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LETTER OF ACCEPTANCE

Malang, 13 August 2019

Dear Wahyu Widowati
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Congratulation,

On behalf of the ISSMART 2019 scientific committee, we are pleased to inform you that your submitted full-paper entitled

"Comparative Analysis of Wharton's Jelly Mesenchymal Stem Cell (WJ-MSCs) Isolated Using Explant and Enzymatic Methods"

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Head of Committee 1st ISSMART 2019

Eko Suyanto, M.Sc

Comparative Analysis of Wharton's Jelly Mesenchymal Stem Cell (WJ-MSCs) Isolated Using Explant and Enzymatic Methods

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Abstract. Wharton's Jelly is one of the best sources for mesenchymal stem cells. Human Wharton's Jelly Mesenchymal Stem Cells (hWJ-MSCs) have high proliferation, multi-lineage differentiation potential, and do not produce any teratogen or carcinogen. These characteristics make hWJ-MSCs become suitable for regenerative medicine. Some methods were developed to isolate hWJ-MSCs from umbilical cord, such as explant method and enzymatic method. This study aims to characterize hWJ-MSCs which are isolated by two different methods, explant attachment method and enzymatic method. hWJ-MSCs isolation was performed through explant method and enzymatic method using trypsin, hyaluronidase and collagenase type 1 with certain ratio of concentration. Isolated hWJ-MSCs was characterized using flow cytometer to detect the expression of CD44, CD90, CD105, CD73 and negative lineage. MSCs differentiation assay was performed to analyze adipogenic, chondrogenic and osteogenic cells lineage. We successfully isolated hWJ-MSCs from umbilical cord through enzymatic and explant methods. Immunophenotyping assay through flow cytometry analysis showed high purity of WJ-MSCs. The isolated hWJ-MSCs from both methods showed positive expression of CD44, CD90, CD105, and CD73. The isolated hWJ-MSCs exhibited capacity to differentiate into adipocyte, chondrocyte, and osteocyte cells. hWJ-MSCs isolated through explant and enzymatic method have high proliferation capacity and be able to differentiate into three different lineage cells. Both methods explant attachment and enzymatic methods are efficiently produced hWJ-MSCs.

Keywords : Wharton's Jelly Mesenchymal Stem Cell, adipogenic, chondrogenic, osteogenic

1. Introduction

Cell therapy and tissue engineering have been increasingly used and promising therapies [1]. Human mesenchymal stem cells (hMSCs) are the powerful tools because self-renewing, multi potent differentiation capability to differentiate into adipocytes, osteoblasts, chondrocytes [2][3], strong immunosuppressive properties and can be used for autologous and allogeneic therapy [4][5], and possible differentiation potential into hepatocytes, cardiomyocytes, skeletal myocytes and neurons [3] [6]. The hMSCs are easy to acquire, harvesting process and culture, fast in vitro expansion, feasible for autologous and allogenic therapy, and paracrine function [1].

The hMSCs were successfully isolated from adult tissue including bone marrow, peripheral blood, adipose tissue, skeletal muscle, synovium, dental pulp [7], hair follicle (HF) [8][9], cornea [9][10], and neonatal birth-associated tissues including umbilical cord blood, umbilical cord, placenta, decidua, chorion villi, chorion membrane, amniotic fluid and Wharton's Jelly [7][11]. Ontologically, stem cells that derived from earlier phases of life possess a higher proliferative capacity as foetal MSCs replicate much faster than adult BM-MSCs [12][13].

Umbilical cord tissue is one of the major sources of MSCs since isolation of umbilical cord MSCs (UCMSCs) is easier, does not require an invasive procedure and has a higher proliferation potential compared with other sources [3], has a higher proliferation potential compared with other sources [3][15]. Moreover, umbilical cord is usually discarded after birth so the collection of cells without any ethical conflict or moral concerns [3][7][13][15][16], low risk of infection, non-carcinogenesis, multipotency [7], exhibit low immunogenicity and low immunity after cyto-therapy [5]. Wharton's jelly (WJ) was proven as the best MSC source among various compartments of UC, WJ is perivascular regions surrounding the vessels, arteries, veins, the media and the adventitia compartment of the walls of UCB vessels [18][19]. WJ-MSCs have a higher proliferation rate and self-renewal capacity than adult tissue-derived MSCs [2][20], and favourable for tissue engineering purpose [21].

