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CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM WHITE ADIPOSE TISSUE OF *Macaca fascicularis*

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ABSTRACT

Mesenchymal stem cells (MSC) derived from white adipose tissue are considered to be an appropriate therapeutic candidate for various human diseases due to their natural ability to maintain homeostasis. They have also been considered for use as a therapeutic alternative due to their sufficient availability and ease of harvesting. MSC play an important role in the process of adipogenesis and energy metabolism that is directly related to the pathogenesis of various degenerative diseases and metabolic syndromes. The purpose of this study was to obtain MSC cultures from white adipose tissue biopsied from three adult males *Macaca fascicularis* and to evaluate these cultures qualitatively and quantitatively regarding their characteristics and ability to differentiate. Polymerase chain reaction (PCR) was used to confirm the expression of mesenchymal stem cell-specific gene markers, and a specific growth medium was used to differentiate these cells into adipocytes, chondrocytes, and osteocytes. The results obtained showed that the MSC culture from *M. fascicularis* WAT had a fibroblast-like morphology with a spindle shape. The MSC markers of CD73, CD90 and CD105, were also expressed. The MSC culture was also able to differentiate into adipocytes, chondrocytes, and osteocytes. Based on these results, white adipose tissue from *Macaca fascicularis* was found to have potential as a source of MSC.

Key words: *Macaca fascicularis*, mesenchymal stem cells, white adipose tissue

ABSTRAK

Sel punca mesenkimal (SPM) bersumber jaringan adiposa putih dianggap sebagai kandidat terapi yang tepat untuk berbagai macam penyakit manusia berdasarkan potensi alaminya untuk mempertahankan homeostasis dan telah menjadi perhatian untuk digunakan sebagai alternatif terapeutik karena ketersediaannya yang mencukupi dan kemudahan memanennya serta berperan penting dalam proses adipogenesis dan metabolisme energi yang berkaitan langsung patogenesis berbagai penyakit degeneratif maupun sindrom metabolik. Penelitian ini bertujuan untuk memperoleh kultur SPM bersumber jaringan adiposa putih yang dibiopsi dari tiga ekor Macaca fascicularis jantan dewasa dan mengevaluasi kultur tersebut secara kualitatif maupun kuantitatif terkait karakteristik dan kemampuannya berdiferensiasi. Polymerase cahin reaction (PCR) digunakan untuk mengkonfirmasi ekspresi dari marka gen khusus sel punca mesenkimal dan media penumbuh spesifik digunakan untuk mendiferensiasikan sel tersebut menjadi adiposit, kondrosit, dan osteosit. Hasil yang diperoleh menunjukkan kultur SPM asal jaringan adiposa putih M. fascicularis memiliki morfologi fibroblast like dengan bentuk spindle. Marka SPM yaitu CD73, CD90 dan CD105 juga terkespres. Kultur SPM tersebut juga mampu berdiferensiasi menjadi adiposit, kondrosit dan osteosit. Berdasarkan hasil tersebut maka JAP asal Macaca fascicularis terbukti memiliki potensi sebagai sumber SPM.

Kata kunci: Macaca fascicularis, sel punca mesenkimal, jaringan adiposa putih

INTRODUCTION

Stem cells are cells with the ability to self-renew and differentiate into specific cell lines. Stem cells also have an expression of specific markers on the surface of their membrane that contribute to the process of tissue regeneration and are able to secrete various soluble molecules in a paracrine manner. Along with the development of science and technology related to stem cells, especially mesenchymal stem cells (MSC), they also provide a new hope in curing various degenerative diseases (Meyer *et al*., 2018; Putra, 2019).

Mesenchymal stem cells have immune-privileged potential and have a low risk for tumor and teratoma formation (Chagasstelles and Nardi, 2011). MSC can be an option for clinical therapy because of its ease in vitro expansion and the ability to differentiate into various cell types (Putra, 2019). MSC from adipose tissue has gained a lot of attention as a therapeutic alternative (Meyer *et al*., 2018). The International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy propose three minimum criteria that adipose tissue-sourced MSC must have, they are: plastic adherence; the presence of CD73, CD90, and CD105 gene expression; and having the potential to differentiate into adipocytes, chondrocytes, and osteocytes (Palumbo *et al*., 2018).

