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Gingival-Derived Mesenchymal Stem Cell from Rabbit (*Oryctolagus cuniculus*): Isolation, Culture, and Characterization

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Abstract

Objective This study aims to confirm whether the GDMSCs isolated from rabbit's (*Oryctolagus cuniculus*) gingiva are mesenchymal stem cells (MSCs).

Materials and Methods This study design was partly quasi-experimental with an observational design. GDMSCs were isolated from the gingiva of healthy male rabbits (*O. cuniculus*) (n = 2), 6 months old, and 3 to 5 kg of body weight. The specific cell surface markers of MSCs; clusters of differentiation (CD), namely, CD44, CD73, CD90, CD105, and CD200 expressions; and hematopoietic stem cell surface markers CD34 and CD45 were examined using flow cytometry and immunohistochemistry with immunofluorescence. The osteogenic differentiation of isolated GDMSCs was examined using alizarin red staining.

Results GDMSCs in the fourth passage showed a spindle-like formation and fibroblast-like cells that attached to the base of the culture plate. GDMSCs were MSCs that positively expressed CD44, CD73, CD90, CD105, and CD200 but did not express CD34 and CD45 when examined using flow cytometry and immunohistochemical analysis. GDMSCs had osteogenic differentiation confirmed by calcified deposits *in vitro* with a red–violet and brownish color after alizarin red staining.

Conclusion GDMSCs isolated from the rabbits (*O. cuniculus*) were confirmed as MSCs *in vitro* documented using immunohistochemistry and flow cytometry. GDMSCs can differentiate into osteogenic lineage *in vitro* that may be suitable for regenerative dentistry.

Keywords
- gingival-derived mesenchymal stem cells
- cluster of differentiation
- osteogenic differentiation
- regenerative dentistry
- tissue engineering

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Introduction

Cell-based therapy using mesenchymal stem cells (MSCs) has gained much attention. MSC therapy has become a new hope for enhancing tissue regeneration, stimulating the healing of tissue defects, and cell replacement in lost cells. The study regarding regenerative dentistry using MSCs has gained momentum.[1,2] MSCs have good proliferation and multipotential differentiation. Thus, MSCs secrete various growth factors that help tissues regenerate and are suitable for wound healing.[3]

MSCs have been successfully extracted from various tissues, such as the umbilical cord, hair follicles, and adipose tissue, including orofacial tissues.[4-8] MSCs can be derived from orofacial tissue, including the dental pulp of permanent or deciduous teeth, the periodontal ligament, and the gingiva.[9-13] One of the potential and superior sources of MSCs from the oral cavity was in the gingiva. The gingiva was known as a hidden gem and a precious source of MSCs. Gingival-derived mesenchymal stem cells (GDMSCs) were chosen because they are homogeneous, nonteratogenic, easily isolated, and stable in phenotype. GDMSCs are stem cells that can be isolated from gingival lamina propria, the free gingival margin, attached gingiva, and hyperplastic gingiva without a history of periodontal disease using aseptic techniques to avoid infection and inflammatory contamination.[14,15] GDMSCs have many beneficial uses over MSCs that originate from other sources as they occur in large numbers and are easily collected with minimally invasive cell isolation techniques.[16]

GDMSCs expressed positive MSC cluster of differentiation (CD) markers, namely, CD73, CD90, CD105, CD44, CD146, CD166, CD271, STRO-1, and vimentin.[14,15] GDMSCs can differentiate in osteogenic, adipogenic, and chondrogenic cells. Osteogenic differentiation is evidenced by the formation of calcified deposits with alizarin red staining or through the microscopic transmission of electrons that show the cellular properties of osteoblasts.[16]

Osteogenic differentiation of GDMSCs is also evidenced at the mRNA level through the increased expression of runt-related transcription factor 2 (RUNX2); this is a marker of the early phase of osteogenic differentiation, alkaline phosphatase (ALP), which plays a role in hard tissue mineralization and osterix and is a specific transcription factor for osteoblasts.[17,18] GDMSCs can divide into a mesenchymal lineage, such as osteogenic differentiation, that expresses RUNX2, osteocalcin, osteopontin, osteonectin, and ALP; they can also express chondrogenic differentiation, namely, SOX9 and aggrecan.[19-23] In addition, GDMSCs are known to increase the expression of collagen type I and collagen type III.[16,24,25]