Clinical therapies require a large number of cells, so many strategies are used to improve the quality and quantity of stem cells [2][22]. Isolation methods were categorized into two major methods; enzymatic method and explant culture method. The procedure of enzymatic method are proteolytic enzymes for cells separation from tissue; the single cell suspension is then cultured in appropriate medium for further cell proliferation [9]. Most studies used collagenase digestion alone or in association with different proteases to accelerated degradation of extracellular matrix proteins [21]. The studies used a panel of enzymes to induce the release of cells (collagenase/trypsin or collagenase/hyaluronidase/trypsin) [21][24]. The explant method is based on the migratory ability of the cells [21], the original tissue is cut into smaller pieces which are placed in culture dishes or flasks, and cells start to migrate out of tissue and adhere to the culture surface [9]. These two techniques are different by many aspects such as time to reach isolated cells and economy, however in the case of MSCs isolation, it seems that both of them provide acceptable cell yield [9][23]. The choice method is critical for cells isolation which it can influence cells yield, viability, integrity, and function [21][23]. The cells isolation method was affected by multiple parameters including tissue source, isolation method and medium composition [24].

This research was done to compare between enzymatic and explant method for human Wharton's Jelly-derived Mesenchymal Stem Cells (hWJ-MSCs) including immunophenotyping, differentiation capacities, population doubling capacity from various sources and passages.

2. Methods

2.1. hWJ-MSCs Isolation with Explants Procedure

Fresh human umbilical cords (UC; n=3) were collected from women aged 25-40 years after normal vaginal delivery, have signed the informed consent using the guidelines approved by Ethics Committee in collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Indonesia [2][5][7]. The UC was washed with PBS (Biowest, X0515-500) supplemented with antibiotic and transferred to laboratory using transport medium containing phosphate buffer saline (PBS) (Biowest, X0515-500), 1% Antibiotic-Antimycotic (Ab-Am) (Biowest, L0010-100), 1% Amphotericin B (Amp B) (Biowest, L0009-100), 0.1% Gentamicin (Gibco,

15750060), 1% Nanomycopulitin (Biowest, LX16-100), and transferred immediately to the laboratory to be processed.

UC was washed again by PBS, and the vessel was removed from the UC. After vessel removal, Wharton's jelly was dissected into small segments (1-2 mm) and transferred onto 100 mm petri dishes. The explants were cultured in MEM-α (Biowest, L0475-500) supplemented with 20% fetal bovine serum (FBS) (Biowest, S1810-500). Cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37^0C for 3 weeks after explantation, when fibroblast like and spindle-shaped adherent cells migrated from the tissue fragments, the adherent cells and tissue fragments were detached using Trypsin-EDTA solution (Biowest, L0931-500) followed by washing 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. The culture flask was left undisturbed for 3 - 4 days and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium changed every 2-3 days thereafter [2][5][7].

2.2. hWJ-MSCs Isolation with Enzymatic Digestion

Wharton's Jelly fragments were washed again with PBS 1x three times and cut into 1-2 mm, and then crushed using sterile blade. The crushed tissues then were inserted into a 50 ml falcon tube (TPP, 91050) containing the mixed enzyme solution contains 4 mg/ml Collagenase Type I (Gibco, 10114532), 1 mg/ml Hyaluronidase (Sigma, 515397), Trypsin 0,1% (Biowest, L0931-500), and then rocked 3 h, 300 rpm, 37 ˚C, and 5% CO_2 condition followed with overnight incubation, 37 °C, 5% CO_2. Sample was filtered using 70 µm cell strainer (Corning, 431752) then centrifuged at 1600 rpm for 10 min (MPW-250R). Supernatant was discarded and the pellet was washed with PBS 1x and centrifuged at 1600 rpm for 5 min. Washed cells were cultured in MEM-α containing 10% FBS and 1% Ab-Am, 1% Amp B, 0.1% Gentamicin, 1% Nanomycopulitin in 100 mm dish in a humidified 37 ◦C, 5% CO_2 incubator. After 3-week culture, we harvested the P0 cells. The medium was changed twice a week [25].

