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ISOLATION AND CHARACTERIZATION OF SKIN DERIVED MESENCHYMAL STEM CELL (SMSCs) FROM NEW ZEALAND RABBIT, *ORYCTOLAGUS CUNICULUS* : A *IN VITRO* STUDY

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ABSTRACT : Mesenchymal stem cells (MSCs) exhibit high proliferation and self-renewal capabilities and are critical for tissue repair and regeneration. Furthermore, they can be isolated from a variety of tissues and have many potential applications in the clinical setting. In this study, Skin Derived Mesenchymal Stem Cells (SMSCs) were isolated from a New Zealand rabbit and analyzed for the skin sampling area, separation method, culture conditions, primary and passage culture times, and cell surface markers. The result shows that SMSCs have surface markers of mesenchymal stem cells by immunofluorescence of CD105, CD90, CD73, CD44, and CD200 and the CD105, CD90, and CD73 positive markers were also confirmed with Flow cytometry. Furthermore, negative markers of CD45 and CD34 did not show in immunofluorescence but CD45, CD34, and CD133 were confirmed with Flow cytometry.

Key words : SMSCs, flow cytometry, immunofluorescence, mesenchymal stem cells.

INTRODUCTION

Mesenchymal Stem Cells (MSCs) have considerable regeneration abilities that are beneficial in tissue engineering. The MSCs are multipotent stromal cells which have osteogenic, chondrogenic, and adipogenic abilities and are regarded as readily available regenerative cells that are able to mobilize to the certain signal of various cell response in the body. Furthermore, they have a high proliferative growth capacity.

These cells are well known for their potential therapeutic value in clinical use because of their high proliferative capacity, ability to differentiate into multiple lineages and ability to migrate into injured organs and cancers (Panes *et al*, 2016; Mohr and Zwacka, 2018).

The skin is the most easily accessible solid tissue source and has been shown to be a promising source of various adult stem cell or progenitor cell populations, such as epidermal stem cells (Kim *et al*, 2004), follicle stem cells (Xi *et al*, 2003) and dermal progenitors (Shyer *et al*, 2017).

This organ has a crucial role in protecting the body against external factors, such as mechanical strokes and infections. During the maintenance of the body homeostasis, the integrity of the skin is provided via fluid balance, flexibility, thermal regulation and keratinocytes exhibiting high mitotic activity.

In the current review, we aimed to summarize the characteristic properties of MSCs and their potential clinical use in skin wound healing. Thereby, an alternative therapy using MSCs is exhibited with advantages in the dermatology area.

MATERIALS AND METHODS

This study received the ethical clearance approval letter with number 289/HRECC.FODM/XII/2017 for animal subjects from the Faculty of Veterinary Medicine, Universitas Airlangga Surabaya, East Java, Indonesia Ethics Research Committee. The research was conducted at an experimental laboratory within the Stem Cell Research and Development Centre, Universitas Airlangga, Surabaya, East Java, Indonesia. The exploration of SMSCs involved the use of the dorsal skin of an adult

male white New Zealand rabbit. The immunofluorescence and flow cytometry were performed at passage 4 to examine and measure the expression of CD34, CD44, CD45, CD73, CD90, CD105 and CD133.

Rabbit Skin Derived Mesenchymal stem cell isolation

SMSCs were isolated from a healthy white New Zealand male rabbit that was 6 months old at the time of the test and weigh 2-3kg. A 4 × 4cm segment was taken from the dorsal skin. Before sampling, the rabbit was sedated with Ketamine and Xylazine and the dorsal area was shaved clean. The samples were then washed with a NaCl saline solution to cleanse blood and debris. The tissue was digested with Collagenase type IV (Worthington, USA) 0.075% at 37°C for 45 minutes. The resulting cell suspensions were seeded and cultured in 600mm culture dishes (Iwaki) in Alpha MEM Medium (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA) and 1% L-Glutamine (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), and 1% Amphotericin B (Sigma-Aldrich, USA) at 37°C in a 5% CO_2 incubator. The medium was changed every 3 days and after 90% of confluency, the cells were passaged.

