and CD105, which are known to be expressed in MSCs (Table 3 and Figure 1). The expression of surface marker did not differ between the different cell culture conditions.

Isolation and expansion of WJ-MSC

The WJ-MSCs were isolated for the proliferation assay. WJ-MSCs were then cultured in a medium supplemented with huPL-ABO and FBS. Approximately 3 weeks after cultivation, fibroblast-like adherent cells migrated from the tissue fragments. Microscopic analysis confirmed that both FBS-

and huPL-ABO-supplemented cells shared similar morphology and density (Figure 2). At Passage 4 (P4), WJ-MSCs cultured in all culture conditions also displayed a fibroblast-like morphology (Figure 2). However, cells cultured with huPL-ABO appeared thinner, longer, and had higher density compared with FBS-supplemented cells, which resulted in higher cell number harvested from the same surface area.

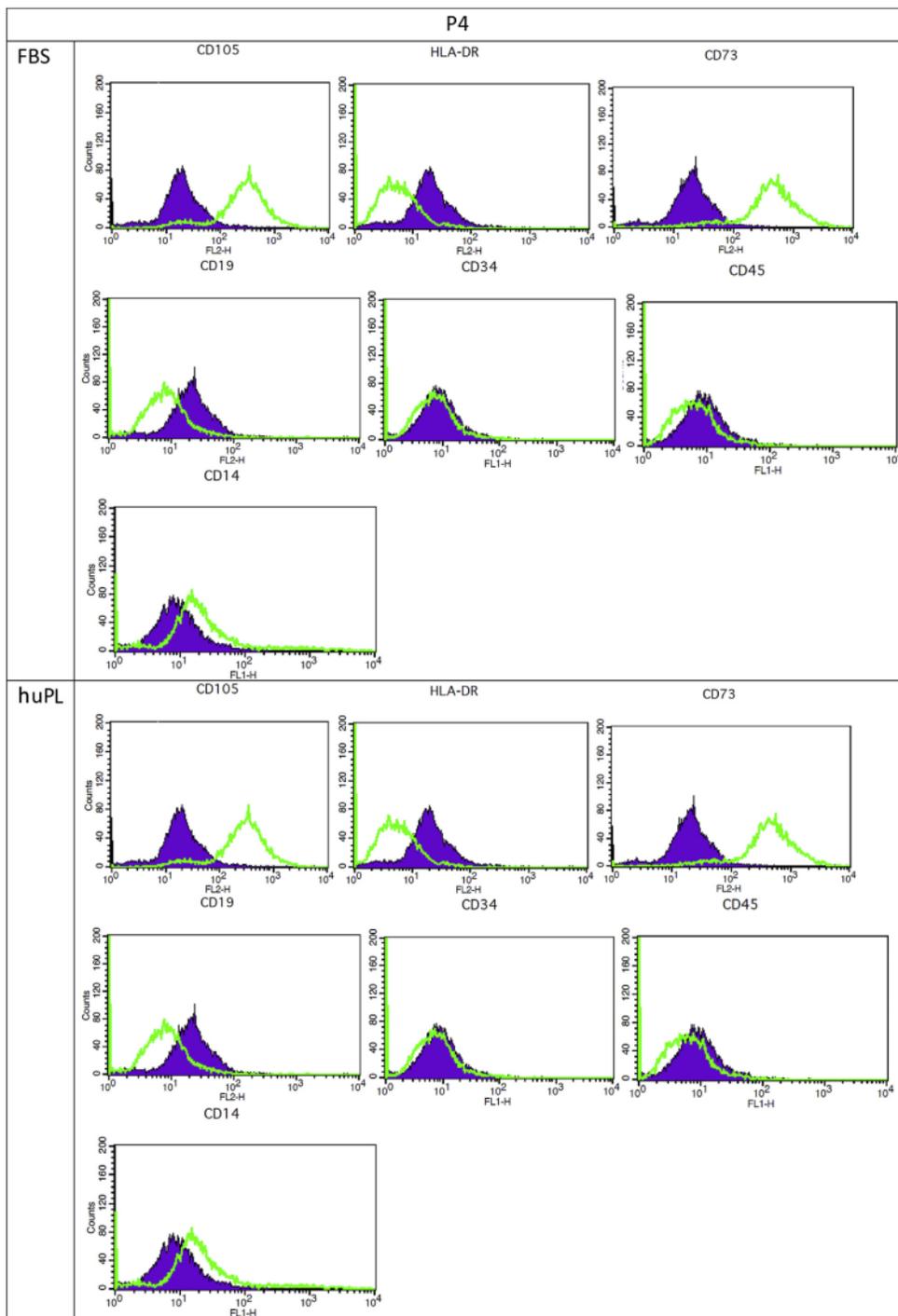
The optimal concentration of huPL-ABO in the culture medium was determined by culturing WJ-MSCs with various concentrations of huPL-ABO, and the PDT was calculated. The optimal concentrations of huPL-ABO in the culture medium were 0.250 mg/mL and 0.125 mg/mL (Figure 3). Because of insignificant differences between the concentrations in terms of WJ-MSCs PDT, 0.125 mg/mL was used as the concentration of huPL-ABO in all comparisons between FBS and huPL-ABO. PDT was calculated from P1 to P8 to compare the effect of the two supplements on the proliferative capacity of WJ-MSCs. The PDT was lower for WJ-MSCs cultured in huPL-ABO than in FBS, but significant difference was observed only for P8 ($p < 0.05$; Figure 4). The PDT of WJ-MSCs cultured in huPL-ABO supplementation was more stable and less prone to fluctuations through passages compared with those cultured in FBS supplementation. The high and stable cell proliferation rate of WJ-MSCs in the huPL-ABO-supplemented culture medium resulted in higher cumulative cell number compared with those cultured in the FBS-supplemented medium (Table 4).

