MCBS Office <mcbs_office@cellbiopharm.com>
Kepada:wahyu@amubbrc.co.id,wahyu_w60@yahoo.com
27 Agu jam 08.58

Dear Dr. Wahyu Widowati,

Regarding manuscript "Isolation, Characterization, Proliferation and Differentiation of Synovial Membrane -Derived Mesenchymal Stem Cells (SM-MSCs) from Osteoarthritis Patients" that has been accepted in Molecular and Cellular Biomedical Sciences.

Now we are in the process of typesetting and lay-outing, however we need the raw version of Figure 3 (Qualitative analysis of SM-MSCs differentiation and the Negative Control) in better quality. Can you provide us with it? It's okay if you send us the raw pictures without size mark and notes in .jpeg or .png.

Thank you so much for your attention. Hopefully we can hear from you before August 31, 2019. We wish you a nice day.

Best Regards,

MCBS Office

Isolation, Characterization, Proliferation and Differentiation of Synovial Membrane - Derived Mesenchymal Stem Cells (SM-MSCs) from Osteoarthritis Patients

Abstract

Background: Mesenchymal Stem Cells (MSCs) are the cells which has high renewal capacity and be able to differentiate into some types of cells. MSCs can be obtained from several tissues including bone marrow, synovial membrane, blood, adipose tissue, and periosteum. The proliferation and self-repair ability of MSCs are the advantages to use as stem cells – based therapy of various diseases. The purpose of this study is to determine the characterization, differentiation and proliferation of Synovial Membrane – derived Mesenchymal Stem Cells (SM-MSCs).

Materials and Methods: The cells proliferation capacity was determined by cell counting using trypan blue, characterization of MSCs (CD90, CD44, CD105, CD73, CD11b, CD19, CD34, CD45 and HLA-DR) using flow cytometry analysis, and differentiation capability into three lineage cells was determined by alcian blue, alizarin red, and oil red O staining.

Results: The type culture of SM-MSCs was adherent and showed positive surface marker of CD90, CD44, CD105, CD73 and negative of CD11b, CD19, CD34, CD45, HLA-DR. Based on the result, SM-MSCs P3 showed differentiation potency into adipogenic, chondrogenic, and osteogenic lineage cells. The population doubling time of SM-MSCs has ~~in~~increased from P3 to P~~8~~4. The population doubling time of SM-MSCs P3 was ~~6.26~~1.69 days and SM-MSCs ~~P4~~ P8 was ~~3.33~~3.64 days.

Conclusion: The results indicated that SM-MSCs isolated from OA patients has been successfully characterized and having three basic criteria to be defined as MSCs.

Keywords: Synovial Membrane, Mesenchymal Stromal Cells, Adipocyte, Chondrocyte, Osteocyte.

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Introduction

Osteoarthritis (OA) is the most common disease in the joints associated with disability and pain. (1) The prevalence of OA increases due to aging and an increase in related factors such as obesity. Radiographic evidence of knee osteoarthritis is present in about 30% of men and women over the age of 65 years. Estimates around the world are that 9.6% of men and 18.0% of women over the age of 60 have symptomatic osteoarthritis. About 80% of those with OA will have limitations in movement, and 25% cannot carry out their daily activities. (2)(3) OA is one of degenerative disease that occurs the most in elderly population. This disease affects joints and bones around it, and leading to chronic disability.(4) The main target of OA is cartilage tissue, and the starting point for OA is cartilage damage or loss.(5) The rate of chondrocytes regeneration as the component of cartilage tissue was decreased in older people (6), as a result, therapy is needed to cure OA. Unfortunately, the existing OA therapy has been used drugs administration and surgery for total joint replacement.(7) This therapy, is not effective because long-term drug administration causes negative effects on the other organs such as kidneys, digestive system and cardiovascular disorders.(8, 9) Consequently, an alternative therapy is necessary to regenerate damaged articular cartilage.

Chondrocytes are the main cellular component of cartilage which is most widely used for OA therapy. Autologous chondrocytes implantation (ACI) is a biomedical treatment cell-based for OA patients, the procedure takes place in three stages including cartilage isolation, cell grown in-vitro and cell implantation to patient.(10) But this therapy still has various disadvantages, for example surgery that causes cartilage damage, dedifferentiation in chondrocytes and has lower regeneration potency.(11) Therefore, another approach is required for cartilage regeneration in people with OA ~~autologically.~~use cell-based therapy.

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Stem cells can be utilized in tissue regeneration, for they have the ability to self-renew and differentiate as at the time of embryonic stage, in which cells can proliferate into tissue or certain organs during the developmental process.(12) Mesenchymal Stem Cells (MSCs) can differentiate into adipocytes, chondrocytes and osteocytes.(13, 14) MSCs can be isolated from various tissues such as bone marrow (BM-MSCs), adipose tissues (AT-~~S~~MSCs), umbilical cords (UC-MSCs), Wharton's jelly (WJ-MSCs), synovial tissues (ST-MSCs), and other tissues.(15, 16)

MSCs isolated from synovial membrane (SM) have ability to differentiate into chondrocytes that very suitable for cartilage regeneration therapy. MSCs are able to fuse with cartilage defects, proliferate, regenerate articular cartilage, reduce~~ing~~ the concentration of synovial fluid from prostaglandin (17) and reduce~~ing~~ the progressive nature of OA.(18) Future research should be directed at addressing the gap in diagnostics and ~~biomarkers~~therapy for OA. Hence, research on the potential of SM from OA patients in its use as autologous therapy necessary to be done. The objective of this study is to determine the character~~ization~~, differentiation and proliferation of synovial cells of membranes P4 & P8 isolated from patients with osteoarthritis.

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~~Osteoarthritis (OA) is one of degenerative disease that occurs the most in elderly population. This disease affects joints and bones around it, and leading to chronic disability.(1) The main target of OA is cartilage tissue, and the starting point for OA is cartilage damage or loss.(2) The rate of chondrocytes regeneration as the component of cartilage tissue was decreased in older people (3), as a result, therapy is needed to cure OA. Unfortunately, the existing OA therapy has been drugs administration and surgery for total joint replacement.(4) This is not effective because long-term drug administration causes negative effects on the other organs such as kidneys, digestive system and cardiovascular disorders.(5, 6) Consequently, an alternative therapy is necessary to regenerate damaged articular cartilage.~~

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Materials and Methods

~~MSCs Isolation from Synovial Membrane~~

MSCs Isolation from Synovial Membrane

Synovial membrane were obtained from the knee in patients ~~(n=3)~~ with ~~osteoarthritis~~