2.3. hWJ-MSCs Markers Detection using Fluorescence Activated Cell Sorting (FACS)

The hWJ-MSCs were evaluated using surface marker detection at Passage 3 (P3) and P8 to confirm MSCs characterization, hWJ-MSCs at 80% confluence were harvested and dissociated with trypsin-EDTA and centrifuged at 300 x g for 10 min. The pellet was resuspended with PBS 1x, FBS, and cells were counted with a hemocytometer. Between $10x10^4$ – $20x10^4$ cells in 500 µL PBS were introduced into FACS (Macsquant Analyzer 10, Miltenyi). The cells were stained with CD90, CD105, CD4, CD73 and their isotype control in hWJ-MSCs analysis kit and the procedure was according to manufacture protocol (BD stem flowTM kit, 562245). The experiments and measurement of surface marker were performed in triplicate [2][5][7].

2.4. Morphology Analysis of hWJ-MSCs

The hWJMSCs cultures were routinely visualized using an inverted research microscope and the phenotype and growth of primary and passaged cells were monitored Photomicrographs were taken with a digital camera model of Inverted Microscope (Olympus, CKX41-F32FL) under inverted phase-contrast optics [2][5][7][26].

2.5. hWJ-MSCs Differentiation

For osteogenic differentiation, hWJ-MSCs were seeded at density $5x10^3$ cells in 24 well plate (Nunc, 72296-18) using StemPro Osteogenesis Differentiation Kit (Gibco A10072-01) for 3 weeks. Calcium deposits were visualized using Alizarin red S (Sigma, A5533). For chondrogenic differentiation, hWJ-MSCs were seeded at density $5x10^3$ cells in 4 well plate (Nunc, 176740) using StemPro Chondrogenesis Differentiation Kit (Gibco, A10071-01) for 3 weeks. Chondrocytes were visualized using Alcian blue (Sigma, A5268). Adipogenic differentiation of hWJ-MSCs was done using StemPro Adipogenesis Differentiation Kit (Gibco, A10070-01) for 3 weeks. Oil Red O (Sigma, O0625) to confirm lipid droplets [5][7].

2.6. Passaging hWJ-MSCs Cell Proliferation Analysis

Passaging hWJ-MSCs were counted and passaged at a confluence of 80%. Briefly, cultured cells were detached by trypsin (Biowest, L0931-500), then incubated for 1-3 min at 37ºC, completed medium consisting of 80% MEM-α, FBS 20%, 1% Antibiotic and anti-mycotic and 1% heparin were added to stop trypsin and centrifuged (MPW-2000) at 1600 rpm, 5 min at 240 C. The pellet of cells was resuspended with trypan blue solution (Sigma, 25200072) and diluted 1:1 dilution. Then, cells were counted by a haemocytometer (Neubauer,17849). Population Doubling (PD) was counted at every passage with the formula:

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

where NI is the inoculum cell number and NH the cell harvest number. Then, to determine cumulative PD data by added the PD at the previous passage. The PD time (PDT) was determined by the formula [2][27]:

PD time= (t (time))/(PD (in days))

3. Results and Discussion

3.1. Morphology of Isolated MSC

MSCs from Wharton's Jelly was successfully isolated through both explant and enzymatic methods. The isolated cell from both methods showed Spindle-shaped and fibroblast-like MSCs and clearly observed under inverted microscope. The homogenous monolayer hWJ-MSCs attached in plastic flask and cultured until passage 8. The morphological of primary hWJ-MSCs changed in the higher passage, resulted in big Spindle-shaped MSCs.