[fx2]To study the optimal proliferation period closely, proliferation assay of WJ-MSCs ($n = 5$) for all culture conditions was carried out at P4 (Figure 5). Cells were seeded at the same density of 2×10^2 cells/cm². Starting on Day 3, cell numbers were counted and compared on each day with Day 9. Proliferation kinetics of the growth curve showed a

Table 3 Surface marker expression of WJ-MSCs cultured with either FBS or huPL-ABO as analyzed by flow cytometry at P4 and P8, $n = 3$ (mean percentage ± standard deviation).

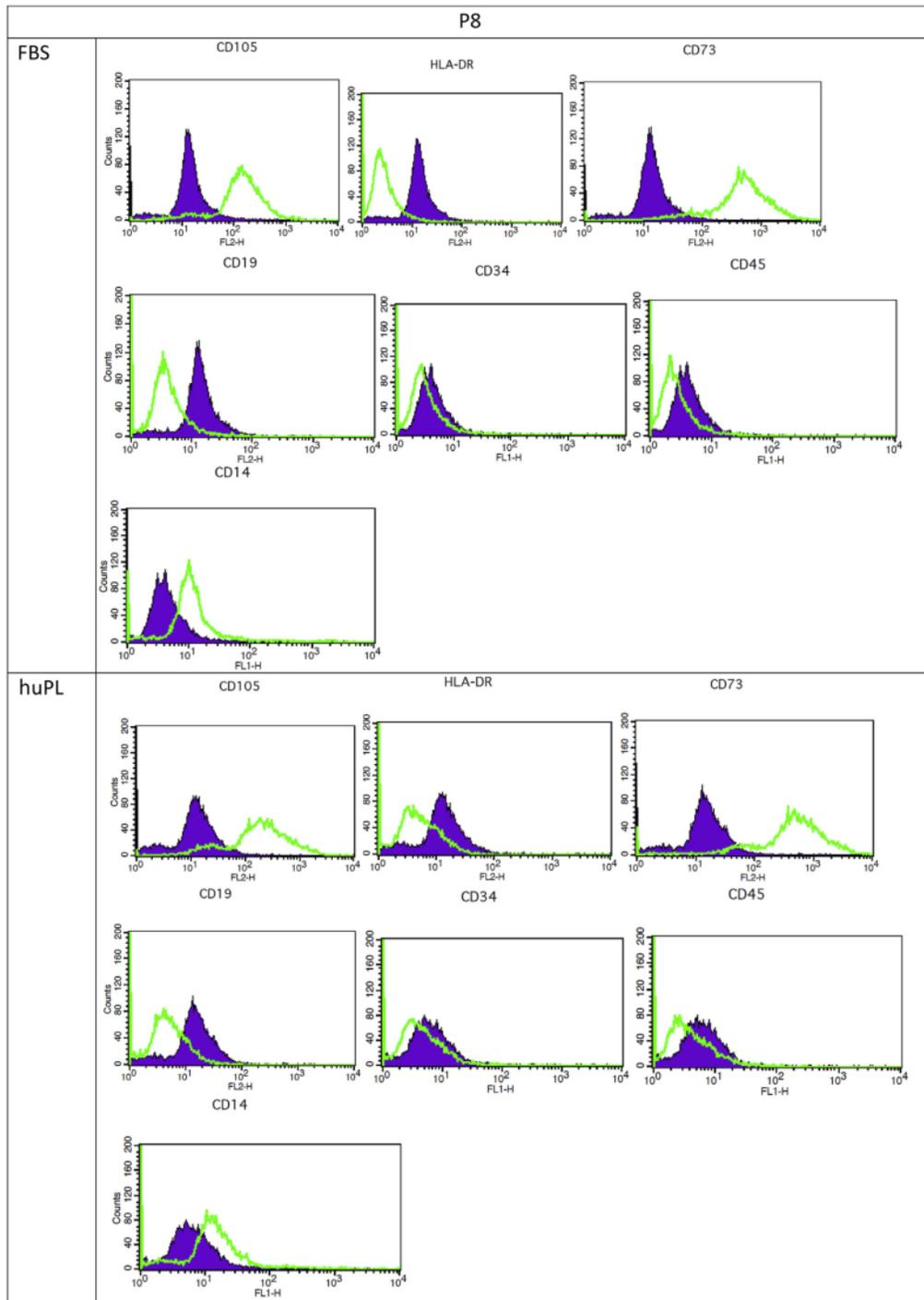
Surface marker	FBS		huPL-ABO	
	P4	P8	P4	P8
CD73	97.4 ± 0.2	96.4 ± 0.5	99.0 ± 0.1	97.4 ± 2.3
CD105	95.8 ± 0.8	93.2 ± 3.9	97.2 ± 2.0	97.8 ± 1.6
HLA-DR	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CD14	1.2 ± 1.0	1.3 ± 1.1	1.3 ± 1.1	1.4 ± 1.5
CD19	0.0 ± 0.0	0.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
CD34	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CD45	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