OA (n=3) ~~in~~from Dr. M. Djamil Hospital, Padang, West Sumatera, Indonesia. The patients have signed the informed consent using the guidelines approved by Research Ethics Committee, Faculty of Medicine, University of Andalas Padang, West Sumatera, Indonesia ~~.~~ with ethical numbers 226/KEP/FK/2019. Synovial membranes then washed using phosphate buffer saline (PBS) (Gibco, 14200075, Massachusetts, USA), 1% antibiotic and antimycotic (ABAM) (Gibco, 15240062, Massachusetts, USA) and 1% Amphotericin B (Gibco, 15290026, Massachusetts, USA). Synovial membrane cut into small pieces and cleansed from the fat and skin tissue. The synovial membrane placed into a 50 ml centrifuge tube (SPL, 50015, Pocheon-si, South Korea) containing Collagenase enzyme type I (Gibco, 17100017, Massachusetts, USA), Hyaluronidase (Sigma Aldrich, H-3506, Massachusetts, USA ~~Singapore~~) and Trypsin-EDTA 0.1% (Gibco, 25200072, Massachusetts, USA). The tube stored in CO_2 resistant shaker (Thermo Fisher Scientific, SHKE2000CO2, Massachusetts, USA) at 300 rpm for 16 hours in the incubator (Thermo Fisher Scientific, 8000DH, Massachusetts, USA) of 5% CO_2 and 37°C.(16) The remaining synovial membrane was removed, and the supernatant was centrifuged (MPW-260R, Warsaw, Poland) at 1600 rpm for 5 min. The supernatant was removed, and the pellet was mixed with complete medium: Mem-α (Gibco, 10950080, Massachusetts, USA) which supplemented by 10% Fetal Bovine Serum (FBS) (Gibco, 10270106, Massachusetts, USA), and 1% antibiotic-antimycotic (ABAM) (Gibco, 15240062, Massachusetts, USA), 1% Amphotericin B (Gibco, 15290026, Massachusetts, USA) addition. SM-MSCs was cultured at density of 2 x 10^6 cells in T-flask 25cm^3 (TPP, 90026, Trasadingen, Switzerland~~Singapore~~). The cells were incubated on T25 Flask (TPP, 90026, Trasadingen, Switzerland~~Singapore~~) at 5% CO_2 and 37°C incubator.(20).

SM-MSCs ~~CD~~ Surface Markers Characterization

SM-MSCs that reached 80% confluency were harvested (P4 & P8) to analyze the positive and negative Cluster of differentiation (CD) markers using flowcytometry (Macsquant,

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Analyzer 10, California, USA). The cells was stained with hMSCs analysis kit components (CD90 FIT C, CD105 PerCP-Cy5, CD73 APC, CD34 PE, CD116 PE, CD19 PE, CD45 PE, HLA-DR PE and CD44 PE) (BD stem flow^{TM}kit, 562245, New Jersey, USA) and the procedure was according to the manufacture protocol. The experiments and measurement were performed in triplicate.(18, 19)

Differentiation Capability of SM-MSCs

In osteogenic differentiation, SM-MSCs were seeded at density 5 x 10^3 cells in 24 well plate (Nunc, 72296-18, Massachusetts, USA) using StemPro Osteogenesis Differentiation Kit (Gibco A10072-01, Massachusetts, USA) for 3 weeks. Calcium deposits as the osteogenic differentiation marker were visualized using Alizarin Red S (Sigma Aldrich, A5533, Massachusetts, USA~~Singapore~~). For chondrogenic differentiation, SM-MSCs were seeded at density 5 x 10^3 cells in 4 well plate (Nunc, 176740, Massachusetts, USA) using StemPro Chondrogenesis Differentiation Kit (Gibco A10071-01, Massachusetts, USA) for 3 weeks. Glycosaminoglycan as the chondrogenic differentiation marker were visualized using Alcian Blue (Sigma Aldrich, A5268, , Massachusetts, USA~~Singapore~~). Adipogenic differentiation of SM-MSCs was performed by StemPro Adipogenesis Differentiation Kit (Gibco, A10070-01, Massachusetts, USA) for 3 weeks. Oil Red O staining was used (Sigma Aldrich, 00625~~-~~, Massachusetts, USA ~~Singapore~~) to confirm lipid droplets in the culture as the marker of adipogenic differentiation.(13, 22)

Population Doubling Time of SM-MSCs

Fourth until eighth (P4 – P8) passage of SM-MSCs in T75 flask were detached by 3 mL of trypsin-EDTA 0.25% (Gibco, 25200072, Massachusetts, USA) and was incubated at 5% CO_2 and 37°C incubator for 3 minutes. The complete medium was added to stop the trypsin and the cells were centrifugated (MPW-260R, Warsaw, Poland) at 1600 rpm for 5 min. The obtained pellet was added with 1 ml complete medium. The cells were counted by a

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hemocytometer (Neubauer, 17849, Lauda-Königshofen, Germany) using Trypan Blue (Sigma Aldrich, 25200072, Massachusetts, USA~~Singapore~~) 1:1 as the stain. Population Doubling (PD) was counted at every passage with the formula as follows (21):

$$PD = LOG (Harvest/Seeded)/ LOG_2$$

The PD time (PDT) was determined by the formula as follows (18):

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

Statistical Analysis?

Statistical was analyzed with Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software. Value was provided as Mean ± Standard Deviation. Significant diversity between the groups were determined using the Analysis of variance (One Way ANOVA) followed by Tukey's HSD Post-hoc Test.

~~Synovial membrane were obtained from the knee in patients (n=3) with osteoarthritis in Dr. M. Djamil Hospital, Padang, West Sumatera, Indonesia. The patients have signed the informed consent using the guidelines approved by Research Ethics Committee, Faculty of Medicine, University of Andalas Padang, West Sumatera, Indonesia. Synovial membranes then washed using phosphate buffer saline (PBS) (Gibco, 14200075), 1% antibiotic and antimycotic (ABAM) (Gibco, 15240062) and 1% Amphotericin B (Gibco, 15290026). Synovial membrane cut into small pieces and cleansed from the fat and skin tissue. The synovial membrane placed into a 50 ml centrifuge tube (SPL 50015) containing Collagenase enzyme type I (Gibco, 17100017), Hyaluronidase (Sigma, H-3506) and Trypsin-EDTA 0.1% (Gibco, 25200072). The tube stored in CO_2 resistant shaker (Thermo, SHKE2000CO2) at 300 rpm for 16 hours in the incubator (Thermo, 8000DH) of 5% CO2 and 37°C.(16) The remaining synovial membrane was removed, and the supernatant was centrifuged (MPW-260R) at 1600 rpm for 5 min. The supernatant was removed, and the pellet was mixed with complete medium: Mem-α (Gibco, 10950080) which supplemented by 10% Fetal Bovine Serum (FBS) (Gibco, 10270106), and~~

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1% antibiotic-antimycotic (ABAM) (Gibco, 15240062), 1% Amphotericin B (Gibco, 15290026) addition. SM-MSCs was cultured at density of 2 x 10^6 cells in T-flask 25cm^3 (TPP, 90026). The cells were incubated on T25 Flask (TPP, 90026) at 5% CO_2 and 37°C incubator.(17).