GDMSCs have better proliferation properties than bone marrow stem cells and dental pulp stem cells and are morphologically stable and nonteratogenic, although they can originate from healthy tissue, hyperplastic, or inflammatory tissue.[16] This nonteratogenic nature is caused by the expression of tumor necrosis factor-related apoptosis-inducing ligand by the GDMSCs, which plays a role in the apoptosis and necrosis of cancer cells.[26,27] In this study, we would like to describe the isolation, culture, and characterization of GDMSCs isolated from the rabbits' (*Oryctolagus cuniculus*) gingiva. Thus, the purpose of this study is to confirm that the GDMSCs isolated from these rabbits (*O. cuniculus*) are MSCs by examining the expression of the surface markers CD44, CD73, CD90, CD105, and CD200 as specific cell surface markers of MSCs. CD34 and CD45 as hematopoietic stem cell (HSC) surface markers were examined by flow cytometry and immunohistochemistry with immunofluorescence, and the osteogenic differentiation of isolated GDMSCs was examined using alizarin red staining.

Materials and Methods

Experimental Study Design and Ethical Clearance Approval

This study design was partly quasi-experimental with an observational design. It was ethically approved by the Faculty of Veterinary Science, Airlangga University, Surabaya, Indonesia and Ethical Clearance Committee of Health Research with the appointment number 2.KE.017.02.2020.

The animal subject experienced the adaptation for a week and was placed at 21 to 23°C with controlled humidity (50 ± 5%) in a 12-hour artificial light cycle (8 AM to 8 PM); each animal was in an individual cage. Every animal was fed with standard pellets and given water *ad libitum*, with a husk replacement every 3 days. GDMSCs were isolated from the gingiva of healthy male rabbits (*O. cuniculus*) (n = 2), 6 months old, with a 3 to 5 kg of body weight (bw).

GDMSCs Extraction from the Rabbit

The animal was under anesthesia induced by a combination of ketamine 50 to 60 mg/kg of bw (Ket-A; Kepro, Holland) and xylazine 5 to 10 mg/kg of bw (Xyla; Intercheme, Holland). The rabbit's free gingival margin in the mandibula region between two central incisors was disinfected with 10% povidone iodine (OneMed; Sidoarjo, Indonesia) applied with a sterile cotton stick. The free gingival margin was gently handled with the sterile needle holder clamp and then dissected vertically with a sterile scalpel blade. The gingiva was then cut horizontally with a sterile sharp blade. After the target gingiva had been extracted, it was immersed in 100 U/mL penicillin-G, 100 µg/mL streptomycin, and 100 µg/mL kanamycin (Sigma Aldrich, United States) as a washing solution and then put into phosphate-buffered saline (PBS) (Sigma Aldrich) as a medium to transport the stem cell culture to the laboratory for further processing (►**Fig. 1**).

GDMSCs Isolation and Culture

The gingiva obtained was cut into small pieces; 1 mg/mL of the enzyme trypsin was added and then cultured in Dulbecco's modified Eagle medium (Life Technologies, GIBCO BRL) with the addition of 20% fetal bovine serum (Biochrome AG, Germany), 5 mg/mL glutamine (Gibco Invitrogen), 100 U/mL penicillin-G, 100 µg/mL streptomycin, and 100