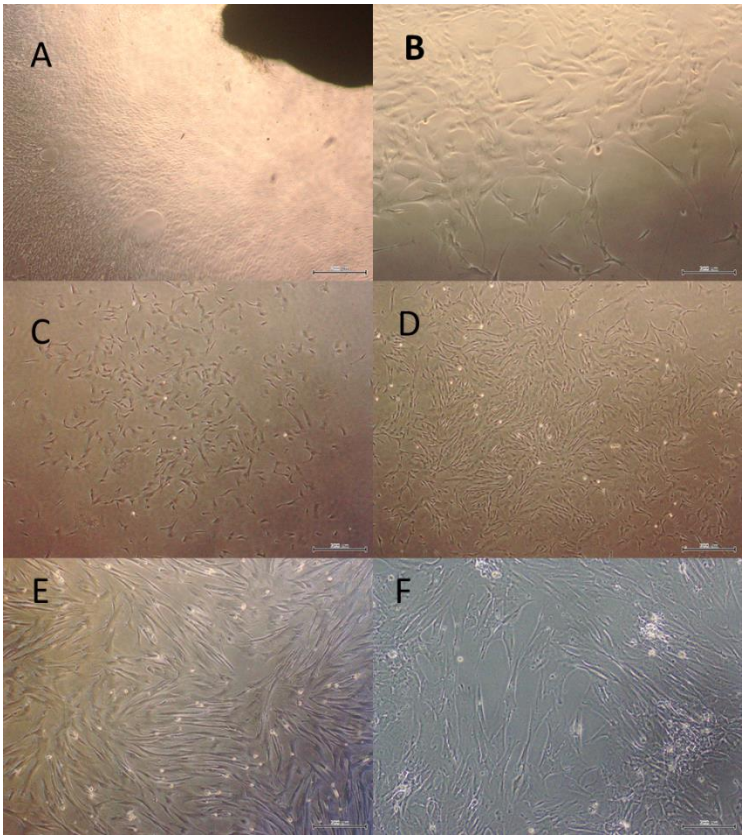

Figure 1. Morphology of isolated hWJ-MSCs in homogenous monolayer adherent flask. A and B are isolated hWJ-MSCs through explant method (40x mag). C and D are isolated hWJ-MSCs through enzymatic method (40x mag.). E and F hWJMSCs in Passage 8 (E, mag 40x; F, mag.. 100x).

3.2. hWJ-MSCs Markers Detection

CD90, CD105, CD73 and CD44 were used to characterize the immunophenotypes of isolated hWJ-MSCs from both methods. The immunophenotypes of hWJ-MSCs were analysed by flow cytometry. The results showed in figure 2. In general, the expression of all markers was higher in explant method than enzymatic one. The expression of isolated hWJ-MSCs P 3 through enzymatic method were 92.03% for CD90, 91.91 % for CD44, 90.76 % for CD105 and 96.64 % for CD73, when the expression of isolated hWJ-MSCs (P3) through explant method 96.75 % for CD90, 96.20 %for CD44, 98.3 % for CD105, and 96.53 % for CD73. In higher passage (P8) the isolated MSCs still exhibit MSCs characteristics (Figure 2, Tabel 1.). The isolated MSCs from both methods showed a very little expression (0-1.5%) of negative lineage (CD34, CD11b, CD19, CD45, HLA-DR) See Figure 3.