SM-MSCs CD Markers Characterization

SM-MSCs that reached 80% confluency were harvested (P4 & P8) to analyze the positive and negative CD markers using flowcytometry (Macsquant, Analyzer 10). The cells was stained with hMSCs analysis kit components (CD90-FIT-C, CD105-PerCP-Cy5, CD73-APC, CD34-PE, CD116-PE, CD19-PE, CD45-PE, HLA-DR-PE and CD44-PE) (BD stem flow™ kit, 562245) and the procedure was according to the manufacture protocol. The experiments and measurement were performed in triplicate.(18, 19)

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~~for 3 minutes. The complete medium was added to stop the trypsin and the cells were centrifugated (MPW 260R) at 1600 rpm for 5 min. The obtained pellet was added with 1 ml complete medium. The cells were counted by a hemocytometer (Neubauer, 17849) using Trypan Blue (Sigma, 25200072) 1:1 as the stain. Population Doubling (PD) was counted at every passage with the formula as follows (18):~~

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

~~**Statistical Analysis..?**~~

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Results

SM-MSCs Characteristic

The culture of SM-MSCs at 4th passage as the result of cells isolation from knee-joint of OA patients showed in Figure 1. SM-MSCs exhibited adherent type of culture, elongated and resulted in spindle-shaped cells. In the Table 1. and Figure~~.~~ 2, the cells isolated from synovial membrane of OA patients at 4th and 8th passage was showed positive for CD90, CD44, CD105, and CD73, and negative expression for negative lineage such as CD34, CD45, CD11b, CD19 and HLA-DR.~~.~~ The two different passages, ~~4th passage~~P4 (early) and ~~8th passage~~P8 (intermediate) of the cells showed no difference CD markers expression. Both ~~passage~~ P44 and ~~passage 8~~P8 were detected to have the same level expression of certain positive and negative surface markers.

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Differentiation of MSCs

The result of SM-MSCs differentiation into 3 different types of cells such as adipocyte, chondrocyte and osteocyte have shown in Figure 3. There was no difference result of differentiation between ~~passage~~ P4 (early) and P~~passage~~ 8 (intermediate) as indicates from the

qualitative staining result. Osteogenesis was confirmed by the accumulation of calcium deposit which detected by alizarin red S, and the positive result showed by the red color. Alcian blue staining is specific polyvalent dye that is used to identify acidic polysaccharides such as glycosaminoglycans as well as the marker of chondrogenesis. As seen at the table, the cells were associated in the process of differentiation and stained as blackish blue. Oil droplet formation is one of adipogenic differentiation marker and was stained by Oil Red O. Oil Red O is a diazo dye that is used to stain the triglycerides and lipid formation.(10, 20, 19, 21)

Population Doubling Time of SMMSCs

Population doubling time (PDT) and cumulative population doubling (CPD) have shown in Table 2. SM-MSCs isolated from the synovial shows that PDT and CPD increase along with the increase of passage and was significantly different among the passages except the 5^{th} and the 6^{th} passage were defined as not significantly different at $P<0.05$. The PDT and CPD can be seen in Ttable 2 and were used to measure the MSCs proliferation capacity and define as a specific time for cells to undergo the cell division. ~~PD time~~PDT from P3-P8 was ranged 1,69 to 3,64 days.

Discussion

SM-MSCs ~~Synovial membrane-derived mesenchymal stem cells from grade IV osteoarthritis of~~ the knee of OA patient with grade IV are used because they are waste that is not used anymore. OA patient with grade IV has low quality of stemness, MSCs quantity and quality decrease with aging, limiting the potential efficacy of MSCs for therapy ~~–~~the elderly population (22~~**Block *et al*., 2017**~~). We try to develop~~ed research~~ method by utilizing ~~unused~~waste materials and low quality. There are 3 basic criteria of the cells to be define as MSCs based on the

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International Society for Cellular Therapy, such as: 1) have the adherent type of culture, 2) have positive expression for several CD markers such as CD73, CD90, CD44 and CD105 and showed negative expression for CD14, CD34, CD45 and ~~Human Leucocyte Antigen (~~HLA-DR~~)~~, 3) have differentiation capability into 3 different lineage cells, osteocyte, chondrocyte, and adipocyte. The result indicates that the cells isolated from the synovial membrane of OA patients have conformed with the first basic criteria of MSCs, have adherent fibroblast-like cell population. ~~.~~ (21,22,23,27~~, **Ha et al., 2012**~~)

~~The second criteria for MSCs is the positive and negative expression of specific surface markers owned by the cells. MSCs naturally have positive surface marker of CD90, CD73 and CD105 and negative surface marker of CD45, CD34, CD11b, CD19, and HLA-DR. Several studies also confirmed that MSCs have positive surface marker of CD44.(21, 22) The cells isolated from synovial membrane of OA patients also conformed with the second basic criteria of MSCs.(26)~~

Markers characterization of the cells results in same finding with the previous research that the ~~stem cells~~MSCs isolated from different sources was also exhibited high expression of CD90, CD44, CD105, CD73 and low expression of CD34, CD45, CD14, CD19, and HLA-II both early (P4) and intermediate passage (P8). (21,13,25,24) ~~(18, 10, 20, 19)~~ It was also conformable with previous research that MSCs from various sources, including AT-MSCs, had positive markers for CD73, CD90, and CD105 and negative markers of CD11b or CD14, CD19, CD34, CD45, and HLA-DR in their cell-surface immunophenotyped.(28~~3~~) Several studies also confirmed that MSCs have positive surface marker of CD44.(21, 26~~2~~) The cells isolated from synovial membrane of OA patients also conformed with previous study which SM-MSCs had positive CD44. (28~~6~~)

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~~From the differentiation analysis result after 3 weeks of incubation, the cells that isolated from synovial membrane showed the ability to differentiate into 3 different lineages (adipocytes, osteocytes and chondrocytes) of cells as the third basic criteria to define MSCs.~~ According to the results, the cells isolated from the synovial membrane was considered as MSCs ~~because exhibited 3 basic criteria to be defined as MSCs, adherent to the plastic substrates, positive and negative expression to the certain CD markers, and~~ be able to differentiate into 3 types of cells (osteocyte, chondrocyte, adipocyte)~~.~~ Therefore, the method used in the cells isolation has successfully used to produce SM-MSCs and considered as the first step of application in stem cell mediated therapy for OA patients.

The PDT and CPD difference in the 5th and the 6th passage might caused by the proliferation capacity of cells were decreased at the higher passage. As indicated by MSCs derived from other sources such as ~~Bone Marrow Mesenchymal Stem Cells (~~BM-MSCs~~)~~ and ~~Adipose Tissue derived Mesenchymal Stem Cells (~~AT-MSCs~~)~~ have relative shorter culture time, the cell growth was slower at P10 – P11, at P14 – P16 in umbilical cord blood MSCs (UCB-MSCs).(2~~1~~94) The PDT result are supported by previous research conducted by Widowati *et al*. (2~~1~~18) which explained that WJ-MSCs induced by normoxia and hypoxia 2,5% and 5% have increase in proliferation time from early passage to ~~higher~~ older passage. The validation SM-MSCs from various grade OA patients is needed to confirm the isolation and characterization method. The validation of SM-MSCs for OA therapy in animal~~. But, OA treatment has limitation. Limitation for drug treatment is an innate ability to heal degenerated cartilage limited by the avascular nature of cartilages (28).~~is needed to continue models should eventually follow for further study.