Characterization of SMSCs by immunofluorescence

The cells that have been 90% monolayer confluency were made into single cells with triple express and incubated at 37°C, 5% CO_2 for 5 minutes. The detached cells were centrifuged at 2000rpm for 5 minutes. The pellets were added to 1ml of αMEM growth medium (Sigma Aldrich®, USA). SMSCs were seeded 2×10^4 and cultured in a coverslip (Iwaki) and incubated at 37°C in a 5% CO_2 incubator for 3 days. The coverslip was harvested and fixated with absolute methanol for 5 minutes, then washed twice with PBS (Sigma-Aldrich, USA) tween and permeabilized with 0.5% TritonX100 (Sigma-Aldrich, USA) for 5 minutes, then blocked with 1% BSA (Sigma-Aldrich, USA) for 30 minutes. After the blocking proses, SMSCs were stained with anti-CD34, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti-CD200 and anti-CD105. Monoclonal antibodies (Bioss) were added to each sample and then incubated at 37°C for 1 hour. The immunofluorescence (IF) examination on the coverslip with 50% glycerin was dropped above the glass object and also stained with DAPI for cell nucleus staining. The results were observed using a fluorescence microscope with a 100x magnification (Automated Fluorescence Microscope, BX63, Olympus®, USA). The result color showed fluorescence, meaning that there are positive surface markers, while the DAPI staining showed the blue color of the stained cell nuclei.

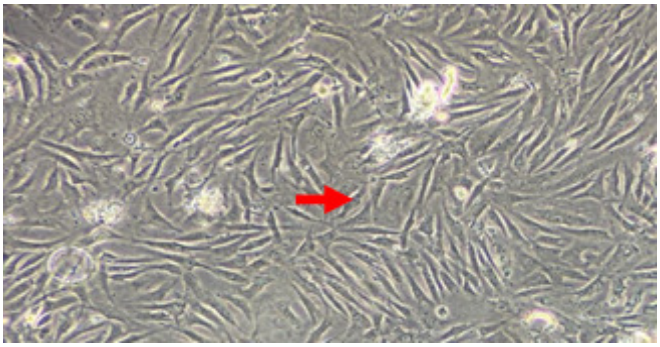

Fig. 1 : The morphology of SMSCs showed a spindle-shape, fibroblast-like cell and adherence to plate under an inverted microscope.

The negative conclusion of IF markers showed that the stained cells emitted a low fluorescent color.

Characterization of SMSCs by flow cytometry

The characterization of SMSCs using flow cytometry was done based on the research of Fedik *et al* SMSCs (passage 4) that had been 90% monolayer were made single cells with triple express. Triple express was added and incubated at 37°C, 5% CO_2 for 5 minutes and the detached cells were centrifuged at 2000rpm for 5 minutes. The cells supernatant were incubated with specific monoclonal antibodies (10mg/mL), conjugated with Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE) and PerCP. The anti-CD34, anti-CD133, anti-CD45, anti-CD73, anti-CD90, anti-CD105 primary monoclonal antibodies were used. The cells were then diluted in 4ml PBS, centrifuged and resuspended with 600ml phosphate buffered saline–formaldehyde 2% for 1 hour on ice in a dark room. The isotype controls used were immunoglobulin G (IgG1) FITC and IgG1 PE monoclonal antibodies for 45 minutes on ice in a dark room. The cells were fixed in a fluorescence-activated cell sorting solution after washing. The analysis was performed with flow cytometry (Epics-XL/MCL, Beckman Coulter, Fullerton, CA, USA).

RESULTS

SMSCs were isolated from a healthy white New Zealand male rabbit that was 6 months old and 2-3kg in weight. A 4x4cm segment was taken from the dorsal skin and incubated at 37°C in a 5% CO_2 incubator. The medium was changed every 3 days and after 90% of confluency, the cells were passaged. SMSCs showed a spindle-shape, fibroblast-like cell under an inverted microscope (Fig. 1). The SMSCs expressed higher positive MSCs markers (Fig. 2) than the negative control, emitting low HSCs markers (Fig. 3). The CD73 expression has the highest mean among other positive MSC markers examined using immunocytochemistry (Fig.

