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Comparison of osteo/odontogenic differentiation of human adult dental pulp stem cells and stem cells from apical papilla in the presence of platelet lysate

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ABSTRACT

Introduction: Human dental pulp cells (DPSCs) and stem cells from apical papilla have been used for the repair of damaged tooth tissues. Human platelet lysate (PL) has been suggested as a substitute for fetal bovine serum (FBS) for large scale expansion of dental stem cells. However, biological effects and optimal concentrations of PL for proliferation and differentiation of human dental stem cells remain to be elucidated.

Methodology: DPSCs and SCAP cells were isolated from impacted third molars of young healthy donors, at the stage of root development and identified by markers using flow cytometry. For comparison the cells were cultured in media containing PL (1%, 5% and 10%) and FBS, with subsequent induction for osteogenic/odontogenic differentiation. The cultures were analyzed for; morphology, growth characteristics, mineralization potential (Alizarin Red method) and differentiation markers using ELISA and real time -polymerase chain reaction (qPCR).

Results: The proliferation rates of DPSCs and SCAP significantly increased when cells were treated with 5% PL (7X doubling time) as compared to FBS. 5% PL also enhanced mineralized differentiation of DPSCs and SCAP, as indicated by the measurement of alkaline phosphatase activity, osteocalcin and osteopontin, calcium deposition and q-PCR.

Conclusion: Our findings suggest that using 5% platelet lysate, proliferation and osteo/odontogenesis of DPSCs and SCAP for a short period of time (15 days), was significantly improved. This may imply its use as an optimum concentration for expansion of dental stem cells in bone regeneration.

1. Introduction

Since year 2000, after the discovery of adult stem cells from the dental pulp (DPSCs) (Gronthos, Mankani, Brahim, Robey, & Shi, 2000), several types of dental stem cells have been isolated successively from mature and immature teeth including; stem cells derived from the apical papilla (SCAP) (Sonoyama et al., 2008), stem cells derived from exfoliated deciduous teeth (SHED) (Miura et al., 2003), stem cells from human periodontal ligament (PDL) (Seo et al., 2004), and mesenchymal stem cells from tooth germs (Morsczeck et al., 2009; dAquino et al., 2011). It is considered that these stem cells are undifferentiated mesenchymal cells present in dental tissues and characterized by their; colony forming capacity, unlimited self-renewal and their multipotent differentiation potential (Gronthos et al., 2000) to give rise to distinct cell lineages such as osteoblasts, adipocytes, odontoblast, smooth muscle cells and neurons (Gronthos et al., 2002; Batouli et al., 2003; Iohara et al., 2004; Arthur, Rychkov, Shi, Koblar, & Gronthos, 2008; Kadar et al., 2009; Loomba et al., 2012). Ref : (Nakashima, 2005).

Some studies highlighted the ability of DPSCs to express chondrogenic markers (Balic, Aguila, Caimano, Francone, & Mina, 2010Karaoz et al., 2010) and some proteins involved in melanogenesis at some stages of *in vitro* differentiation (Paino et al., 2010). In addition, DPSCs are able to form capillary-like structures when cultured with VEGF (Marchionni et al., 2009). Moreover, DPSCs could change into cardiomyocytes which were used for the repair of myocardial infarction (Gandia et al., 2008).

Dental stem cells have been used for tissue- engineering studies to evaluate their potential in clinical applications. Sharpe and

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Young (2005) were pioneers in the use of stem cells in dental tissue engineering. DPSCs were loaded onto scaffolds of collagen sponges, hydroxyapatite (HA), chitosan, biocoral, or PLGA and implanted into animal model to study their ability for bone regeneration (Zhang et al., 2006; Graziano et al., 2008; Yang et al., 2009; Yang et al., 2012). All the mentioned groups reported that DPSCs loaded on scaffolds were able to form bone *in vivo*, except Zhang et al. (2006) who reported that DPSCs loaded onto spongeous collagen, in mice, were not able to form hard tissues. It is very important to mention that all the studies were done on different animal models. There have been few clinical trials that gave clear evidence of the possibility for bone regeneration in humans by using DPSCs ref (dAquino et al., 2009; Giuliani et al., 2013).

SCAP are suitable for cell-based regeneration and preferentially for root formation (Sonoyama et al., 2008). The cultures of SCAP, isolated from impacted third molars, were able to differentiate into odontoblast-like cells with an active mineralization and migratory potential, leading to an organized three-dimensional dentin-like structures *in vitro* and the ability to produce dentin *in vivo* (Sonoyama et al., 2008; Laino et al., 2011).

Recently, platelet lysate (PL) has attracted major attention for its possible clinical use, due to the detection of anti-fetal bovine serum (FBS) antibodies in most patients infused with MSCs cultures in FBS. This interest has led to the development of therapeutic protocols based on non- transfusional use of hemo-components such as; allogenic and autologous human plasma or serum, cord blood serum or human platelet lysate (HPL). HPL has been used as a substitute for FBS in humans because of its abilities; to stimulate MSC proliferation, maintained their differentiation potential and immunophenotypic characteristics. PL has also been used as an alternative for animal serum in human adipose mesenchymal stem cells (hASCs) culture (Doucet et al., 2005; Kocaoemer, Kern, Kluter, & Bieback, 2007; Schallmoser et al., 2007; Bieback et al., 2009; Capelli et al., 2011; Crespo-Diaz et al., 2011; Govindasamy et al., 2011). Moreover, PL has been reported to increase the proliferation rate of osteoblasts, fibroblasts and periodontal ligament cells (Okuda et al., 2003; Soffer, Ouhayoun, & Anagnostou, 2003). However, the optimum concentration and the effect of PL on SCAP and DPSCs stem cells have not been evaluated yet.