Conclusion~~..~~ ~~2~~

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The results of this experiment indicated that SM-MSCs isolated from OA patients has been successfully characterized. SM-MSCs ~~-~~have high expression of CD90, CD44, CD105, CD73 and low expression of CD34, CD45, CD14, CD19, HLA-II, be able to differentiate into osteocyte, chondrocyte, adipocyte and have the adherent type of culture. ~~and having three basic criteria to be defined as MSCs.~~

Conflicts of Interest

The authors declares no conflict of interest

Acknowledgment

The authors gratefully acknowledge the financial support from the the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia. (Research Grant Hibah Kompetensi). This research was supported by Grant for Professor from Andalas University. This research was also supported by Aretha Medika Utama Biomolecular and Biomedical Research Center (AMU-BBRC), Bandung, Indonesia. The authors also would like to thank Annisa Amalia, Dwi Surya Artie, Dewani Tediana Yusepany, Anisa Siwianti from AMU-BBRC for their valuable assistance.

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Figures/Tables

Table 1. Antigen surface markers of synovial membrane mesenchymal stem cells

Passage	CD90	CD44	CD105	CD73	Negative Lineage
P4	92,52 ± 0,15	87,02 ± 0,58	98,34 ± 0,02	95,15 ± 0,59	0,42 ± 0,18

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P8	92.59 ± 0.11	87.88 ± 0.49	98.33 ± 0.02	95.67 ± 0.42	0.72 ± 0.18

*Data are presented as mean ± standard deviation of surface markers of synovial membrane mesenchymal stem cell. Negative lineage marker containing CD45 , CD34, CD11b, CD19, HLA-DR.

Table 2. PDT and CPD of Synovial Membrane Mesenchymal Stem Cells

Passage	PDT (d)	CPD
P3	1.69 ± 0.04[a*]	1.69
P4	1.79 ± 0.02[b]	3.46
P5	2.36 ± 0.03[c]	5.82
P6	2.33 ± 0.03[c]	8.15
P7	3.15 ± 0.01[d]	11.30
P8	3.64 ± 0.03[e]	14.94

*Data were presented as mean ± standard deviation. Different small letters (a, b, c, d, e) in the same column are significant at $P < 0.05$ (Tukey HSD post hoc test).

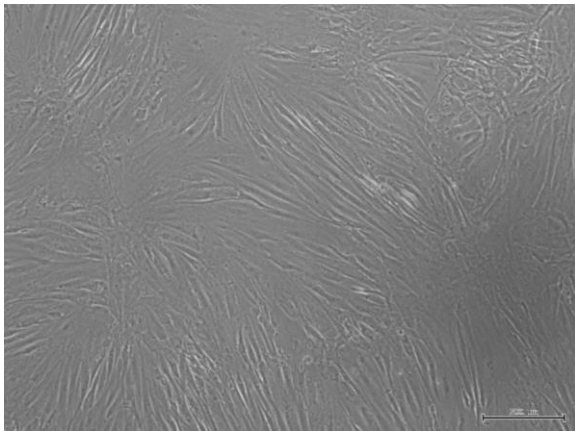

Figure 1. The culture of SM-MSCs at 4th passageP4 which have adherent characteristic and of culture

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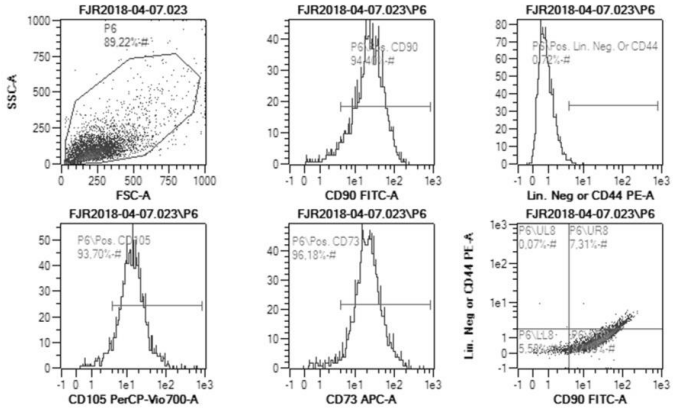

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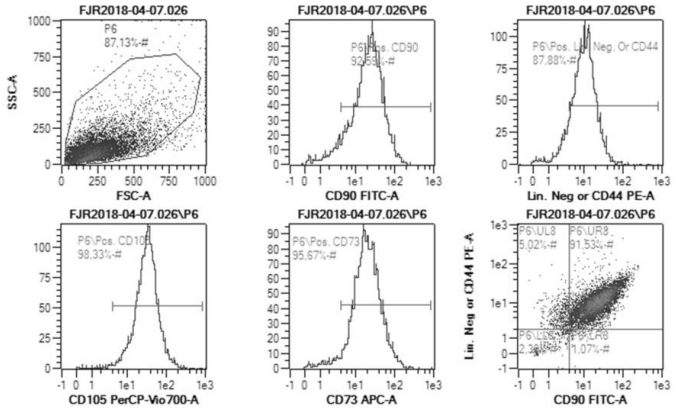
P6\Pos. CD90	94.40	3928	1.46e5	146.50
P6\Pos. Lin. Neg. Or CD44	0.72	30	1.12e3	1.12
P6\Pos. CD105	93.70	3899	1.45e5	145.41
P6\Pos. CD73	96.18	4002	1.49e5	149.26



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Path	%-#	Count	Count/mL	Count/µL
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P6\Pos. CD90	92.59	8327	1.67e5	166.54
P6\Pos. Lin. Neg. Or CD44	87.88	7903	1.58e5	158.06
P6\Pos. CD105	98.33	8843	1.77e5	176.86
P6\Pos. CD73	95.67	8604	1.72e5	172.08

Figure 2. Analysis CD markers expression on SM-MSCs P4 by flow cytometer. SM-MSCs was confirmed as MSCs because exhibited high expression for positive marker, CD73=95.15%; CD90=92.52%; CD105=98.34%, and low expression of negative lineage (0.72%), b) SM-MSCs also exhibited positive expression for CD44 as the additional CD markers to define MSCs.