Adipose tissue is a heterogeneous tissue that plays a role in adipogenesis (Cohen and Spiegelman, 2016). Adipose tissue functions as a place to store energy reserves and plays a role in maintaining metabolic and energy homeostasis (Karundeng *et al*., 2014). Adipose tissue consists of two types, white adipose tissue (WAT) and brown adipose tissue or (Cohen and Spiegelman, 2016). The WAT functions as an important endocrine organ for controlling energy and

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lipid homeostasis, which is a major determinant of body weight and insulin sensitivity in humans, and the conditions of lipodystrophy and cachexia, are also associated with complications of the metabolic syndrome (Vegiopoulos *et al.*, 2017).

Adipose tissue in humans can be used as a source of MSC but often its use is restricted by ethical problems. Therefore, animals are needed as an alternative source of MSC and as models for research support. The selection of animals as sources of MSC and the use of model animals must be accompanied by the application of the 3R rule (replacement, reduction, and refinement). *Macaca fascicularis* is a primate with a large population in nature but it is also classified as an endangered species by the IUCN (2020) and is included in Appendix II of the CITES (2019). *M. fascicularis* is similar enough in its anatomy and physiology to humans that it can serve as a source of MSC and animal model for degenerative and infectious diseases (Sajuthi *et al.*, 2016).

The absence of reports regarding the potential of *M. fascicularis* WAT as a source of MSC, as well as research on incidences of degenerative diseases and metabolic syndrome due to adipose tissue dysfunction should be of particular concern at this time. The MSC sourced from the WAT of *M. fascicularis* which has been expanded in vitro is expected to be useful in regenerative treatments for degenerative diseases and metabolic syndrome in animals and humans. The purpose of this study was to generate and characterize MSC cultures sourced from *M. fascicularis* WAT biopsies and evaluate the differentiation ability of these cultures. The information found in this study is useful as a learning model for the treatment of degenerative diseases and metabolic syndrome in animals and humans.

MATERIALS AND METHODS

White Adipose Tissue Collection

This study was conducted at the Research Animal Laboratory, Microbiology and Immunology Laboratory as well as the Biotechnology Laboratory of the Primate Animal Study Center, Bogor Agricultural University Research and Community Service Institute from November 2019-June 2020. The WAT samples were biopsied from three adult male *M. fascicularis* aged 6-8 years old and weighing 5-7 kg. The procedures that were carried out on the research animals were approved by the Institutional Animal Care and Use Committee from the Center for Primate Studies, Bogor Agricultural University number IPB PRC-19-B008. The WAT samples were obtained from biopsies of the subcutaneous tissue around the umbilical cords of the animals. The samples were collected after the animals had been anesthetized with a combination of ketamine 15 mg/kg and xylazine 0.5 mg/kg. Postoperatively, the animals were given analgesics and anti-inflammatory drugs with ketoprofen 2 mg/kg and tramadol 1 mg/kg and the antibiotic amoxicillin 11 mg/kg.

White Adipose Tissue-Sourced Cell Culture and Subculture

After obtaining the white adipose tissue samples through the biopsy method, they were then washed using phosphate-buffered saline (PBS). After washing the samples, 0.75% collagenase was added with the same volume as the PBS. Lastly, the samples were incubated at 37° C for 30 minutes. The tissue suspension and enzymes were resuspended until the tissue was destroyed and single-nucleated cells were obtained. A Dulbecco's Modified Eagle's Medium DMEM medium containing 20% Fetal Bovine Serum (FBS) was added and centrifuged at 350 g for 15 minutes to obtain cell pellets. The pellets were then washed using PBS and centrifuged for an additional 10 minutes. After being centrifuged for 10 minutes, the collected pellets were resuspended in the media, and the cells were counted using a hemocytometer. The cell population was cultured on a 6 well culture plate in an incubator with a temperature of 37° C and 5% CO_2 in the MSC conditioned selection medium. Subculture was carried out on cells that had grown on 6-well culture plates with confluence above 90%. Furthermore, the medium on the plate was removed and the cells were rinsed using PBS, then trypsination was carried out with 0.125% trypsin until the cells were released and formed single cells. The suspension was centrifuged and washed with PBS, then centrifuged again at 390 x g for 10 minutes. Cell pellets were then resuspended in the media for counting. The cell population was cultured on a 6-well culture plate in an incubator at 37° C and 5% CO_2 in a cell conditioned medium.