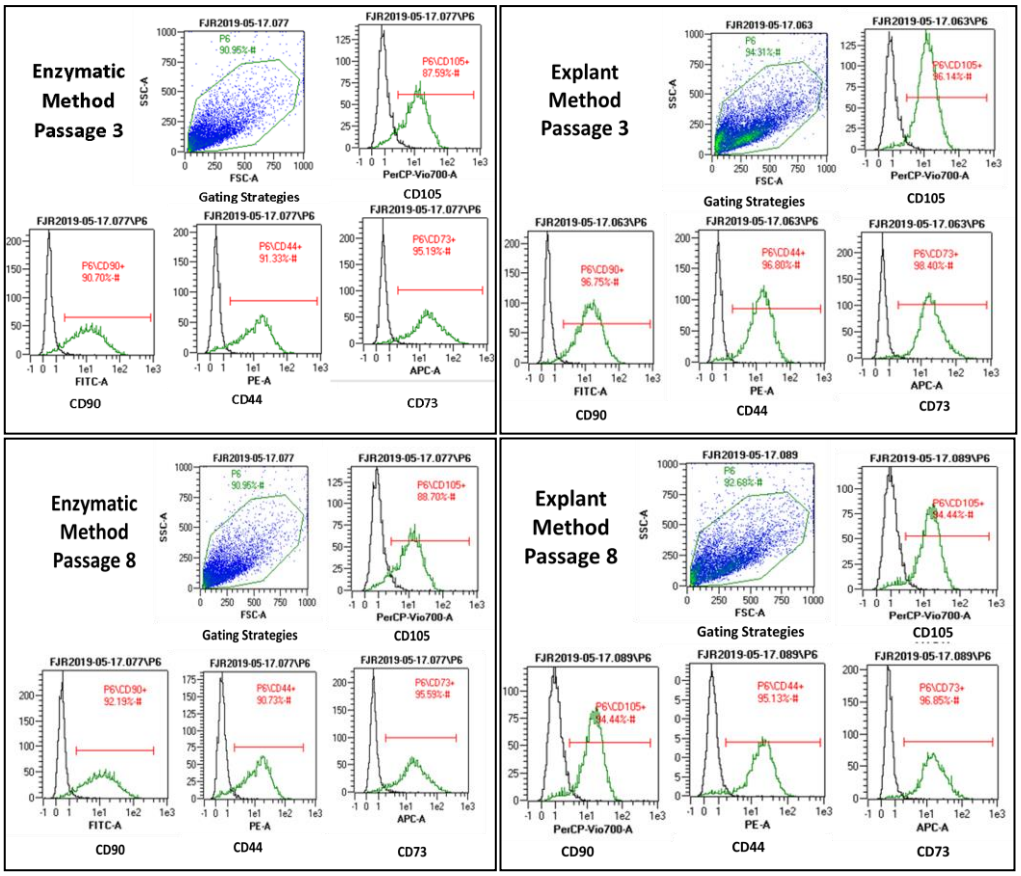

Figure 2. Immunophenotyping of isolated hWJ-MSCs in both methods at passage 3 and 8. The isolated hWJ-MSCs showed positive expression of CD90, CD105, CD44 and CD73 (green line). To avoid positive false, the isotype of each markers was analysed (black line).

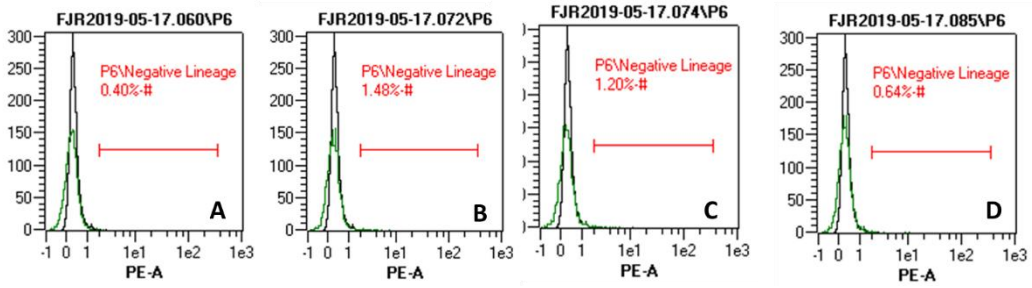

Figure 3. Negative Lineage Cocktail (Immunophenotyping of isolated hhWJ-MSCs in both method in passage 3 and 8. The isolated hWJ-MSCs showed negative CD34, CD11b, CD19, CD45, LA-DR expression (green line). To avoid positive or negative false, the negative isotype cocktail was analysed (black line). A=Explant P3, B=Enzymatic P3, C=Explant P8, D=Enzymatic P8.

Table 1. Marker expression of isolated hWJMSCs

Isolation Methods and Passage	Marker Expression (%)				
	FITC-CD90	PE-CD44	PerCP-CD105	APC-CD73	Neg. Lin.
Enzymatic P3	92.03±0.47[a]	91.91±0.51[a]	90.76±0.52[a]	96.64±0.67[a]	1.21±0.28[a]
Explant P3	96.91±0.26[b]	96.51±0.29[b]	96.81±0.35[b]	98.36±0.21[b]	0.40±0.12[a]
Enzymatic P8	91.42±0.81[a]	91.23±0.94[a]	90.56±0.66[a]	95.92±0.46[a]	1.29±0.29[a]
Explant P8	95.42±1.64[b]	94.39±1.61[b]	94.46±1.64[b]	96.65±1.20[a]	0.68±0.11[a]

*The data was shown in mean±standard deviation. Different subscript alphabet in the same column was statistically significant differences among treatment (isolation method and passage) through ANOVA and Tuckey Analysis with P<0.05

Statistical analysis of marker expression showed that the expression of CD90, CD105, and CD73 was higher in the MSCs isolated through explant method than enzymatic one. But, the expression of those marker still high (>90%) in enzymatic method-isolated MSCs. Other research shows that the expression of MSCs positive marker (CD90, CD105, CD73 and CD90) were between 85-99% [16][28][29].