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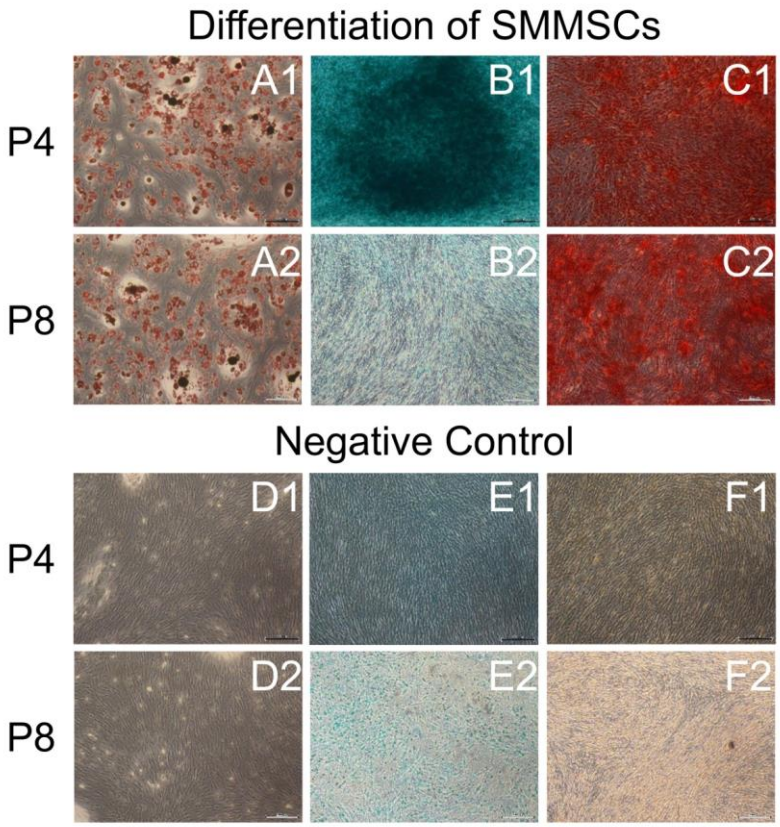

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507 **Figure 3.** Qualitative analysis of SM-MSCs differentiation at passage 4 and 8 by Alcian Blue, Alizarin Red S,
508 and Oil Red O staining for chondrogenic differentiation, osteogenic differentiation, and adipogenic differentiation,
509 respectively.
510 *A1 : Differentiation Adipocytes P4
511 A2 : Differentiation Adipocytes P8
512 B1 : Differentiation Chondrocytes P4
513 B2 : Differentiation Chondrocytes P8
514 C1 : Differentiation Osteocytes P4
515 C2 : Differentiation Osteocytes P8
516 D1 : Control Adipocytes P4
517 D2 : Control Adipocytes P8
518 E1 : Control Chondrocytes P4
519 E2 : Control Chondrocytes P8
520 F1 : Control Osteocytes P4
521 F2 : Control Osteocytes P8
522

RESEARCH ARTICLE

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Isolation, Characterization, Proliferation and Differentiation of Synovial Membrane-derived Mesenchymal Stem Cells (SM-MSCs) from Osteoarthritis Patients

Marlina[1], Rizki Rahmadian[1], Armenia[1], Wahyu Widowati[2], Rizal[3], Hanna Sari Widya Kusuma[3], Satrio Haryo Benowo Wibowo[3], Wahyu Setia Widodo[3], Ika Adhani Sholihah[3]

[1]*Faculty of Pharmacy, Andalas University, Padang, Indonesia*
[2]*Medical Research Center, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia*
[3]*Biomolecular and Biomedical Research Centre, Aretha Medika Utama, Bandung, Indonesia*

Background: Mesenchymal stem cells (MSCs) are the cells which has high renewal capacity and and are capable for differentiating into some types of cells. MSCs can be obtained from several tissues including bone marrow, synovial membrane, blood, adipose tissue and periosteum. The proliferation and self-repair ability of MSCs are the advantages to use as stem cells-based therapy of various diseases. The aim of this study was to determine the differentiation, characterization and priliferation of synovial membrane-derived MSCs (SM-MSCs).
Materials and Methods: The cells proliferation capacity was determined by cell counting using trypan blue, characterization of MSCs (cluster of differentiation (CD)90, CD11b, CD73, CD34, CD19, CD45, CD105 and human leukocyte antigen-DR isotype (HLA-DR)) using flow cytometry analysis, and differentiation capability into three lineage cells was determined with red alcian blue, oil red O and alizarin staining.
Results: The type culture of SM-MSCs was adherent and showed positive CD44, CD105, CD73, CD90 and negative of CD19, HLA-DR, CD11b, CD45, CD34 surface marker. Based on the result, SM-MSCs P3 showed differentiation potency into adipogenic, chondrogenic, and osteogenic lineage cells. The population doubling time of SM-MSCs has increased from P3 to P8. The population doubling time of SM-MSCs P3 was 1.69 days and SM-MSCs P8 was 3.64 days.
Conclusion: The results indicated that SM-MCSCs from osteoarthritis patients are able to differentiate into osteocytes, chondrocytes, adipocytes and highly express of CD105, CD73, CD90, CD44 and negative for CD34, CD45, CD14, CD19.

Keywords*: synovial membrane, mesenchymal stromal cells, adipocyte, chondrocyte, osteocyte*

Introduction

Osteoarthritis (OA) is the most common disease in the joints associated with disability and pain.[1] The prevalence of OA increases due to aging and an increase in related factors such as obesity. Radiographic evidence of knee OA is present in about 30% of men and women over the age of 65 years. Estimates around the world are that 9.6% of men and 18.0%

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Corresponding Author:
Wahyu Widowati
Medical Research Center, Faculty of Medicine
Maranatha Christian University
Jl. Surya Sumantri, Bandung, Indonesia
e-mail: wahyu_w60@yahoo.com

Cell and Biopharmaceutical Institute

of women over the age of 60 have symptomatic OA. About 80% of those with OA will have limitations in movement, and 25% cannot carry out their daily activities.[2,3] OA is one of degenerative diseases in old age population. The disease damage joints and bones around it, and leading to chronic disability.[4] The main target of OA is cartilage tissue, and the starting point for OA is cartilage damage or loss.[5] The rate of chondrocytes regeneration as the component of cartilage tissue was decreased in older people[6], as a result, therapy is needed to cure OA. Unfortunately, the existing OA therapy has been used drugs administration and surgery for total joint replacement.[7] This therapy is not effective because long-term drug administration causes negative effects on the other organs such as cardiovascular, kidneys, and digestive system.[8,9] Consequently, an alternative therapy is necessary to regenerate damaged articular cartilage.

Chondrocytes are the main cellular component of cartilage, which is widely used for OA treatment.Autologous chondrocytes implantation (ACI) is a biomedical treatment cell-based for OA patients, the procedure takes place in three stages including cartilage isolation, cell grown *in vitro* and cell implantation to patient.[10] But this therapy still has various disadvantages, for example surgery that causes cartilage damage, dedifferentiation in chondrocytes and has lower regeneration potency.[11] Therefore, another approach is required for regeneration of cartilage in OA patients.