mRNA and RT-PCR Extraction

Extraction of mRNA was performed on the MSC cultures using the RNeasy kit (Qiagen, USA). The concentrations of mRNA were measured using a Nanodrop spectrophotometer (Thermo, USA) at wavelengths of 260 and 280 nm. Samples of mRNA with a concentration of 10ng were then reverse transcribed into cDNA strands using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). The mRNA extraction and reverse transcriptase PCR procedures adhere to the company's standard procedures. The reverse-transcribed cDNA strands obtained from these procedures were used in PCR.

Stem Cell Marker Detection

PCR amplification was performed to detect stem cell populations (CD73, CD90, and CD105). PCR reagents using goTaq PCR mix (Promega, USA) consisted of 25 µM MgCl, 400 µM dATP, 400 µM dTTP, 400 µM dCTP, 400 µM dGTP, nuclease-free water, and primers (forward and reverse) added to 2 µL of cDNA samples. The PCR amplification stages consisted of denaturation at 94° C for 30 seconds, annealing at 50° C according to the temperature melting (Tm) of each primer (Table 1) to be used for 30 seconds, and elongation at 72° C for 30 seconds, all of which was repeated for 40 cycles. The PCR results were electrophoresed on 2% agarose gel in

1x ethylenediaminetetraacetic acid (EDTA) tris acetate (TAE) buffer solution containing ethidium bromide. Electrophoresis was run at a voltage of 100 volts for 45 minutes. Visualization of the electrophoresis results was carried out using the Gel Doc documentation tool.

Adipogenic, Chondrogenic and Osteogenic Differentiation

MSC with a concentration of 3000/cm^2 were grown on adipogenic, chondrogenic, and osteogenic differentiation media respectively. Cells were cultured in this medium for 21 days and the medium was changed every three days. The cultured cells were incubated at 37° C with 5% of CO_2 concentration. The differentiated stem cells after 21 days were washed with PBS and fixed using a fixative solution. The results of fixation on the adipogenic media were then washed with ion-free water and incubated in Oil Red O working solution for 15 minutes, and then washed again using ion-free water. The results of fixation on the chondrogenic medium were then washed with diH_2O and incubated in 3% of acetic acid for three minutes and stained with 1% of Alcian blue for 60 minutes. Then the cells were rinsed using PBS, and stained with 0.1% of nuclear fast red for 20 minutes. The cells were then washed again using PBS. Fixation results on the osteogenic media were first incubated for 30 minutes, and then the cells were washed using PBS and incubated for another 45 minutes in alizarin red. The cells were then washed again and PBS was added. All stained cells were then observed under a microscope. The staining results were analyzed using a scoring technique that refers to DeRycke *et al.* (2009), score - (no staining), + (less than 10% of stained cells), ++ (10%-50% of stained cells), and +++ (more than 50% of stained cells).

Data Analysis

The data from research results in the laboratory was analyzed descriptively and the resulting data is presented in the form of images.

RESULTS AND DISCUSSION

The WAT cell cultures resulted in cell adhesion to the surface of the substrate in a plastic culture bottle, which means these cells have plastic adherence properties with cell morphology similar to the morphology of MSC, namely a fibroblast-like spindle shape (Figure 1). After 48 hours, the cell population became homogeneous and the subcultures and colonies