CD90 or Thy-1, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein. This protein has a number of activities such as neuritis outgrowth, T-cell activation, apoptosis in carcinoma cells, tumour suppression, wound healing. Recent studies also indicate that this protein regulates fibroblast focal adhesion, cytoskeleton organization, and cell migration. In MSCs, this protein participated in self-renewal and differentiation [30]. Endoglin (CD105) is a component of the transforming growth factor-beta receptor (TGF-βR) complex. This protein expressed in primitive hematopoietic cells indicate that this molecule has effect in cell cycle and cell survival and differentiation [31]. CD44 antigen is a glycoprotein that has function in cell–cell interactions, cell migration and adhesion [32]. And the last, CD73 or ecto-5′-nucleotidase (ecto-5′-NT, EC 3.1.3.5) is a glycosyl-phosphatidylinositol (GPI)-linked well-known as lymphocyte differentiation antigen. The protein had a function as a co-signaling molecule on T lymphocytes and as an adhesion molecule. This protein help lymphocyte to bind to endothelium [33]. The different expression of this marker in both enzymatic and explant method may affect the proliferation of MSCs. The expression of negative lineages (CD34 PE, CD11b PE, CD19 PE, CD45 PE, HLA-DR PE) was measly expressed. This means that isolated MSCs has high purity.

3.3. Multilineage Differentiation

The assessment of differentiation capacity of hWJ-MSCs toward three lineages (adipocyte, chondrocyte, and osteocyte) was done. All isolated hWJ-MSCs can be differentiate toward all three lineages. The result showed in Figure 4.

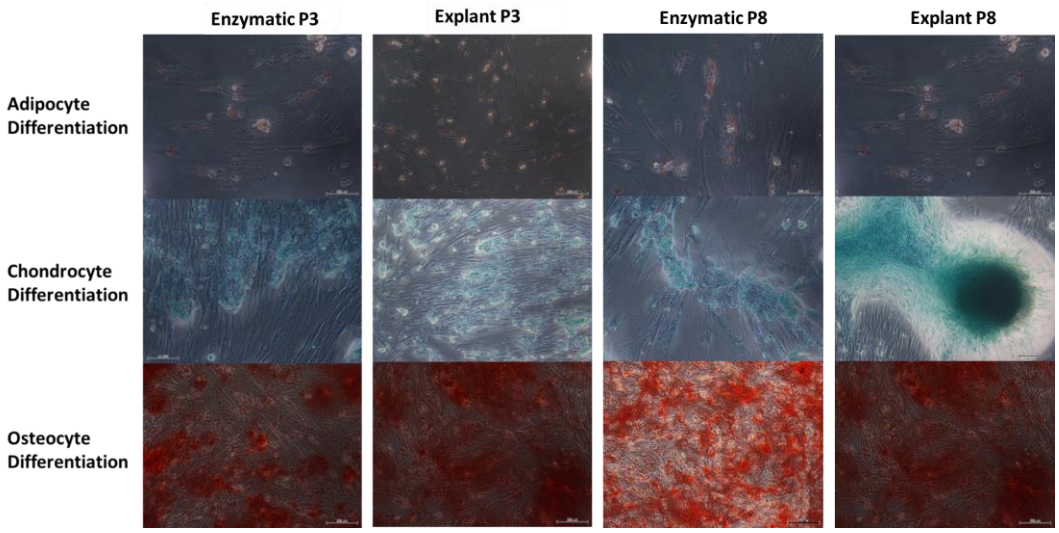

Figure 4. Oil-red-o staining to detect adipogenesis, Alcian blue staining to detect chodrogenesis and, Alizarin-red staining to detect osteogenesis (Mag.100x) in multilineage differentiation of Isolated MSCs from both enzymatic and explant method.

3.4. Population Doubling Time (PDT)

To evaluate the self-renewal capacity of both isolated hWJ-MSCs we used population doubling time (PDT) assay and the result showed in Figure 4. PDT is time for cells to divide become two individuals. The lower PDT value means the higher proliferation capacity of the cells. The result of this assay showed in Figure 4.