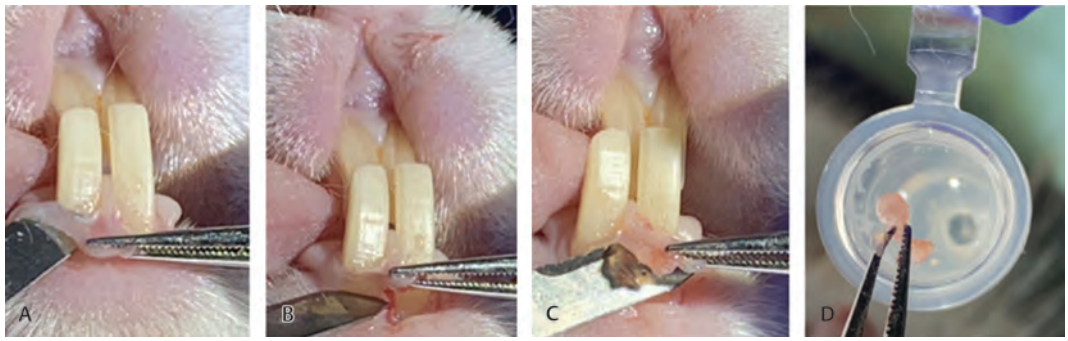

Fig. 1 GDMSCs extraction from the rabbit's gingiva. (**A**) The free gingival margin was gently handled with the sterile needle holder clamp. (**B**) The gingiva was cut vertically with a sterile scalpel blade. (**C**) The gingiva was then cut horizontally with a sterile sharp blade. (**D**) After the target gingiva had been dissected, it was put into PBS as a medium to transport the stem cell culture to the laboratory for further processing. GDMSCs, gingival-derived mesenchymal stem cells; PBS, phosphate-buffered saline.

µg/mL kanamycin (Sigma Aldrich). Three days after culture, the GDMSCs culture medium was removed to eliminate cell debris and unattached cells. In addition, a new culture medium was added to the GDMSCs culture. When the confluent cells were observed, cell passaging was achieved by the addition of 0.05% trypsin-EDTA. The cells were washed, collected, and cultured again in 60 or 100 mm tissue culture dishes. Confluent cells were subjected to passaging again into fourth passage according to the study requirements.[28]

GDMSCs Characterization by Means of Immunohistochemistry with Immunofluorescence

GDMSCs were coated with coverslips, and after incubation at 37°C for 1 to 2 hours, they were fixed using 10% formaldehyde for 15 minutes. Then the coverslips were rinsed four times with PBS and dried for a few minutes. Polyclonal antibodies labeled fluorescein isothiocyanate (FITC) CD200, CD105, CD73, CD90, CD45, CD44, and CD34 (Bioss Antibodies Inc.; Woburn, Massachusetts, United States) were added to cells and incubated for 60 minutes. After that, the cells were rinsed with PBS twice and were ready for analysis using an inverted fluorescence microscope (Olympus; Tokyo, Japan).[28]

GDMSCs Characterization by Means of Flow Cytometry

GDMSCs flow cytometry analysis begins with sample preparation; 1.[10,6] GDMSCs/mL dissolved in 3 mL cold (4°C) PBS is put into a tightly closed falcon tube. A 200 µL aliquot of the GDMSCs suspension is put into test tubes as required. Polyclonal antibodies CD34, CD44, CD45, CD73, CD90, and CD105 labeled FITC (Bioss Antibodies Inc.) are added. They are mixed well and incubated at 4°C for at least 30 to 60 minutes, avoiding direct light. The GDMSCs are then washed with 2 mL cold (4°C) PBS, then centrifuged at 300 to 400 *g* for 5 minutes at 4°C, and the resulting supernatant was discarded. They are incubated for 10 minutes at room temperature, centrifuged at 300 to 400 *g* for 5 minutes at room temperature, and the supernatant was discarded. They are washed with 2 mL room temperature PBS, centrifuged at 300 to 400 *g* for 5 minutes and the supernatant was discarded. The cells are then resuspended in 200 µL cold (4°C) PBS or with 200 µL of 0.5% paraformaldehyde in the PBS. Then the sample is read with an FACSCalibur flow cytometer (FACSCalibur, Becton, Dickinson and Company, Singapore).[28]

GDMSCs Osteogenic Differentiation Assessment

The assessment of GDMSCs osteogenic differentiation ability was performed; 1.[10,6] GDMSCs/mL at the fourth passage were cultivated in a plate culture with a culture medium until it reached 80% confluence. Osteogenic differentiation of GDMSCs was induced with an osteogenic medium consisting of 100 nM MEM α, dexamethasone, 200 µM ascorbic acid, and 10 mM of glycerol 2-phosphate (Osteo-MAX; Merck, United States). The culture medium was changed twice a week for 14 days. The activity of ALP, which is a marker of osteogenic differentiation, was then stained using alizarin red (Sigma-Aldrich). The mineral deposit in the GDMSCs culture after osteogenic induction was examined by an inverted light microscope with ×40, ×100, and ×200 magnifications (Olympus).[29]

Results

In this study, the isolation of GDMSCs from young adult male rabbits (*O. cuniculus*) was performed and continued with culture until fourth passage. The morphology of the GDMSCs in the fourth passage showed a spindle-like formation and fibroblast-like cells that attached to the base of the culture plate. The immunohistochemical method was examined by using immunofluorescence staining labeled FITC with a green color and observed using an inverted fluorescence microscope with 100 magnification. Confirmation that cultured GDMSCs were MSCs was performed by observing positive expressions of CD44, CD73, CD90, CD105, and CD200 (►**Fig. 2**).