FBS = fetal bovine serum; huPL = human platelet lysate; P4 = Passage 4; P8 = Passage 8; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells.



(A)

Figure 1 Representation of flow cytometry measurement comparison results on CD105, CD73, CD14, CD19, CD34, CD45, and HLA-II marker at (A) Passage 4 (P4) and (B) Passage 8 (P8) supplemented with fetal bovine serum (FBS) and human platelet lysate (huPL).



(B)

Figure 1 (continued).

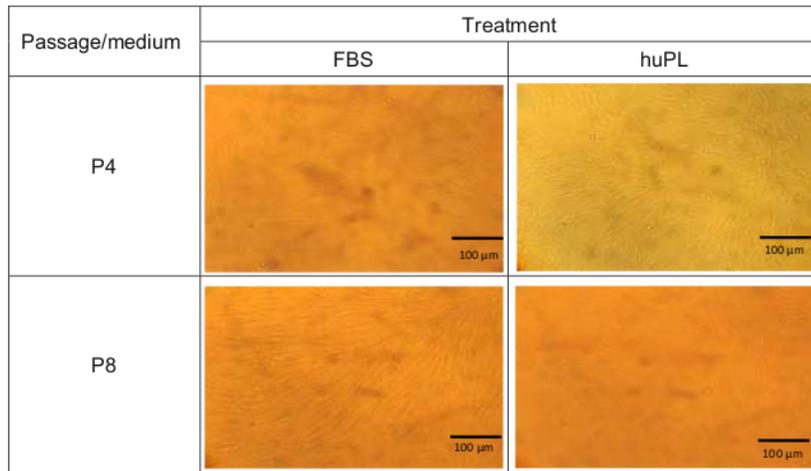


Figure 2 Morphology of Wharton's jelly-derived mesenchymal stem cells from one representative donor at Passage 4 (P4) and Passage 8 (P8) in cultures supplemented with fetal bovine serum (FBS) and human platelet lysate-ABO (huPL-ABO). Magnification 100 \times .