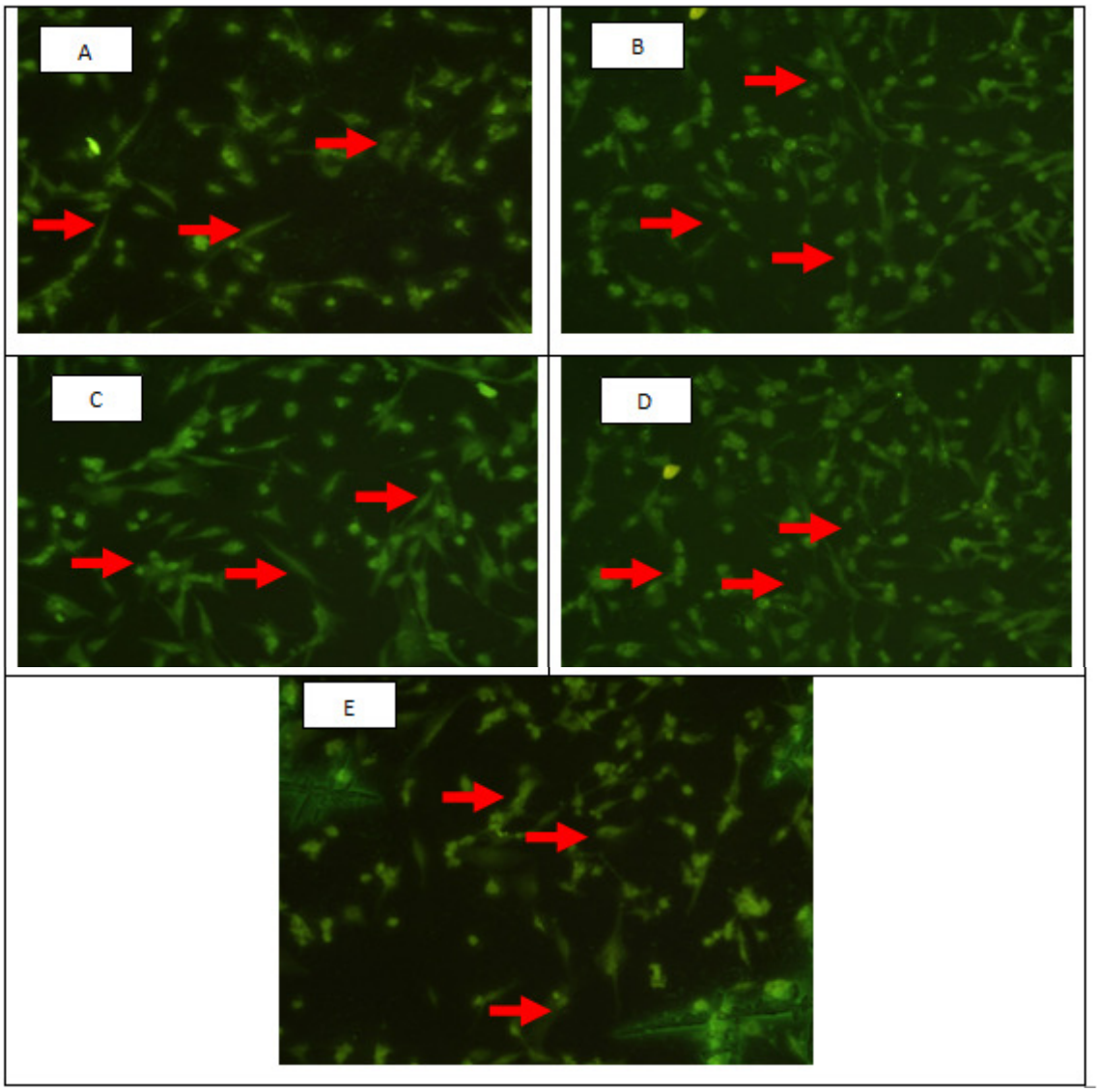

Fig. 2 : SMSCs expressed positive (+) MSCs surface cell markers (Red Arrow). A,(CD200) B. CD90; C (CD105), D (CD73); E, (CD44).