In addition to the characterization of GDMSCs with positive surface markers from MSCs, characterization of GDMSCs with positive surface markers from HSCs was also performed. In the GDMSCs characterization, there were no positive surface markers for HSCs. CD34 and CD45 negative expressions were found in the GDMSCs culture (►**Fig. 3**).

Flow cytometry was also examined to characterize GDMSCs as MSCs for added confirmation. The results of flow cytometry showed that GDMSCs expressed positive

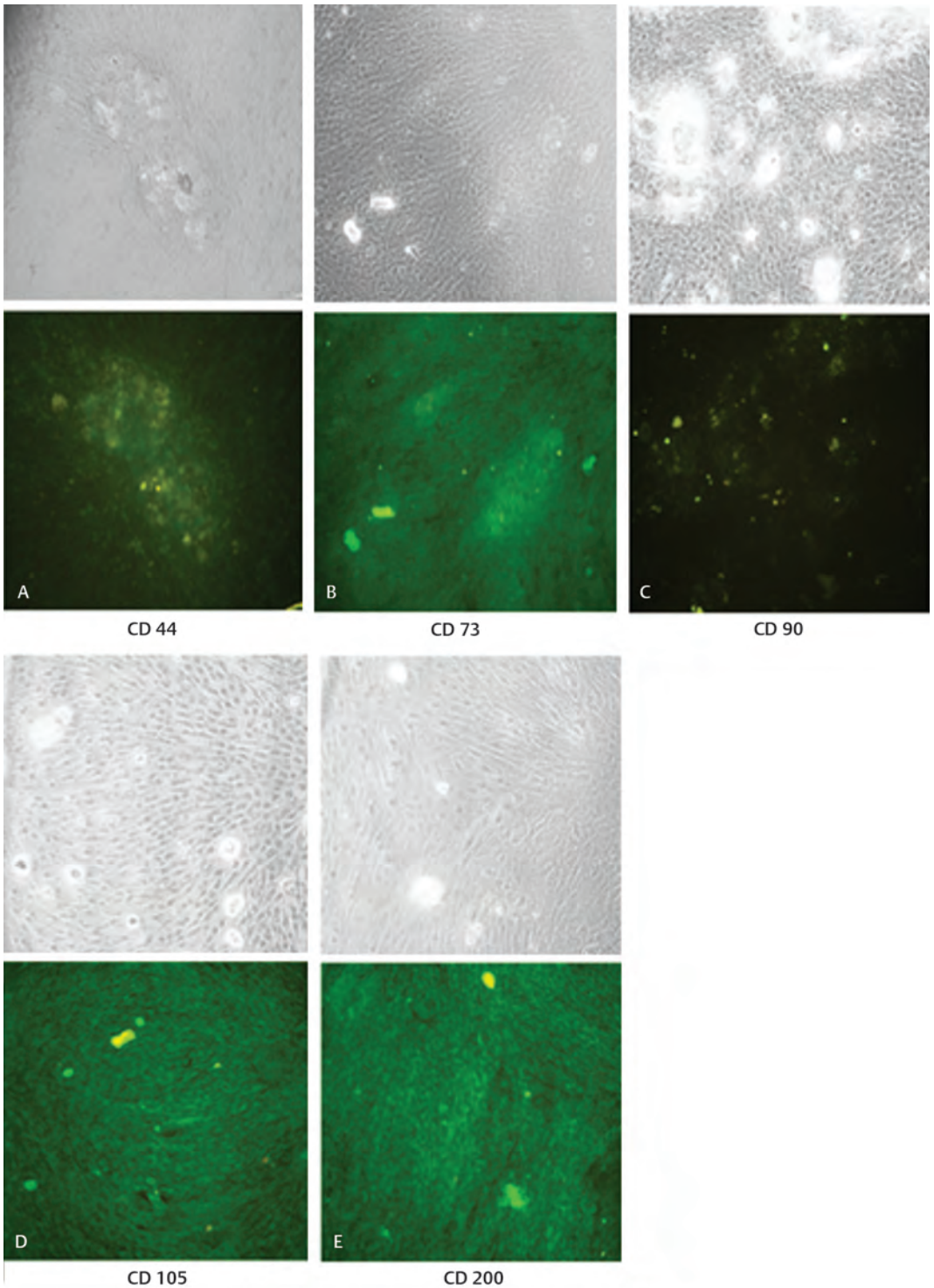

Fig. 2 The morphology of GDMSCs at the fourth passage shows a spindle-like formation and fibroblast-like cells attached to the base of the plate culture. Examination using the immunohistochemical method with green immunofluorescence staining was observed using inverted fluorescence microscopy with ×100 magnification. (**A**) CD44 positive expression; (**B**) CD73 positive expression; (**C**) CD90 positive expression; (**D**) CD105 positive expression; and (**E**) CD200 positive expression. CD, cluster of differentiation; GDMSCs, gingival-derived mesenchymal stem cells.