higher exponential growth of cells in the huPL-ABO cultures starting on Day 5, which reached significant difference ($p < 0.05$) on Day 7, compared with those in the FBS-supplemented cultures. Cultures with huPL-ABO supplementation reached a final cell count of $8.5 \times 10^5 \pm 7.4 \times 10^4$ on Day 7. In comparison, those with FBS supplementation reached a final cell count of $4.7 \times 10^5 \pm 8.7 \times 10^4$ on the same day. Both huPL-ABO- and FBS-supplemented cultures experienced a decrease in cell number on Day 9, as they had already reached a postconfluence period on Day 7 or Day 8. The proliferation of these cells might be even higher if a lower seeding density and a larger growth area were used. The PDT of WJ-MSCs cultured with huPL also showed more stability and less increase through passages compared with those cultured with FBS. The high and stable cell proliferation rate of WJ-MSCs in the huPL-

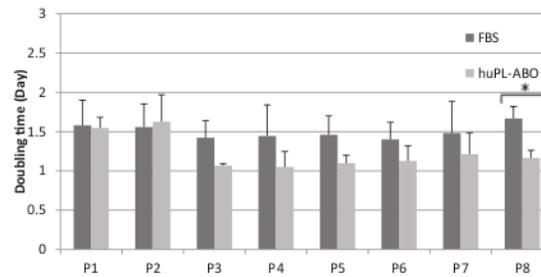


Figure 4 Population doubling time of Wharton's jelly-derived mesenchymal stem cells supplemented with 0.125 mg/mL human platelet lysate-ABO (huPL-ABO) and 20% fetal bovine serum (FBS) for Passage 1 (P1) through Passage 8 (P8). Each column represents the mean \pm standard error of three independent experiments (* $p < 0.05$).

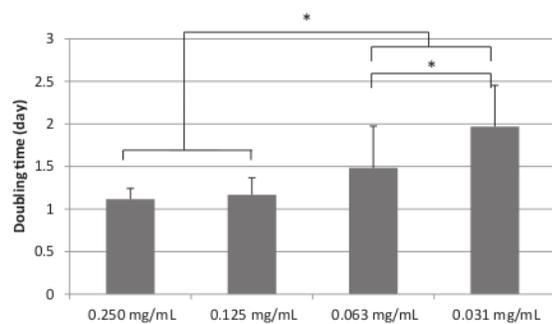


Figure 3 Population doubling time of Wharton's jelly-derived mesenchymal stem cells supplemented with 0.250 mg/mL, 0.125 mg/mL, 0.063 mg/mL, and 0.031 mg/mL human platelet lysate-ABO. Each column represents the mean \pm standard error of three independent experiments (* $p < 0.05$).

Table 4 Cumulative cell number of WJ-MSCs cultured with FBS and huPL-ABO from P1 through P8.

Passage	FBS	huPL-ABO
1	1.95×10^6	2.85×10^6
2	1.08×10^7	1.59×10^7
3	6.40×10^7	2.15×10^8
4	3.60×10^8	2.96×10^9
5	2.40×10^9	3.76×10^{10}
6	1.72×10^{10}	4.46×10^{11}
7	1.12×10^{11}	4.37×10^{12}
8	5.96×10^{11}	4.73×10^{13}

FBS = fetal bovine serum; huPL = human platelet lysate; P4 = Passage 4; P8 = Passage 8; WJ-MSCs = Wharton's jelly-derived mesenchymal stem cells.

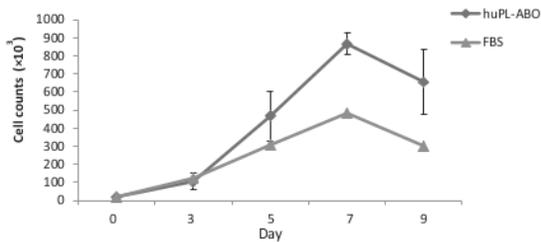


Figure 5 Growth curve assessing the proliferation kinetics of Wharton's jelly-derived mesenchymal stem cells in 20% fetal bovine serum (FBS) and 0.125 mg/mL human platelet lysate-ABO (huPL-ABO) on Day 3, Day 5, Day 7, and Day 9. ($n = 5$).

supplemented culture medium resulted in higher cumulative cell number compared with the same cells cultured in the FBS-supplemented medium (Table 4).

1 Multilineage differentiation capacity of WJ-MSCs

The differentiation capacities of WJ-MSCs cultured in MEM alpha supplemented with FBS and huPL-ABO were evaluated by culturing WJ-MSCs in the differentiation medium (chondrogenic, osteogenic, and adipogenic lineages). After culturing for approximately 7 days, the morphology of chondrocytes, osteocytes, and adipocytes were studied. Cell staining showed that WJ-MSCs cultured in both culture conditions were able to differentiate into chondrocytes, osteocytes, and adipocytes (Figure 6).

