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In vitro comparative analysis of human dental stem cells from a single donor and its neuronal differentiation potential evaluated by electrophysiology

Imran Ullah [a], Raghavendra Baregundi Subbarao [a], Eun-Jin Kim [b], Dinesh Bharti [a], Si-Jung Jang [a], Ji-Sung Park [a], Sharath Belame Shivakumar [a], Sung-Lim Lee [a], Dawon Kang [b], June-Ho Byun [c], Bong-Wook Park [c,*], Gyu-Jin Rho [a,**]

[a] Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] Department of Physiology and Institute of Health Sciences, School of Medicine, Gyeongsang National University, Jinju 660-751, Republic of Korea
[c] Department of Oral and Maxillofacial Surgery, Institute of Health Science, School of Medicine, Gyeongsang National University, Republic of Korea

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ABSTRACT

Aims: The aim of this study was to find out a mesenchymal stem cells (MSCs) source from human dental tissues of the same donor (follicle, papilla and pulp), which exhibits higher neurogenic differentiation potential in vitro.
Main methods: MSCs were isolated from dental tissues (follicle, papilla and pulp) by digestion method. All MSCs were analyzed for pluripotent makers by western blot, cell surface markers by flow cytometry, adipo- and osteocytes markers by RT-qPCR. The neuronal differentiated MSCs were characterized for neuronal specific markers by RT-qPCR and immunofluorescence. Functional neuronal properties were analyzed by electrophysiology and synaptic markers expression.
Key findings: All MSCs expressed pluripotent markers (Oct4, Sox2 and Nanog) and were found positive for mesenymal markers (CD44, CD90, CD105) while negative for hematopoietic markers (CD34 and CD45). Furthermore, MSCs were successfully differentiated into adipocytes, osteocytes and trans-differentiated into neuronal cells. Among them, dental pulp derived MSCs exhibits higher neurogenic differentiation potential, in term of expression of neuronal specific markers at both gene and protein level, and having higher Na^+ and K^+ current with the expression of synaptic markers.
Significance: The three types of dental MSCs from a single donor broadly possessed similar cellular properties and can differentiate into neuronal cells; however, pulp derived MSCs showed higher neurogenic potential than the follicle and papilla, suggesting their use in future stem cells therapy for the treatment of neurodegenerative disorders.

1. Introduction

From last decade, cell based therapies come up as a novel therapeutic option for the treatment of wide variety of neurological disorders i.e. Parkinson's disease, Alzheimer's disease, spinal cord injury [1,2]. Neural stem cells (NSCs) are considered as the most ideal source to be applied in stem cell based therapies; however, NSCs are difficult to harvest from adult origins, so therefore, other stem cells sources are needed to explore [3].

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent stem cells having multilineage differentiation potential and self-renewal capabilities [4]. MSCs had been derived from various human and animal sources. MSCs derived from human dental tissues keep a promising role in the future regenerative medicine because of their ease of collection, and their capacity to undergo self-renewal and multilineage differentiation [1,5]-[7]. As the dental MSCs are isolated from extracted tooth, a medical waste in the routine dental procedures, which increase its importance in cell-based therapies without any ethical issues. To date, five different types of stem cells have been isolated from human dental tissues [8]. Postnatal dental pulp stem cells (DPSCs) were the first human dental stem cells isolated by Gronthos et al. in 2000 [5]. After this first isolation of human dental stem cells, stem cells from human exfoliated deciduous teeth (SHED) [6], human periodontal ligament stem cells (PDLSCs) [9], dental follicle precursor cells (DFPCs) [10] and from the apical papilla (SCAP) [11] were successfully isolated and characterized. Stem cells

* Correspondence to: B-W Park, Department of Oral and Maxillofacial Surgery, Institute of Health Science, School of Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea.
** Correspondence to: G-J Rho, Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea.
E-mail addresses: parkbw@gnu.ac.kr (B.-W. Park), jinrho@gnu.ac.kr (G.-J. Rho).

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isolated from all dental sources exhibits fibroblast like morphology, express cells surface markers and differentiate into mesodermal lineages i.e. osteocytes [12–14] adipocytes (Miura et al., 2003; Jeon et al., 2011) and chondrocytes (Park et al., 2012) as well as neural cells [6,15], muscle cells [6] and hepatocytes [7,16,17]. The properties of MSCs derived from dental tissues were found similar to those of MSCs derived from bone marrow (BM-MSCs) and skin [14,18]. However, as the dental stem cells have a neural crest origin, they have higher neurogenic capacities than other MSCs [19]. It is considered that stem cells derived from dental tissues have analogous properties to that of neural crest [5]. Previously, human dental stem cells were successfully differentiated into neuron like cells, both in vitro and in vivo [1,5,6,15,20]; however, till now the neuronal differentiation abilities of different dental stem cells are not completely deciphered.

In this study we isolated and characterized human dental MSCs derived from follicle, papilla and pulp tissues of the same donor tooth after impacted third molar extraction. Furthermore, we evaluated their in vitro neurogenic capacity by providing suitable induction conditions and assessed their behavior to functionally active neuron like cells with electrophysiological studies.

2. Materials & methods

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and media from Gibco (Life Technologies, USA), unless otherwise specified.