Table 1. Primer used for the MSC marker detection test

Marker	Nucleotide sequence (5'-3')		Reference
	Forward	Reverse	
5'-nucleotidaseecto (NT5E)/CD73	GACCTGGCTTTGTGACAGCAA	CTGACCCTGAGTAATCATGTCAGTCT	Mariya *et al.*, 2017
Thy-1 cell surface antigen (THY1)/CD90	CAGCTCACCCATCCAGTACGA	GTTGGTTCGGGAGCGGTAT	
Endoglin (ENG)/CD105	GACTGTCTTCACGCGCTTGA	GGAAGGCACCAAAGGTGATG3	
Glyceralaldehyde-3-phospate dehydrogenase (GAPDH)	CGGATTTGGTCGTATTGG	TCAAAGGTGGAGGAGTGG	Tian *et al.*, 2010

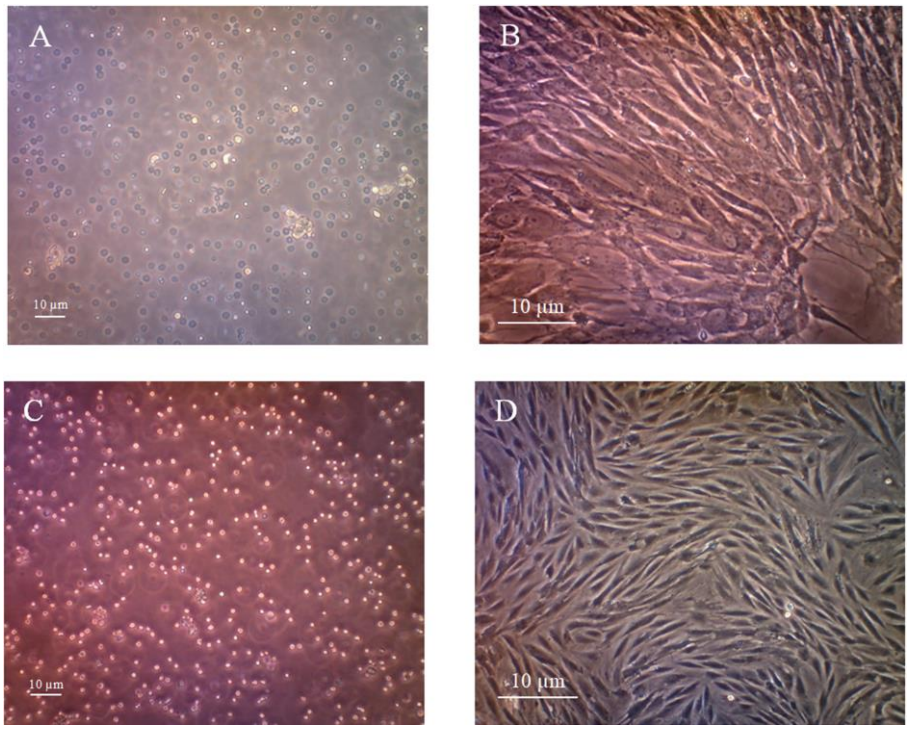

Figure 1. MSC culture and subculture from *M. fascicularis* WAT (A= Primary cells on day 0, B= Primary cells on day 8, C= Preserved cells on day 0, D= Preserved cells on day 8)

became visible. The cell population reached 80% confluence on day 8th, and the number continued to increase as the number of subcultures increased until the 4th subculture when it began to decrease.

The primary cells in this study were obtained by applying mechanical and enzymatic dissociation processes to WAT. The cells were then cultured under certain conditions until they grew to show a fibroblast-like morphology with spindle shape and reached confluence conditions. Due to these processes, it was necessary to harvest cells followed by subculture (passage). The subculture of the cell population was continued by changing the conditioned medium every 3-4 days until the cells experienced senescence. According to Hayflick's theory, the ability of a cell to sustain division will decrease and stop after passing through 40-60 divisions, at which point it will enter into the senescence phase (Putra, 2019).