Discussion

The fact that huPL as an alternative supplement for culture medium can trigger an explosive growth rate at earlier passages makes it an attractive candidate for MSC expansion in clinical settings, because for clinical applications large numbers of MSCs are required in a short period for transplantation into the human body.²⁸ Platelet contains many growth factors, which are stored in α -granules. Inducing lysis in huPL by freeze-thaw cycles has been shown to release the growth factors from platelets, which can stimulate cell proliferation efficiently both *in vivo* and *in vitro*.^{22,29,30} The efficacy of huPL in promoting MSCs expansion was investigated by evaluating its growth factors concentration. This study showed that huPL-ABO is rich in PDGF-AB and TGF- β 1; in addition, it contains VEGF and IGF-1 in lower concentrations. Similar growth factors concentrations were reported by Mooren et al.¹⁹ and Cho et al.¹³ Therefore, it can be concluded that the huPL-ABO production procedures carried out in this study did not affect the effectiveness of platelets to release these growth factors.^{13,19} The concentration of all growth factors examined in this study, except for IGF-1, showed high correlation with protein concentrations of huPL-ABO. Several studies have shown that IGF-1 does not originate from platelets, but is primarily excreted from the liver into the blood plasma, which explains the low correlation of IGF-1 concentration with protein concentration of huPL-ABO in this study.¹³ The high correlation between growth factors and protein

concentration of huPL-ABO and the low variability of growth factors concentration across batches make it possible to create reproducible growth factors concentrations in the culture medium supplemented with huPL-ABO from different batches. According to the study results, the best concentration of huPL-ABO in the culture medium was 0.125 mg/mL.⁴

The study further showed that huPL-ABO acts as a growth-promoting factor in MSCs cultures. Cell culture media supplemented with huPL-ABO gave a higher proliferation rate compared with those supplemented with FBS, especially at late passages. In addition, the medium supplemented with huPL-ABO also had lower PDT and higher cumulative cell number. Growth factors released by platelets in huPL-ABO are effective to stimulate proliferation of WJ-MSCs *in vitro*. This result can be concluded from the growth curve of huPL-ABO-cultured WJ-MSCs, which showed more explosive growth starting from Day 3 compared with FBS-cultured WJ-MSCs, subsequently resulting in higher cell number. Various studies using exogenous growth factors, either alone or in combination, have reported that basic fibroblast growth factor, TGF- β 1, IGF-1, and PDGFs are key factors involved in supporting the expansion of MSCs *ex vivo* and maintaining their multilineage potential for further differentiation.^{31–34} TGF- β 1 is involved in proliferation, differentiation, and migration of MSCs, along with PDGF-AB, which acts as a powerful mitogen. VEGF is involved in angiogenesis and wound healing, whereas IGF-1 is important in controlling cell apoptosis.²⁰ All the growth factors in huPL-ABO play a very important role in maintaining WJ-MSCs proliferation, and in this study all of the growth factors were effectively extracted from the platelets in PRP by the freeze-thaw method. Therefore, the freeze-thaw method is preferable for production of huPL-ABO owing to its high efficiency and safety in releasing growth factors from PRP for clinical applications. The method also does not require any additional substances that might induce an immunologic reaction, and thus it is safe to administer WJ-MSCs grown in huPL-ABO for patients.

To ensure that the characteristics of MSCs are still preserved in the huPL-ABO-cultured WJ-MSCs, we performed immunophenotyping and differentiation capacity assays, and the results were compared with those cultured in FBS. Results of immunophenotyping and differentiation analyses revealed that there are no significant differences between WJ-MSCs cultured in the medium supplemented with huPL-ABO and those in the medium supplemented with FBS. Both cell populations expressed specific MSCs markers CD73 and CD105 (>95%), and lacked expression of CD34, CD45, CD19, and HLA-DR (<2%). MSCs display a variety of cell surface antigens that may vary according to the isolation and expansion method. MSCs usually express CD73, CD90, and CD105 and lack expression of major histocompatibility complex class II surface molecules and endothelial CD31 and hematopoietic-specific antigens (CD34, CD45, and CD14).^{23,25,35} The production of MSCs was extremely donor dependent for all culture conditions tested and could be related to the WJ-MSCs isolation technique. Thus, it is essential to eliminate non-MSCs and contaminated cells before clinical application. Differentiation analysis revealed that WJ-MSCs in both culture conditions were able