CD105 but did not express positive CD45. GDMSCs also expressed MSC surface markers, namely, positive CD44, CD73, and CD90 expressions (►**Fig. 4**).

To find out which GDMSCs in this study were true MSCs, an osteogenic differentiation examination was performed. GDMSCs were cultured in an osteogenic medium until day 14 and then checked for calcified deposits using alizarin red staining. Mineral deposits were found of a red–violet color on GDMSCs, which proved that they had experienced osteogenic differentiation (►**Fig. 5**).

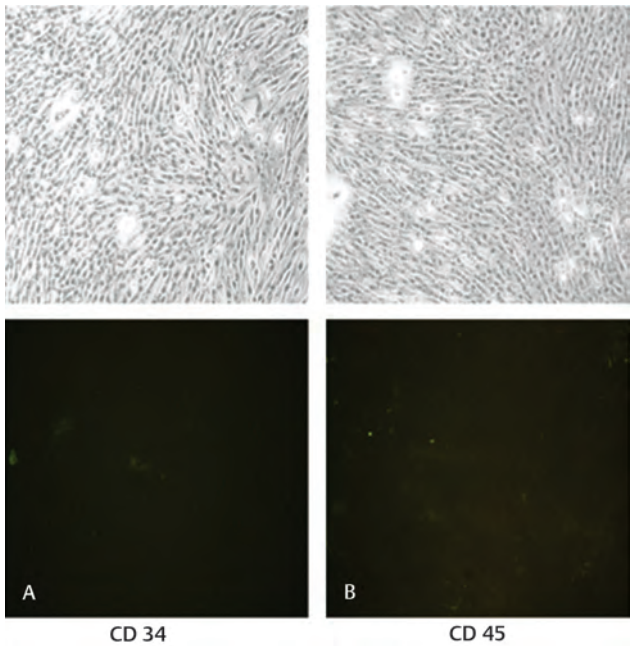

Fig. 3 The results of the characterization of the isolated and cultured cells confirmed GDMSCs did not express the CD's HSC markers. Examination using the immunohistochemical method with green immunofluorescence staining was observed using an inverted microscope with a magnification of ×100. (**A**) CD34 negative expression; (**B**) CD45 negative expression. CD, cluster of differentiation; GDMSCs, gingival-derived mesenchymal stem cells; HSC, hematopoietic stem cell.