Stem cells can be used in tissue regeneration, for stem cells have the ability to differentiate and self-renew at the time of embryonic stage, in which cells can proliferate into tissue or certain organs during the developmental process.[12] Mesenchymal stem cells (MSCs) can differentiate into adipocytes, chondrocytes and osteocytes.[13,14] MSCs can be isolated from various sources such as bone marrow (BM-MSCs), adipose tissues (AT-MSCs), umbilical cords (UC-MSCs), Wharton's jelly (WJ-MSCs), synovial tissues (ST-MSCs), and other tissues.[15,16]

MSCs isolated from synovial membrane (SM) have ability to differentiate into chondrocytes that very suitable for cartilage regeneration therapy. MSCs are able to fuse with cartilage defects, proliferate, regenerate articular cartilage, reduce the concentration of synovial fluid from prostaglandin[17] and reduce the progressiveness of OA.[18] Future research should be directed at addressing the gap in diagnostics and therapy for OA. Hence, research on the potential of SM from OA patients in its use as autologous therapy necessary to be done. The objective of this study was to determine the character, differentiation and proliferation of synovial cells of membranes P4 and P8 isolated from patients with OA.

Materials and methods

MSCs Isolation from SM

SMs were obtained from three knee in patients with OA from Dr. M. Djamil Hospital, Padang, Indonesia. The patients have signed the informed consent using the guidelines approved by Research Ethics Committee, Faculty of Medicine, Universitas Andalas, Padang, Indonesia (No. 226/KEP/FK/2019). SMs then washed using phosphate buffer saline (PBS) (Catalogue #14200075, Gibco, Massachusetts, USA), 1% antibiotic and antimycotic (ABAM) (Catalogue #15240062, Gibco) and 1% Amphotericin B (Catalogue #15290026, Gibco). SMs were cut into small pieces and washed, cleased from skin and fat. The synovial membranes were put into centrifuge tube (Catalogue #50015, SPL, Pocheon, South Korea) containing Collagenase enzyme type I (Catalogue #17100017, Gibco), Hyaluronidase (Catalogue #H-3506, Sigma Aldrich, Massachusetts, USA) and Trypsin-EDTA 0.1% (Catalogue #25200072, Gibco). The tube stored in CO_2 resistant shaker (Catalogue #SHKE2000CO_2, Thermo Fisher Scientific, Massachusetts, USA) at 300 rpm for 16 hours in the incubator (Catalogue #8000DH, Thermo Fisher Scientific) of 5% CO_2 and 37°C.[16] The remaining SM was removed, and the supernatant was centrifuged (MPW-260R, Warsaw, Poland) at 1600 rpm for 5 minutes. The supernatant was removed, and the pellet was mixed with complete medium: Mem-α (Catalogue #32561037, Gibco) which supplemented by 10% Fetal Bovine Serum (FBS) (Catalogue #10270106, Gibco), and 1% ABAM, 1% Amphotericin B addition. SM-MSCs was cultured at density of $2x10^6$ cells in T-flask 25cm^2 (Catalogue #90026, TPP, Trasadingen, Switzerland). The cells were incubated on T-flask 25cm^3 at 5% CO_2 and 37°C incubator.[19]

SM-MSCs Surface Markers Characterization

The SM-MSCs reached 80% confluence were harvested for analyzing the positive and negative cluster differentiation (CD) marksers (Fowcytometry analyzer 10, Macsquant, California, USA). The cells were stained with hMSCs analysis kit (Caltalogue#562245, BD stem flowTM Kit, New Jersey, USA) and the procedure was according to the manufacture protocol. The experiments and measurement were performed in triplicate.[18,20]

Differentiation Capability of SM-MSCs

The osteogenic differentiation, SM-MSCs (P4 and P8) were seeded at density $5x10^3$ in 24 well plate (Catalogue #142475, Nunc, Massachusetts, USA) using StemPro Osteogenesis Differentiation Kit (Catalogue #A10072-01, Gibco) for 3 weeks. Osteocytes were visualized using Alizarin Red S (Catalogue #A5533, Sigma Aldrich).

The chondrogenic differentiation, SM-MSCs (P4, P8) were seeded $5x10^3$ cells 24 well plate using StemPro Chondrogenesis Differentiation Kit (Catalogue# A10071-01, Gibco) for 3 weeks. Chondrocytes were confirmed using Alcian blue (Catalogue#A5268, Sigma Aldrich).

Adipogenic differentiation of SM-MSCs was executed by StemPro Adipogenic Differentiation Kit (Catalogue #A10070-01, Gibco) for 3 weeks. To confrim lipid droplets in the culture as the marker of adiponic differentiation using Oil Red O staining (Catalogue #00625, Sigma Aldrich).[13,21]

Population Doubling (PD) Time of SM-MSCs

P4-P8 of SM-MSCs in T75 flask were detached by 3 mL of trypsin-EDTA 0.25% (Catalogue #25200072, Gibco) and was incubated at 5% CO_2 and 37°C incubator for 3 minutes. The complete medium was added to stop the trypsin and the cells were centrifugated at 1600 rpm for 5 minutes. The obtained pellet was added with 1 mL complete medium. The cells were counted by a hemocytometer (Catalogue #17849, Neubauer, Lauda-Königshofen, Germany) using 1:1 Trypan Blue (Catalogue #T8154, Sigma Aldrich) as the stain. PD was counted at every passage with formula: PD = LOG (Harvest/Seeded)/ LOG_2.[22] Meanwhile, the PD time (PDT) was determined by this formula: PDT = t (time)/PD (in days).[18]

Statistical Analysis

Statistical was analyzed with Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software (IBM Corporation, New York, USA). Value was provided as mean±standard deviation. Significant diversity between the groups were determined using the analysis of variance (One Way ANOVA) followed by Tukey's HSD Post-hoc test.

Results

SM-MSCs Characteristic

Figure 1 showed the culture SM-MSCs at P4 as the result of cells isolation from OA patients. SM-MSCs exhibited adherent of culture, resulted, and elongated in spindle-shaped cells. SM-MSCs exhibited adherent type of culture, elongated and resulted in spindle-shaped cells. In Figure 2 and Table 1, SM-MSCs thet [illegible] isolated from OA patients at P4 and P8 showed negative expression for CD34, CD11b, CD45, CD19 and HLA-DR, yet showed high expression for CD44, CD90, CD73 and CD105. The two different passages, P4 (early) and P8 (intermediate) of the cells showed no difference CD markers expression. Both P4 and P8 were detected to have the same level expression of certain positive and negative surface markers.

Differentiation of MSCs

The result of SM-MSCs differentiation into 3 different types of cells. They were chondrocyte, adipocyte, and osteocyte as shown in Figure 3. There was no difference result of differentiation between P4 and P8 from the qualitative staining result. Osteogenic showed positive result by the red color and confirmed by the accumulation of calcium deposit which detected by Alizarin Red S. Glycosaminoglycan as well as the marker of chondrogenesis could be identified with Alcian Blue staining as specific polyvalent dye that was used to identify acidic polysaccharides. As can be seen in the Figure 3, the cells were associated in the process of chondrogenic differentiation and stained as blackish blue. Oil Red O was used as a dye in adipogenic differentiation which was characterized by the formation of oil droplets. Oil Red O was a diazo dye that was used to stain the lipid formation and triglycerides.[10,19,20,22]

PDT of SM-MSCs

PDT and cumulative population doubling (CPD) data were shown in Table 2. SM-MSCs isolated from the synovial

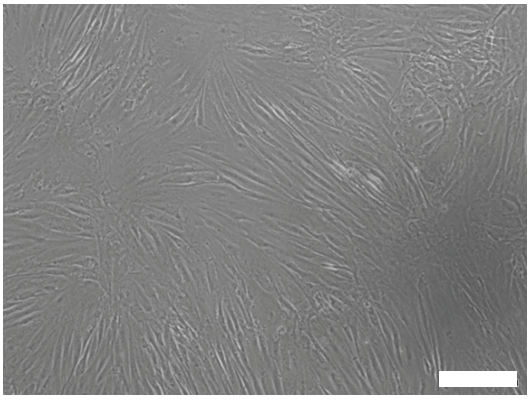

Figure 1. P4 of SM-MSCs culture with adherent characteristic . White bar: 200 μm.