The main objective of this study was to isolate two types of dental stem cells DPSCs and SCAP from impacted third molars and to compare their expansion and differentiation potentials in the presence of PL, as an alternative source of FBS.

2. Materials and methods

2.1. Platelet lysate preparation

For the preparation of platelet lysate (PL), human peripheral blood was taken from donors. All donors were healthy without any clinically evident disease, had not been taking any medications, nonsmokers and non-alcoholic. The collection of samples was performed according to guidelines of the Institutional Review Board. Signed informed consent was obtained from all donors before inclusion in the study. The blood was centrifuged at 1000 rpm for 13 min at 4 °C. The supernatant, made of platelets rich plasma (PRP), was taken and frozen at −80 °C until use. PRP, taken in a different tube, centrifuged at 1000 rpm for 10 min, frozen at −80 °C, and then rapidly thawed at 37 °C. Freezing–thawing process was repeated for two times. The resulting platelet lysate (PL) was centrifuged at 5000 rpm for 10 min at 4 °C to remove platelet bodies. The PL was filtered through 0.22 μ filters to remove platelet membranes.

NOTE Among different studies, the optimum concentration of PL for proliferation and differentiation was variable. This variation could be due to the different preparation methods of PL. In this study, hPL was prepared by subjecting peripheral blood into different freezing–thawing cycles, to lyse platelets bodies and to collect the released growth factors. The platelet bodies were removed by centrifugation to minimize the effect of aggregation on cell culture. Isolated PL was pooled to minimize donor specific effects.

2.2. In vitro *isolation of dental stem cells*

Normal impacted third molar teeth were collected from three donors aged 18, 19 and 24 years at the stage of root development (two thirds of the root completed). All donors were healthy with no clinically evident disease, had not been taking any medications, non-smokers and non-alcohol. Signed informed consent was obtained from all donors before inclusion in the study.

2.3. Cell culture

Cell cultures were established using the enzymatic dissociation method (Gronthos et al., 2000). Briefly, teeth were disinfected by 0.5% chlorohexidine (Sage, USA) and cut around the cementum–enamel junction to reveal the pulp chamber using the hand piece. For each third molar, first the apical papilla tissue was removed, and then the tooth was drilled to remove the pulp tissue from the coronal part of the tooth, so that from each single donor tooth, both DPSCs & SCAP cultures could be established. Each tissue was then digested in a solution of 3 mg/ml collagenase type I (GIBCO, Germany) and 4 mg/ml dispase (GI BCO, Germany) for 1 h at 37 °C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (BD Biosciences, Germany). DPSCs and SCAP were seeded at a density of $10^4/cm^2$ using alpha-Modification of Eagle's Medium [(a-MEM, Lonza, USA), supplemented with 100 mM L-ascorbic acid phosphate (Sigma–Aldrich, USA), 100 mg/ml streptomycin (Invitrogen, USA), 2 mM L-glutamine (Invitrogen, USA), 0.25 mg/ml Amphotericin B (Invitrogen, USA) and 100 units/ml penicillin (Invitrogen, USA)]. For cell growth analysis, cells were seeded at 2×10^5cells/well in 6-well plates. Each well contains a specific concentration of Platelet lysate (PL) as following 1%, 5% and 10%. 10% FBS (Invitrogen, USA) was considered as a positive control and serum free medium was used as a negative control. Then the cells were incubated at 37C in 5% CO_2. The cell number was counted every 24 h by using a hemocytometer. Three replicates for each time point of 24, 48, and 72 h, have been performed respectively.

2.4. Flow cytometry

To identify the surface markers of stem cells, DPSCs and SCAP, cells were trypsinized and harvested at passage 3, then washed twice with PBS. 2×10^6 cells from each type were incubated with different fluorescinated antibodies against isotype controls, FITC-CD24, PE-CD34, PerCP Cy5.5-CD105, FITC-CD90 and PE-CD44 and APC-CD73 for half an hour at room temp. Cell fixative was added to the cells, were centrifuged at 1000 rpm for 5 min and re-suspended in PBS. Antibodies were used in concentrations suggested by the manufactures (BD, USA). The expression profile was analyzed by Fluorescein activated cell sorter {(FACS) Canto (BD, USA)}.

3. *In vitro* mineralization and Alizarin red staining

3.1. Osteo/odontogenic differentiation

α-MEM media were supplemented with 10 mM β-glycerophosphate (Sigma–Aldrich), 50 μg/ml L-ascorbic acid 2-phosphate (Sigma–Aldrich, A), 1 μM dexamethasone (Sigma–Aldrich, USA). These chemicals have the ability to induce the osteo/odontogenic induction for differentiation (differentiation of mesenchymal stem