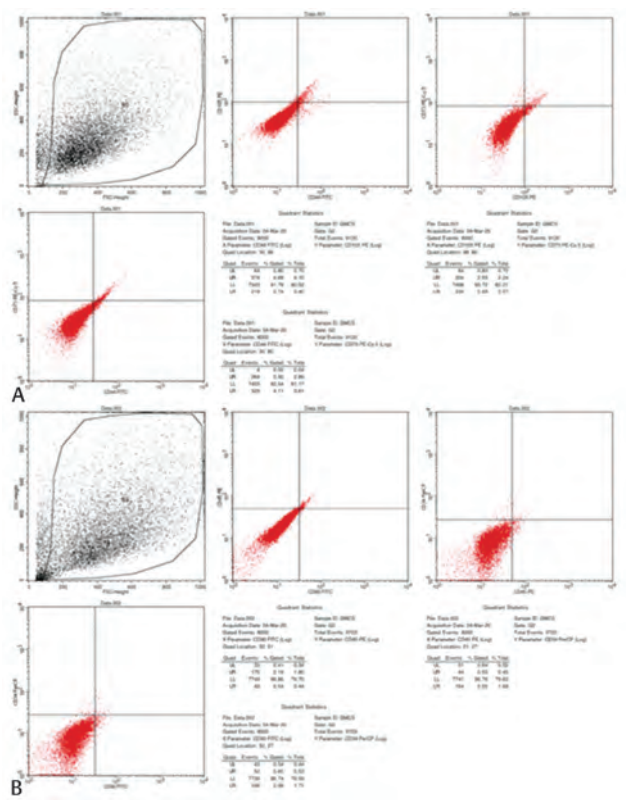

Fig. 4 Characterization of the fourth passage of the GDMSCs with flow cytometry examination. (**A**) GDMSCs expressed CD105 but not CD45; (**B**) GDMSCs also expressed MSC markers, namely, CD44, CD73, and CD90 expressions. CD, cluster of differentiation; GDMSCs, gingival-derived mesenchymal stem cells.

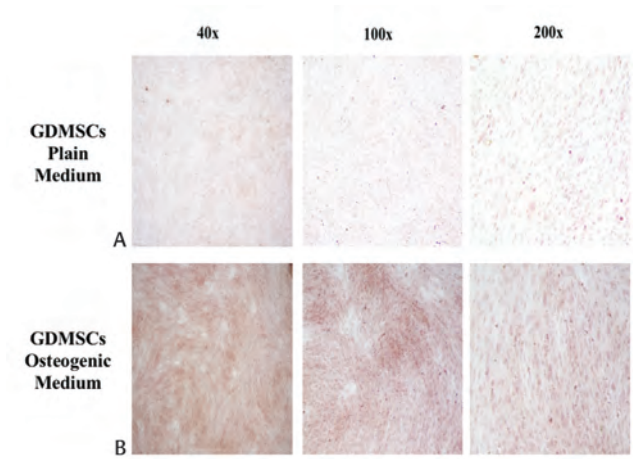

Fig. 5 Alizarin red examination results confirmed osteogenic differentiation after GDMSCs were cultured in an osteogenic medium for 14 days. It appears that there are red–violet and brown calcified deposits observed using a microscope with ×40, ×100, and ×200 magnifications. (**A**) GDMSCs were cultured in a plain medium for 14 days; (**B**) GDMSCs were cultured in an osteogenic medium for 14 days. GDMSCs, gingival-derived mesenchymal stem cells.

Discussion

There are minimal criteria to define MSCs characterization according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy: (1) The cultured MSCs should attach to the base of the culture plate when administered and maintained in the standard culture medium. (2) The specific cell surface of MSCs must be expressed as positive CD105, CD73, and CD90, but with a lack of expression of CD45, CD34, CD14 or CD11b, CD79α, or CD19 and human leukocyte antigen-DR (HLA-DR) surface molecules. (3) MSCs must differentiate to mesenchymal lineages, such as adipogenic, chondrogenic, and osteogenic differentiation *in vitro*.[30]

Both in human and mice MSCs, the CD44 expression can be found. CD44 plays a critical role in the extracellular vesicles of the MSCs biological activity.[31] CD73 expression as one of MSCs' markers has been linked to the regulatory phenotypes of T and NK cells.[32] CD73 is a beneficial molecule and important candidate marker for the multipotent ability of MSCs identification.[33] CD90 or Thy-1 is a glycophosphatidylinositol-linked outer membrane leaflet glycoprotein that is expressed predominantly on MSCs. High CD90 expression has also been related to the undifferentiated status of MSCs. CD90 controls the differentiation of MSCs by acting as an obstacle in differentiation.[34] Endoglin or CD105 is a transforming growth factor-β receptor.[35] One of MSCs' crucial markers is CD105, and numerous studies have mentioned that the expression of CD105 depends on the MSCs source. The osteogenic differentiation of MSCs can be predicted by the CD105 expression.[36] CD200 expression is a native marker for MSCs culture. CD200 is also expressed by T and B cells, dendritic cells, and vascular endothelial cells.[37] The upregulated expression of CD200 was found in MSCs that have osteogenic differentiation ability. In addition, the expression of CD200 was enhanced under the proinflammatory condition,