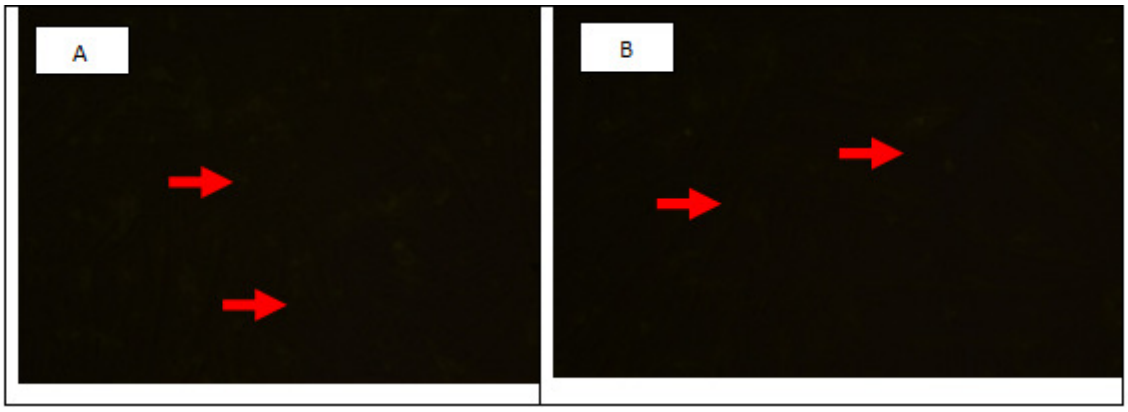

Fig. 3 : SMSCs expressed low HSCs surface cell markers (Red Arrow). A (CD45), B(CD34).

4) and flow cytometry (Fig. 5).

DISCUSSION

Mesenchymal stem cells have generated interest in a wide variety of biomedical disciplines including clinical therapy applications. These cells can be sourced from various tissues like blood, bone marrow (BM), trabecular bone, adipose tissue (AT), dermis, synovium, skeletal muscle, and pericytes (Tuan *et al*, 2003). Skin is the largest organ of the body and has long been researched as a potential source of regenerative cells. For the past

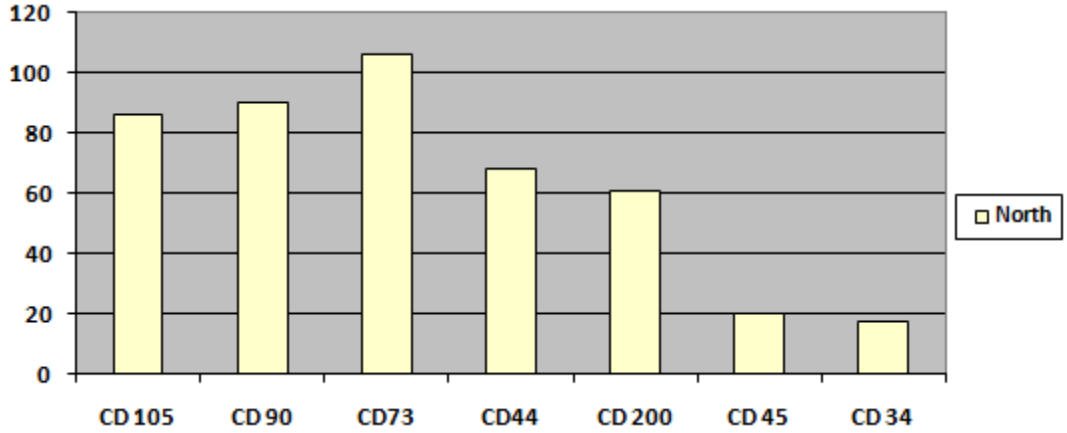

Fig. 4 : The mean of SMSCs expressed positive (+) MSCs surface cell markers CD44, CD73, CD90, CD105, CD200 and negative (-) HSCs surface cell markers CD34 and CD45.