Cell growth in vitro requires physiological conditions similar to in vivo tissue in order to maintain the metabolic functions and biology of the cells in a stable state. Factors that affect the optimum conditions of cultured cells include the physiological conditions of the culture (temperature, pH, osmolarity, and CO_2 pressure) and the microcellular environment of the culture (plastic culture bottles and culture media). The optimum temperature required for a cell culture is 37° C with a pH of 7.2-7.4. The osmolarity factor depends on the concentration of dissolved particles in the culture medium, and the required CO_2 pressure should be 5%. The appropriate culture microcellular environment should consist of a plastic culture bottle, which is a place for cells to grow and attach because it is made of a material that has adhesive properties, and there also needs to be a culture medium present that serves to provide various dissolved nutrient molecules that promote cell growth and metabolism. The culture medium used in this study was DMEM which has higher levels of amino acids and vitamins than other common media. The culture medium used in this study also contained a culture serum in the form of FBS which functioned as a hormonal factor that stimulates cell growth and proliferation in addition to initiating differentiation. FBS also functions as an attachment for cells and is also a stabilizing factor that maintains pH and detoxifies the medium.

Cultured cells have a growth pattern that begins with an adaptation process to the medium in which they are contained, and then there is a period of slow growth followed by exponential proliferation and ends in a stationary state where there is a decrease in growth until it stops. Therefore, a subculture is needed to keep the cultured cells in a state of optimum density. The pattern of cell growth consists of lag (latent), log, and stationary phases. The lag phase is the initial period in which cells in a culture adapt to a niche and typically lasts 12-24 hours. This phase is also known as the latent phase because the cells have not shown any growth activity. The log phase is a phase in which cells grow and proliferate exponentially and can duplicate themselves rapidly with a predetermined time (known as the doubling time). It is in this phase that cell growth fills plastic culture bottles and the cells consume a lot of nutrients. When the cells reach 80% confluence, the cells will enter the stationary phase which can cause the opposite reaction, and because of this it is necessary that a subculture process be carried out. The stationary phase is the phase where the cultured cells stop growing and proliferating. During this phase, the full cell condition might trigger the cells to differentiate into specific mature cells, and some cells may even exit the cycle and go to the G0 phase even though they are still viable. The subculture mesenchymal stem cells in this study successfully expressed the gene markers CD73, CD90, and CD105. The expression of gene markers through PCR testing can be seen by the presence of DNA bands on the gel electrophoresis results (Figure 2). The MSC of the three *M. fascicularis* did not show any differences in the expression of the gene markers CD73, CD90, and CD105. This indicates that WAT from the three *M. fascicularis* can be used as a source of MSC.

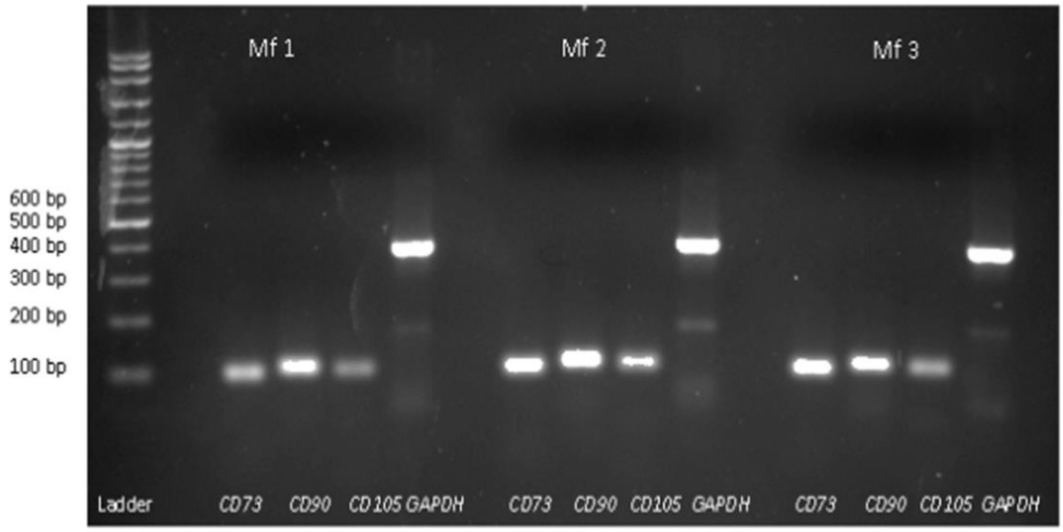

Figure 2. Electrophoregram of MSC marker gene expression markers and house keeping genes of the three *M. fascicularis* (Mf 1, Mf 2, and Mf 3). CD73= 101bp, CD90= 105bp, CD105= 104bp, GAPDH= 352bp