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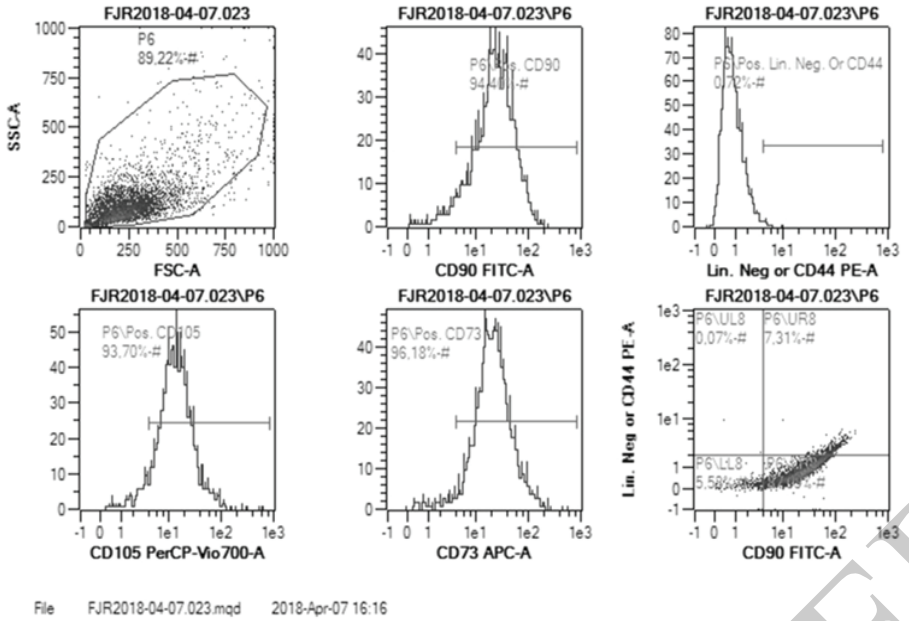

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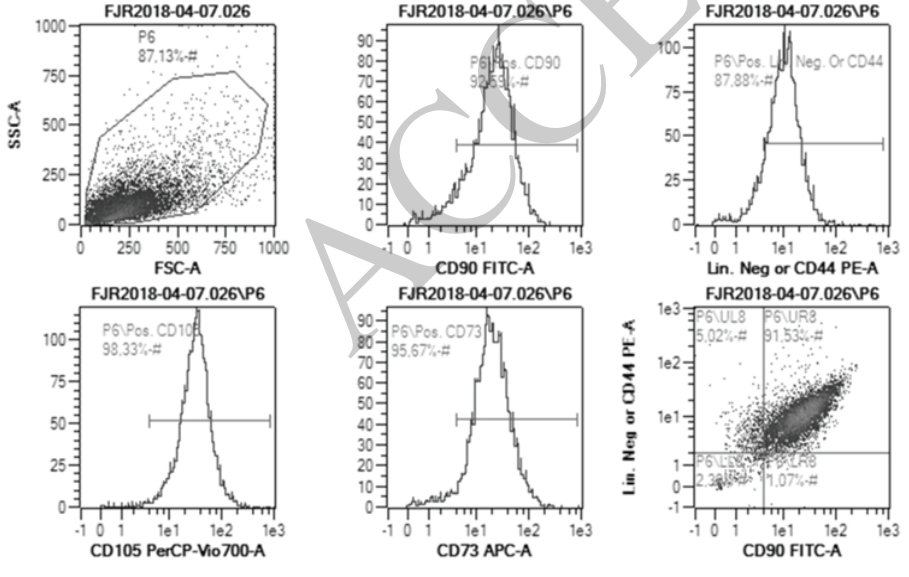
P6\Pos. Lin. Neg. Or CD44	0,72	30	1,12e3	1,12
P6\Pos. CD105	93,70	3899	1,45e5	145,41
P6\Pos. CD73	96,18	4002	1,49e5	149,26



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FJR2018-04-07.026	---	10321	2,06e5	206,42
P6\Pos. CD90	92,59	8327	1,67e5	166,54
P6\Pos. Lin. Neg. Or CD44	87,88	7903	1,58e5	158,06
P6\Pos. CD105	98,33	8843	1,77e5	176,86
P6\Pos. CD73	95,67	8604	1,72e5	172,08

Figure 2. The dot blot expression marker of SM-MSCs analyzed by flow cytometer. SM-MSCs was confirmed as MSCs with high expression for positive marker, CD90: 92.59%; CD73: 95.67%, and CD105: 98.33%. MSCs also low expression of negative lineage. SM-MSCs also exhibited positive expression for CD44 as the additional CD markers to define MSCs.

Table 1. The different eggect of passages toward surface markers of SM-MSCs.

Passage	CD90	CD44	CD105	CD73	Negative Lineage
P4	92.52±0.15	87.02±0.58	98.34±0.02	95.15±0.59	0.42±0.18
P8	92.59±0.11	87.88±0.49	98.33±0.02	95.67±0.42	0.72±0.18

Data are presented as mean±standard deviation of surface markers of SM-MSCs. Negative lineage marker containing CD45, CD34, CD11b, CD19 and HLA-DR.

shows that PDT and CPD increase along with the increase of passage and was significantly different among the passages except the 5th and the 6th passage were defined as not significantly different at $p<0.05$. The PDT and CPD can be seen in Table 2 and were used to measure the MSCs proliferation capacity and define as a specific time for cells to undergo the cell division. PDT from P3-P8 was ranged 1.69 to 3.64 days.