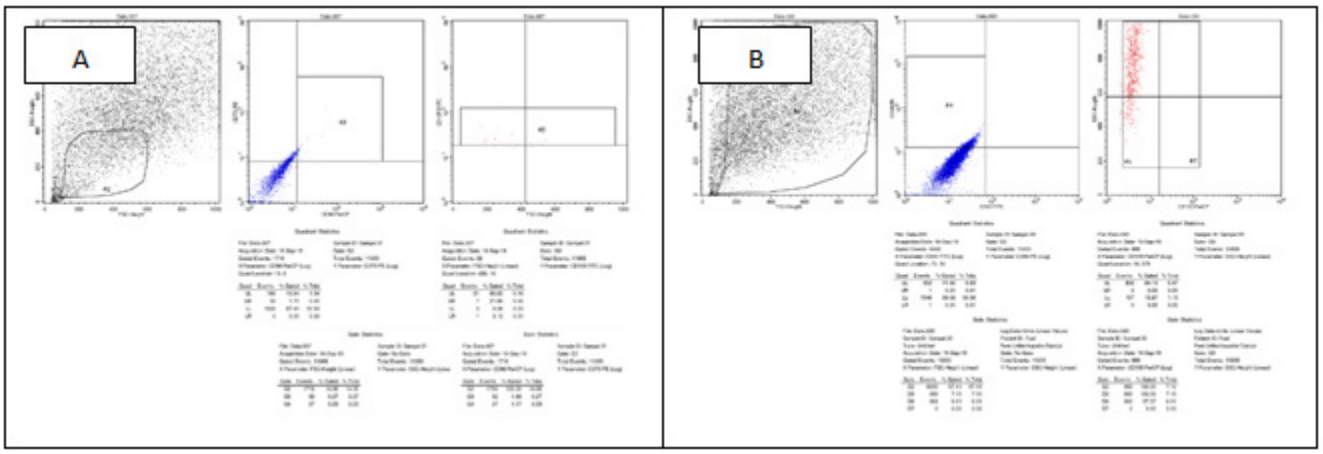

Fig. 5 : Flow cytometry result of SMSCs characterization. A. SMSCs highly expressed CD73, 90 and CD105; B. SMSCs low expressed CD45, CD133 and CD34.

decade, it has been shown that skin-derived multipotent stem cells (SMSCs) are primitive, unique, multipotent stem cells and have multilineage differentiation abilities. These indicated that skin can be a good alternative source of progenitor cells for the study and differentiation (Dongmei *et al*, 2014). According to previous studies, the standard criteria suggested for MSCs characterization included the following: (i) stick to culture plate in culture conditions; (ii) have osteogenic, chondrogenic, and adipogenic ability, (iii) specific marker expression (positive CD73, CD90 and CD105 expression; negative marker of HSCs (Nugraha *et al*, 2018)). The mesenchymal and tissue stem cell committee of the international society for cellular therapy (ISCT) proposed the minimal criteria to define human MSCs. First, MSCs are plastic-adherent cells when they are maintained in standard culture conditions. Second, they should express CD105, CD73, CD90 and lack the expression of CD45, CD34 (Dominici *et al*, 2006). SMSCs showed heterogeneously irregular shapes, and partial colony formations were observed. However, homogeneously shaped and plate-adherent fibroblast-like cells were mainly detected at the end of about 2 weeks (Jun-Ho Byu *et al*, 2012). Our study result detected the CD44, CD73, CD90, CD105 and CD200 positive MSCs markers and the CD34 and CD45 negative expressed HSCs markers using immunofluorescence. Hence, the minimum criteria proposed by ISCT were fulfilled by the MSCs derived in his study. MSCs typically are plastic adherent (La Rocca *et al*, 2009). In addition, flow cytometric analysis for typical markers defining MSCs as proposed by the Mesenchymal and tissue cell committee of the ISCT (La Rocca *et al*, 2009; Dominici *et al*, 2006) was also done. The positive expression of CD29, CD44, CD90, CD105 and CD73 was observed in MSCs derived from all sources while the CD45, CD14, and CD34 markers were negative. However, differences were observed in the percentage of expression among the four sources.

CONCLUSION

SMSCs tissues are promising a future source of stem cells. SMSCs have multipotency abilities with a high rate of proliferation and MSCs characteristics that are advantageous for regenerative medicine therapy through

tissue engineering.

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