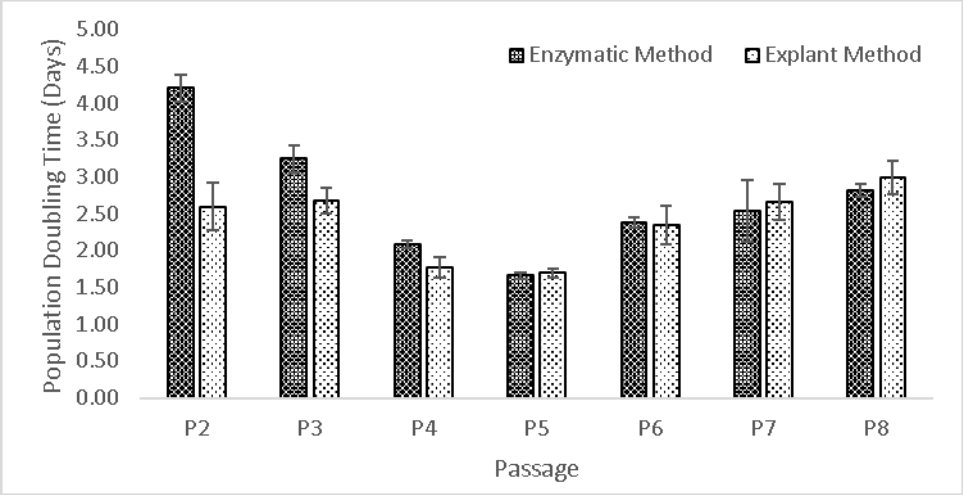

Figure 5. PDT of isolated hWJ-MSCs through explant and enzymatic methods

The PDT of hWJ-MSCs were presented as median±standard deviation. The experiment was done in triplicate The early passage (P2-P3) was high PDT value. The lowest PDT was P4-5, the older passage increase the PDT value

The result of PDT assay showed that in early passage, the explant method has lower PDT. It is mean, in early passage the cells isolated through explant methods has higher proliferation capacity. The lower proliferation capacity in isolated hWJ-MSCs through enzymatic methods may be due to the enzyme treatment. The best proliferation capacity of MSCs was in Passage 5. The increasing passage in line with the lower proliferation capacity of MSCs (Figure 5).

Proliferation assay showed that in early passage, isolated hWJ-MSCs from explant procedure had higher self-renewal capacity. This may be caused by enzymatic digestion in the hWJ-MSCs isolation

process. The cells that isolated may be contains other cells in Wharton's Jelly tissue. After passage 4, the PDT of hWJ-MSCs increase in the increasing of passage. This result in line with the research conducted by Widowati et al., (2014) and Antonius et al., (2015) that the rising passage caused the decreasing proliferation capacity of primary hWJ-MSCs [2][27].

Our results show that both methods can be used for isolation of MSCs from Wharton's Jelly tissues. hWJ-MSCs is one of the best sources of MSCs. It can substitute the Bone Marrow-Mesenchymal Stem Cells (BM-MSCs) since it has similar characteristics. hWJ-MSCs are neither embryonic stem cells nor adult stem cells. hWJ-MSCs have the characteristics of both embryonic stem cells and mesenchymal stem cells. Then, that properties can be used for stem-cells based therapies especially for regenerative diseases [28][34][35].

This study was aimed to compare the characteristics of two different methods that commonly used to isolate hWJ-MSCs, explant and enzymatic methods. The enzymatic method is fastest method, but it uses some enzyme to digest Wharton's Jelly tissues and extract the MSCs efficiently. The enzymes that usually used to digest Wharton's Jelly matrix is Collagenase 1, Hyaluronidase and Trypsin [36]. Collagenase is an enzyme isolated from Clostridium histolyticum and can degrade collagen tissue [37]. Trypsin is a proteolytic enzyme that degrade adherent protein, so the cells can be extracted from the tissue [38]. Hyaluronidase is an enzyme that catalyse the degradation of hyaluronic acid, one of components in Wharton's Jelly Matrix [39]. The combination of that enzymes provided great activity to digest extracellular matrix of Wharton's Jelly and extract MSCs efficiently.

Other methods to isolate MSCs from WJ tissue is Explant Method. The Tissue was cropped and chopped into small pieces., about 1 mm2. The figure was easiest method to isolate MSCs. The MSCs will outgrow from attached tissue. The disadvantage of this method is the inability to determine the number of the cells isolated from the tissue, so the determination of population doubling time at Passage 1 can be measured [36].

We suggest further works to obtain a quick method to obtain high purity of MSCs with xenofree medium. In this study, the isolation and characterization of WJ-MSCs was achieved with high purity and obtained at a more suitable time, but it still used FBS, which was xenogeneic, and that xeno genic medium may be has bad effect in hWJMSCs culture. In our study, the morphology of hWJ-MSCs in higher passage (P8) showed large shape hWJMSCs and had low self-renewal capacity (Figure 1 and Figure 4). That was caused by the use of FBS in the medium. Tonti and Manello (2008) stated that the use of FBS can cause immune reactions and transmission of pathogen [40].

4. Conclusion

In Conclusion, the enzymatic and explant methods can be used for isolation of hWJMSCs resulted in high purity of hWJ-MSCs. The explant method is the easiest one, and resulting higher purity of MSCs than the enzymatic method.

5. Acknowledgements

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