Discussion

SM-MSCs from the knee of OA patient with grade IV are used because they are waste that is not used anymore. OA patient with grade IV has low quality of stemness, MSCs quantity and quality decrease with aging, limiting the potential efficacy of MSCs for therapy the elderly population.[21] We try to develop method by utilizing waste materials and low quality. There are 3 basic criteria of the cells to be characterized as MSCs based on the International Society for Cellular Therapy, such as: 1) have differentiation capability into osteocyte, adipocyte, and chondrocyte; 2) have the adherent type of culture; 3) have positive expression for several CD markers such as CD44, CD73, CD90, and CD105 and showed negative epression for CD45, CD14, CD34, and HLA-DR.[21-24] Markers characterization of the cells results in same finding with the previous study showed that the MSCs isolated different sources was also showed high expression of CD73, CD44, CD90, CD105 and low expression of CD19, CD45, CD34, CD14 and HLA-II both early (P4) and intermediate passage (P8).[13,22,25] It also appropriate with previous study that MSCs from various sources, such as AT-MSCs had positive markers for CD90, CD73, and CD105. AT-MSCs had negative markers of CD14 or CD11b, CD34, CD19, CD45, and HLA-DR in their cell-surface immunophenotyped. Several studies also proved that MSCs have positive surface marker of CD44..[22,26,,27] The cells isolated from SM of OA patients also conformed with previous study which SM-MSCs had positive CD44.[26]

Table 2. PDT and CPD of SM-MSCs.

Passage	PDT (d)	CPD
P3	1.69±0.04[a]	1.69
P4	1.79±0.02[b]	3.46
P5	2.36±0.03[c]	5.82
P6	2.33±0.03[c]	8.15
P7	3.15±0.01[d]	11.3
P8	3.64±0.03[e]	14.94

Data were presented as mean±standard deviation. Different small letters (a,b,c,d,e) in the same column are significant at $p<0.05$ (Tukey HSD post hoc test).

According to the results, the cells isolated from the SM was considered as MSCs be able to differentiate into 3 types of cells (osteocyte, chondrocyte, adipocyte). Thus, the method has succesfully used to produce SM-MSCs and considered as the first step of application mediated therapy for OA patiens.

The PDT and CPD difference in the 5th and the 6th passage might caused by the proliferation capacity of cells were decreased at the higher passage. As indicated by MSCs derived from other sources such as BM-MSCs and AT-MSCs have relative shorter culture time, the cell growth was slower at P10-P11, at P14-P16 in umbilical cord blood MSCs (UCB-MSCs).[28] The PDT result are supported by previous research which explained that WJ-MSCs induced by normoxia and hypoxia 2,5% and 5% have increase in proliferation time from early passage to older passage.[22] The validation SM-MSCs from various grade OA patients is needed to confirm the isolation and characterization method. The validation of SM-MSCs for OA therapy in animal is needed to continue models should eventually follow for further study.

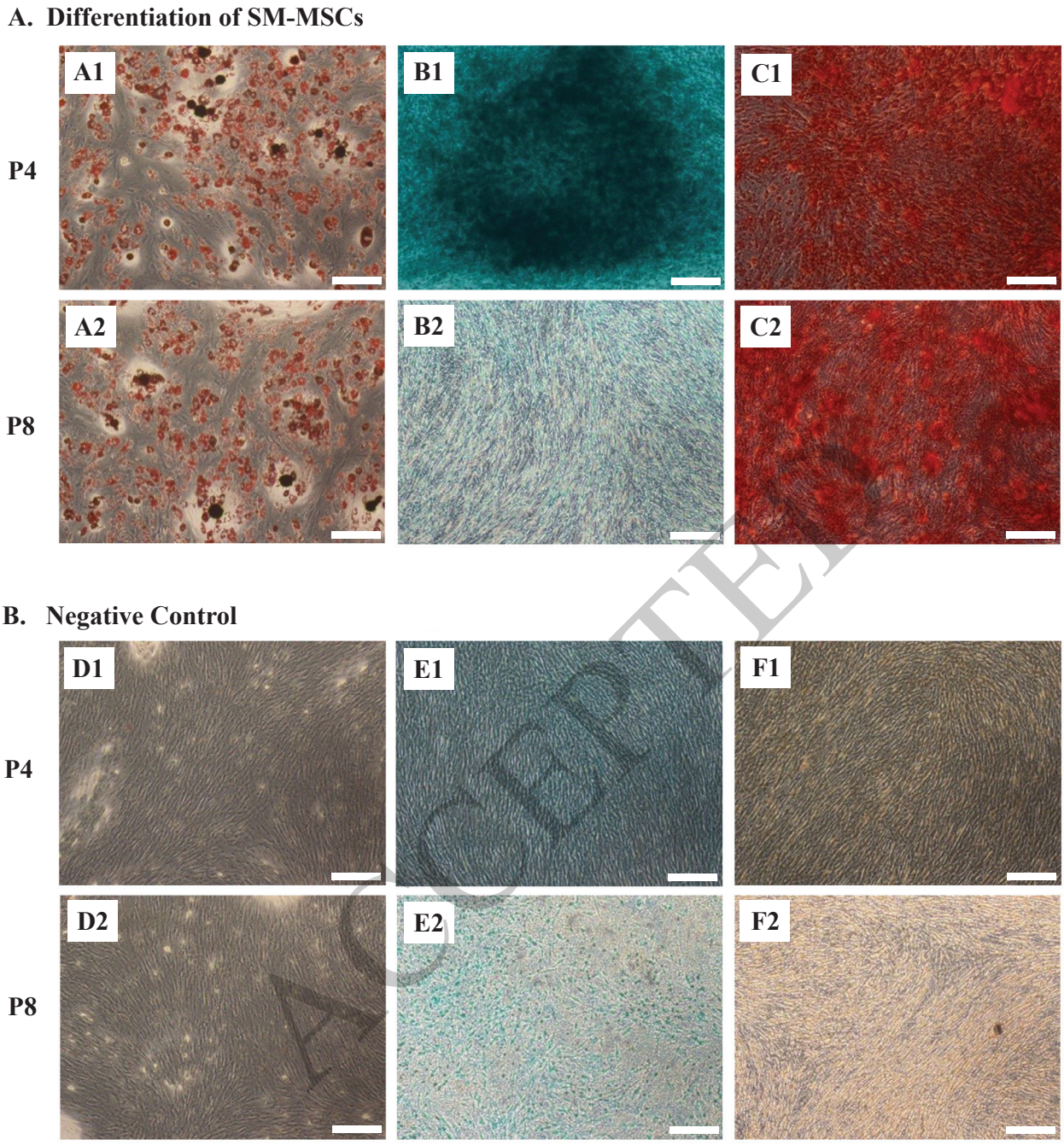

Figure 3. Morphology analysis of osteogenic, chondrogenic, adipogenic differentiation of SM-MSCs at P4 and P8. A1: Differentiation adipocytes P4; A2: Differentiation adipocytes P8; B1: Differentiation chondrocytes P4; B2: Differentiation chondrocytes P8; C1: Differentiation osteocytes P4; C2: Differentiation osteocytes P8; D1: Control adipocytes P4; D2: Control adipocytes P8; E1: Control chondrocytes P4; E2: Control chondrocytes P8; F1: Control osteocytes P4; F2: Control osteocytes P8.

Conclusion

The results of this experiment indicated that SM-MSCs isolated from OA patients has been successfully characterized. SM-MSCs have high expression of CD90, CD44, CD105, CD73 and low expression of CD34, CD45, CD14, CD19, HLA-II, be able to differentiate into osteocyte, chondrocyte, adipocyte and have the adherent type of culture.

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