Clusters of differentiation (CD) are markers on the cell surface that function as receptors and play an important role for cells (Harsan *et al*., 2015). CD73 and CD105 are markers of MSC that play a role in the formation of the cytoskeleton and cell morphology (Roura *et al*., 2006). Expressed CD105 also showed high levels of MSC activity in colony forming unit-fibroblasts (CFU-F). Colony forming unit-fibroblasts are a group of cells that morphologically resemble fibroblasts that can form colonies. CD90 is a marker that can be expressed by bone marrow-derived MSC and a stromal vascular fraction (SVF) but SVF does not express CD105. Stromal vascular fractions are cells derived from adipose tissue with heterogeneous characteristics that are able to attach to culture bottles (Putra, 2019).

At the stage of cell differentiation into adipocytes, chondrocytes, and osteocytes, changes in cell morphology were already visible on day 7, although MSC morphology was still found and some cells died. Some of the cells had undergone morphological changes on day 14 and fully differentiated on day 21. Staining using Oil Red O caused adipocytes to turn red, while chondrocytes turned bluish green after being stained with alcian blue. In addition, osteocytes became brownish-orange in color after being stained with Alizarin Red (Picture 3).

Based on its ability to differentiate, MSC is classified as multipotent, which means that it can differentiate into various cells in one embryonic layer. MSC which originally had a fibroblast-like morphology with a spindle shape became cuboidal with a cytoplasm composed of vacuoles containing oil drops. The use of FBS, in general, can stimulate adipogenic induction spontaneously. The use of medium-induced special supplements for adipogenic differentiation such as Mesencult Adipogenic Differentiation Supplement and administration of a number of chemicals such as dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and high concentrations of insulin can also promote differentiation into adipogenic cells (Niemela *et al*., 2008). Staining using Oil Red O on adipocytes caused the cuboidal cells that form fat deposits to turn red. This happens because Oil Red O is a dye with lysochrome properties which means it is fat-soluble.

Chondrocytes are cartilage cells that cover the matrix or solid material. Cartilage contains a mixture of glycosaminoglycans and protein in its basic substance which gives it a strong character even though it has a greater degree of elasticity and compressibility than bone (Sloane, 2004). The results of the differentiation of MSC into chondrocytes show that these cells have oval, polygonal, or stellate morphology which when reaching 80% confluency will aggregate and then form nodules/aggressors. Staining of chondrocytes was done by using alcian blue dye which is a polysaccharide dye. Alcian blue will color glycosaminoglycans which will cause chondrocytes to appear bluish-green.

The use of a MSC differentiation medium added with several chemicals such as dexamethasone, ascorbic acid, glycerol phosphate, and FBS can induce MSC to differentiate into osteocytes. Identification of osteocyte morphology was easier to do with alizarin red staining. This dye detects calcium deposits produced by osteocytes. The redder the osteocyte, the more calcium is deposited in it. Calcium concentration can affect the morphology of MSC into osteocytes through cell-to-cell matrix interactions (Nakamura *et al*., 2010).

The scoring of the differentiation results showed that more than 50% (+++) of MSC that differentiated into adipocytes, and 10%-50% (++) of MSF that