2.1. Isolation and initial culture of MSCs from human dental follicle, papilla and pulp

MSCs derived from human dental follicle, papilla and pulp were isolated and cultured from a single tooth sample as described previously [14,17,44]. Briefly, human third molar were collected from adult male ($n = 7$, aged 14–18 years) at Dental hospital of Gyeonsang National University under approved medical guidelines (GNUH IRB-2012-09-004), after obtaining the informed consents from patients. Samples were rinsed with Dulbecco's phosphate buffer saline (DPBS) containing 1% penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively; Pen-Strep). Dental follicle was separated from the tooth surface and papilla was plucked from the apical part of the tooth by using sterile scalpel. Dental pulp tissue was separated from the pulp chamber of dental crown following fracture with bone forceps. The tissues were chopped into pieces and then digested in DPBS supplemented with 1 mg/ml collagenase type I in a 37 °C incubator with frequent gentle agitation for 40 min. After digestion, the cell suspensions were filtered through a 100 and 40 μm nylon cell strainer (BD Falcon, Bedford, MA, USA) in order to harvest single cell suspension, and further digestion was prevented by adding Advanced Dulbecco's modified eagle's media (ADMEM) supplemented with 10% fetal bovine serum (FBS). The suspensions were centrifuged at 500 ×*g* for 5 min. Supernatants were discarded and the pellets were reconstituted in ADMEM supplemented with 10% FBS (10% ADMEM). Total of 1×10^5 cells were initially seeded into 10 cm culture dishes containing 10% ADMEM. Culture dishes were kept at 37 °C in a humidified incubator containing 5% Co_2 in air. Upon confluence, cells were dissociated with 0.25% (*w*/*v*) trypsin EDTA solution and sub-cultured till passage 3. Cells from passaged 3 were used for further characterization and analysis.

2.2. Analysis of pluripotent markers by western blotting

For western blot analysis of pluripotent markers (Oct4, Sox2 and Nanog), protein lysate was prepared from third passage of all cells (Follicle, Papilla and Pulp) using RIPA buffer (PIERCE, Rockford, IL, USA) containing protease inhibitor and further quantified using BCA protein assay kit (PIERCE). Each protein sample (20 μg) were separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h at 100 V and transferred to polyvinylidene difluoride membrane (PVDF, Biorad, Hercules, CA, USA) for 2 h at 200 V. Furthermore, membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (1×-TBS) for 1 h at room temperature, followed by washing in 0.1% TBST. The membranes were incubated with primary antibodies of goat anti-Oct4 (1:200, 43–50 kDa, Santa Cruiz, State, USA), mouse anti-Nanog (1:200, 35 kDa, Santa Cruiz), rabbit anti-Sox2 (1:200, 34 kDa, Santa cruiz) and rabbit anti-β-actin (1:1000, Cell signaling, MA, USA) for overnight at 4 °C. After three times washing with 0.1% TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:1000, Santa Cruiz), rabbit anti-goat (1:1000, Santa Cruiz) and goat anti-rabbit (1:1000, Santa Cruiz) secondary antibodies for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Supersignal® West Pico chemilluminescent substrate, PEIRCE, Rockford, IL, USA). The membranes were then exposed to X-ray film in dark and bands were analyzed.

2.3. Cell surface antigen profiling by flow cytometry

Before performing other experiments, cells derived from human dental follicle, papilla and pulp were characterized for the presence of mesenchyme markers (CD13, CD29, CD 44, CD73, CD 90, CD105) and absence of hematopoietic marker CD 34 and CD 45 using flow cytometry (BD FACSClibur, Becton Dickinson, San Jose, CA, USA) as previously described by [17]. Briefly, cells at ~80% confluence were fixed with 3.7% formaldehyde for 30 min, followed by incubation with fluorescence isothiocyanate (FITC) conjugated mouse Anti-CD34, CD44, CD45 and CD90. For analysis of CD 105 (mouse monoclonal, Santa Cruiz Biotechnology Inc., CA, USA), CD13 (goat polyclonal, Santa Cruiz), CD29 (mouse monoclonal, BD Bioscience 561795) and CD73 (mouse monoclonal, BD Bioscience 550256) expression, cells were treated with primary antibody for 1 h at 4 °C. The cells were then washed with DPBS and treated with secondary antibody FITC conjugated goat anti mouse IgG (BD, Pharmingen™), donkey anti-goat (Santa Cruiz) for 1 h at 4 °C in dark. Total of 10,000 FITC labeled cells were measured by flow cytometer using CellQuest software (BD, Beckson Dickinson). The standard was established by isotype match control.

2.4. In vitro lineage differentiation

Adipogenic and osteogenic differentiation of three types of dental MSCs were performed by using previously described protocols [14,17]. Briefly, MSCs were cultured in 10% ADMEM supplemented with lineage specific factors for 21 days and medium was changed twice a week. Adipogenic medium consisted of 1 μM dexamethasone, 10 μM insulin, 100 μM indomethacin and 500 μM Isobutylmethyl Xanthine (IBMX). For the detection of lipid droplets, differentiated cells were stained by Oil red O solution for 30 min. Osteogenic medium consisted of 50 μM ascorbate-2-phosphate, 10 mM glycerol-2-phosphate and 0.1 μM dexamethasone. Cells were stained with Alizarin red and von Kossa for the detection mineralization and calcium deposition, respectively. The cells cultured in 10% ADMEM were used as negative control.

2.5. Neurogenic differentiation of MSCs from dental follicle, papilla and pulp

Cells culture flasks or wells were coated with Geltrex LDEV (Gibco) for 2 h at 37 °C, and washed with DPBS before cells were added. Neurogenic differentiation potential of MSCs was conducted as described previously by Arthur et al. (2008) [28], with minor modifications. MSCs are taken from passage 3 and were grown in 10% ADMEM in 6 wells and 12 wells coated chamber slides. When cells reached at 70–80%, the ADMEM media was changed with neural basal A media (Invitrogen; Carlsbad, CA, USA) supplemented with 1% Pen-Strep, 20 ng/ml epidermal growth factor (EGF), 30 ng/ml basic fibroblast growth factor (bFGF) and 1× B27 supplement for 3 weeks. Media