while the osteogenic expression was inhibited. The nuclear factor-κB took control of the expression of CD200 under the proinflammatory or pro-osteogenic conditions.[38] On the contrary, CD34 and CD45 are HSCs' specific surface markers. CD34 is a glycosylated transmembrane protein that has a role in a cell's adhesion and HSCs' migration.[39] At the same time, CD45 or lymphocyte (a common antigen) is a receptor-linked protein tyrosine phosphatase that is expressed and plays an important role in all leucocytes' functions.[40]

The results of our study are in accordance with the International Society for Cellular Therapy standard criteria; GDMSCs express a specific set of cell surface markers, such as positive CD73, CD90, and CD105, whereas negative expressions of CD14, CD34, CD45, and HLA-DR are considered MSCs.[30] The cultured GDMSCs within this study showed the spindle-like cell formation or fibroblast-like cell shape that attached to the base of the culture plate. This result is also similar to previous studies that isolated GDMSCs from Wistar rat's gingiva.[28] The other sources of MSCs, such as those derived from hair follicles, the umbilical cord, adipose tissue, and dental pulp, also positively presented the specific cell surface markers of MSCs.[5,6,8,9,11]

GDMSCs have a potential proliferation rate characterized by Oct-4, Nanog, and SOX2 expression that play an important role in the self-renewal and survival of MSCs.[41,42] In addition to the proliferation potential of GDMSCs, their migration capability is also good. The GDMSCs can migrate into injury tissue and stimulate regeneration. The homing mechanism of GDMSCs is related to C-X-C chemokine receptor type 4 expression that binds to chemokine stromal cell-derived factor-1.[43-45]

In this study, we demonstrated that GDMSCs could be differentiated into the osteogenic lineage. The mineral deposit was found after staining the GDMSCs culture alizarin red in an osteogenic medium. This result is in line with the International Society for Cellular Therapy standard criteria that stated MSCs must differentiate into the mesenchymal lineage. The previous studies found that GDMSCs cultured in an osteogenic medium with platelet-rich fibrin administration enhance the osteogenic differentiation markers such as osteocalcin and ALP.[21] The previous study by Vidoni et al and Cristaldi et al also confirmed that GDMSCs possessed osteogenic differentiation.[25,29] The osteogenic differentiation capability of GDMSCs suggests MSCs might be useful in the treatment of bone diseases such as periodontitis. Also, GDMSCs play an immunomodulatory role through the expression of Toll-like receptors, inhibition of maturation and excessive activation of dendritic cells, an increase in anti-inflammatory cytokines interleukin-10 (IL-10), IL-6, granulocyte-macrophage colony-stimulating factors, and suppression of proinflammatory cytokines tumor necrosis factor-alpha.[46] These superior properties show the potential of GDMSCs to become a good source of MSCs in regenerative dentistry such as during or postorthodontic treatment that required the osteoimmunology homeostasis. The osteogenic differentiation and immunoregulator capacity of GDMSCs may be useful for postorthodontic relapse prevention by regulating the proinflammatory or anti-inflammatory cytokine, osteoclastogenesis, and osteoblastogenesis.[47,48]

Therefore, the limitation of this study is preliminary study with *in vitro* design. Further study is still required to examine the efficacy of GDMSCs transplantation in the animal model (*in vivo*) experiment.

Conclusion

In summary, we can conclude that GDMSCs isolated from rabbit's gingiva (*O. Cuniculus*) were confirmed as MSCs that expressed positive CD44, CD73, CD90, CD105, and CD200 expression but did not express CD34 and CD45 *in vitro*, documented by immunohistochemistry and flow cytometry. In addition, GDMSCs that have been isolated can differentiate into osteogenic lineage *in vitro* that may be suitable in the field of regenerative medicine related to bone tissue engineering. Further studies are necessary to assess the potential ability of GDMSCs to treat bone defects using stem cell utilization.

Authors' Contributions

F.A.R., D.S.E., and I.B.N. supervised the experiment and manuscript. A.P.N. helped conduct the study and prepare the manuscript. All authors read and approve the final manuscript.

Conflict of Interest

None declared.

Acknowledgments

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