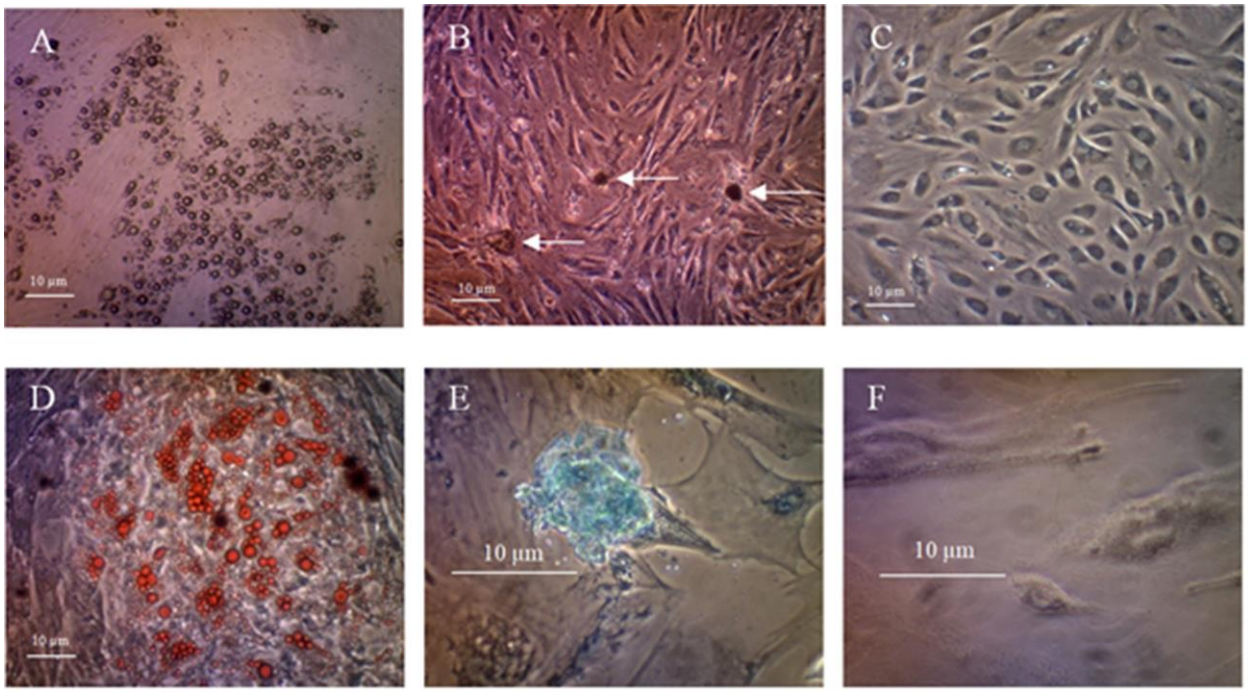

Figure 3. MSC differentiation and staining of differentiation results. A= Adipocytes, B= Chondrocytes, C= Osteocytes, D= adipocytes stained with Oil Red O, E= Chondrocytes stained with Alcian Blue, F= Osteocytes stained with Alizarin Red

differentiated into chondrocytes were successfully stained. Meanwhile, MSC that differentiated into osteocytes only showed the proportion of stained cells by less than 10% (+). The proportion of stained cells corresponded to the rate of cell growth during subculture where adipocytes needed a faster time to reach 80% confluency compared to chondrocytes and osteocytes. The rate of differentiation of MSC also depends on the source of the MSC itself. In this study, the source of MSC used was adipose tissue, so the MSC had more of a tendency to differentiate into adipocytes compared to chondrocytes and osteocytes.

Putra (2019) stated that the amount of MSC contained in WAT is more than stromal tissue.Therefore, adipose tissue is considered to be an excellent source of MSC because of its sufficient availability and the fact that it can be harvested easily. The use of MSC derived from adipose tissue also carries with it a lower risk of complications for patients compared to other sources of MSC. Various research journals have reported that MSC is strongly linked to the restoration and regeneration of various tissues which have suffered from damage and/or degeneration. Some of the disorders that have been treated with MSC include neurodegenerative disorders, cardiovascular lesions, hormonal dysfunction and insufficiency, immune system disorders, musculoskeletal disorders as well as skin and corneal disorders. In this study, MSC from *M. fascicularis* WAT was successfully differentiated into various cell lines, thus showing the clinical potential of MSC as a regenerative medicine.

CONCLUSION

MSC cultures from *M. fascicularis* WAT were successfully generated and were able to meet the following three criteria: plastic adherence with spindle-shaped fibroblast-like morphology, having the expression of CD73, CD90, and CD105 genes, and the ability to differentiate into adipocytes, chondrocytes, and osteocytes.

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