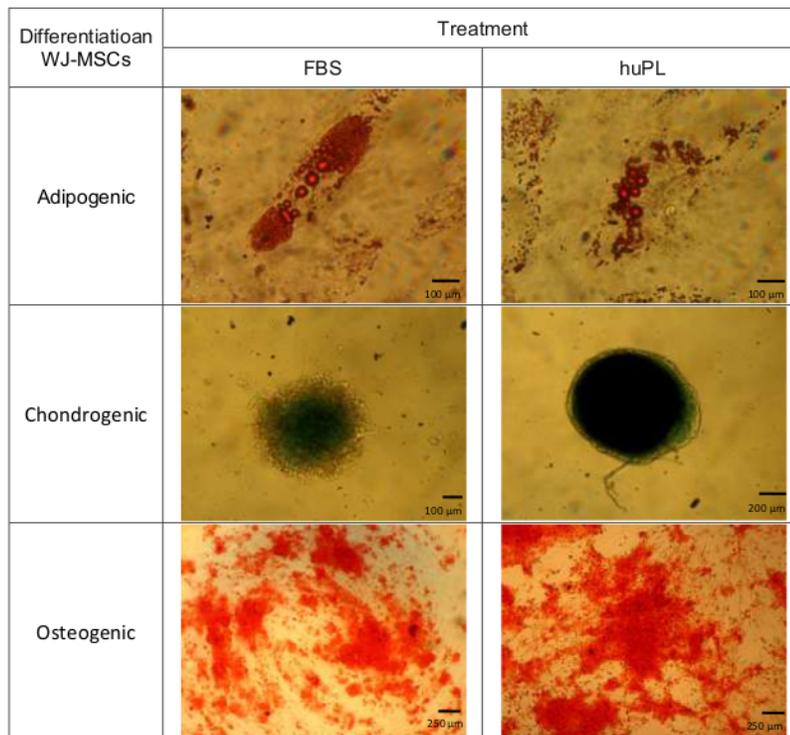


Figure 6 Cell staining for Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) cultured with supplementation of fetal bovine serum (FBS) and human platelet lysate-ABO (huPL-ABO) after incubation in the adipogenic, chondrogenic, and osteogenic differentiation medium for 7 days. Magnification 100 \times .

to differentiate into three differentiation lineages, namely, adipogenic, chondrogenic, and osteogenic, with no significant difference. Some studies have shown that a combination of growth factors in huPL inhibits the osteogenic lineage and stimulates chondrogenic differentiation in MSCs.^{10,13,15,30} However, Ben Azouna et al³⁴ revealed that the inhibitory effect of huPL on chondrogenic differentiation of MSCs had no impact on the real multipotency of MSCs, because they were able to fully differentiate further toward the chondrogenic lineage. The inhibitory effect only caused the MSCs to preserve their stemness and minimized the risk of these cells entering senescence and transformation, which can be of particular interest in clinical applications.^{29,30} However, the concentration of huPL in the culture medium needs to be assessed thoroughly, because even a slight change in the amount of growth factors in the culture medium could lead to variation in results.³⁵

For clinical applications, the proposed culture conditions using huPL-ABO appeared to be more advantageous than using FBS. It can replace FBS for safe propagation of functional MSCs, which thus avoids the risk of transmission of bovine pathogen or immunogen, such as bovine serum albumin and bovine apolipoprotein B-100, during their culture for cell therapy.² It is further recommended to produce huPL-ABO by matching platelets of blood group O with plasma of blood group AB as performed in this study to

avoid possible influences of ABH antigens and isoagglutinins.³⁶ MSCs expanded using an autologous platelet lysate technique show no evidence of malignant transformation *in vivo*, following implantation of MSCs.^{37,38}

In conclusion, our study shows that huPL-ABO produced by the freeze-thaw method is able to enhance the WJ-MSC proliferation rate constantly and decrease the time required to reach confluence compared with the FBS culture condition. huPL-ABO can replace FBS for safe propagation of functional MSCs, thereby avoiding the risk of bovine pathogen or immunogenic transmission, as well as the influence of ABH antigens and isoagglutinins, during their culture for cell therapy. Although still not serum free, we established an efficient and complete xeno-free protocol for propagation of human WJ-MSCs. We propose that the humanized system developed in this study for MSCs expansion could be translated into a clinical expansion protocol.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13

PAGE 14

PAGE 15
