IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
January 2018 Vol.:8, Issue:3
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Effect of Platelet Lysate on Osteogenic and Neurogenic Differentiation of Periodontal Ligament Stem Cells (PDLSCs) and Stem Cells Derived from Exfoliated Deciduous Teeth (SHEDs)

Gita Malathi Kambhampati[1], Ravi Chandra P.V[2], Vishnupriya Satti[1], Phanni Bhushann Meka[1,3], Vijaya Laxmi Kodati[*4]

1) *Department of Genetics, Osmania University, Hyderabad 500007. India.*

2) *Ravi Dental Hospital, Hyderabad, India.*

3) *Dr. Habibullah Life Sciences, CLRD, Hyderabad, India.*

4) *Vasavi Hospital, Hyderabad, India.*

Submission: 23 December 2017
Accepted: 30 December 2017
Published: 30 January 2018

Keywords: Dental pulp stem cells, differentiation, proliferation, gene expression, cell therapy

ABSTRACT

Stem cell like Periodontal Ligament Stem Cells (PDLSCs) and Stem Cells derived from Exfoliated Deciduous Teeth (SHEDs) suggested to be reliable stem cell source for clinical utility to repair damaged tooth tissue. However, the expansion of stem cells using animal serum (FBS) have several disadvantages. Though human platelet lysate (PL) found to be an alternative substitute for FBS, optimum concentration and its biological effect on PDLSCs 0and SHEDs remains to be explored. PDLSCs and SHEDs were isolated from dental tissues and stem cell marker characterization carried out by flow cytometry. Expression of osteogenic and neurogenic differentiation markers evaluated by quantitative Real Time PCR (qRT-PCR). Mineralized potential of PDLSCs and SHEDs was assessed by Alizarin Red Staining method. PDLSCs and SHEDs proliferation was significantly elevated when cells treated with 5% PL compared to 10% FBS. Moreover, both cells showed enhanced mineralization ability and neurogenic differentiation at 5% PL. Moreover, SHEDs expressed increased neurogenic differentiation potential compared to PDLSCs at 5% PL concentration. Our results suggested that at 5% optimum PL concentration both PDLSCs and SHEDs shows increased osteogenic and neurogenic differentiation ability compared to FBS.

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INTRODUCTION

Stem cell biology has emerged as a crucial area for the understanding of tissue engineering and implementation of regenerative/therapeutic medicine. Mesenchymal stem cells (MSCs) from Bone Marrow (BM), Umbilical cord blood (UCB) and adipose tissues have been isolated and characterized successfully in the past decades. Further, dental tissue has been found to be a promising alternative source for multipotent MSC like cells. Dental stem cells were successfully isolated and characterized from dental tissues and termed as post-natal dental stem cells (DPSCs).[1] Further, several researchers isolated and characterized MSC- like cells from deciduous or permanent teeth including; Stem Cells from Exfoliated teeth-SHEDs[2], Periodontal Ligament Stem Cells-PDLSCs[3], Stem Cells from Apical Papilla-SCAP[4]. All these stem cells have unique characteristic features in terms of multilineage differential potential, colony forming ability, self-renewal property to produce different lineages like odontoblasts, osteoblasts, neurons and adipocytes.

Previously, several reporters suggested that SHEDs proliferate much faster than other stem cells like DPSCs and bone marrow derived stem cells. SHEDs cultured in neurogenic medium formed spear like clusters which further differentiate into fibroblast cells when grown on 0.1% gelatin coated plates.[2] Moreover, SHEDs express several neural stem cell like markers such as βIII- tubulin, GAD and NeuN when cultured in neurogenic differentiation medium. Miura et al [2] reported that SHEDs yielded human specific odontoblast like cells when they transplanted into immune compromised mice. These odontoblast like cells morphologically similar to dentin like structures. In an *in vitro* study, it was showed that SHEDs able to repair critical sized calvarial defects by substantial bone formation.[3]

PDLSCs reside in the perivascular space of the matured periodontal ligaments, possess mesenchymal stem cell properties which potentiate periodontal regeneration. PDLSCs though to have the ability to rebuilding the periodontal supporting tissues like alveolar bone and cementum thereby restore the physiological function of teeth. PDLSCs showed elevated expression of tendon specific transcription factors compared to BMMSC under specific culture conditions PDLSCs expressed osteoblastic and cementoblastic markers similar to other dental stem cells.[5,6,7].

Present stem cell based therapies have experimental concerns. Current cell culture protocols use animal derived growth supplements or components which should be substituted with

alternative growth factors. Expanding and differentiating stem cells in Fetal Bovine Serum (FBS) result in allergic reactions, transmission of animal viruses, prions, yeast, bacteria or toxins upon transplantation[8,9,10]. In some patients, anti-fetal bovine serum antibodies have been identified. Recently, human platelet lysate (PL) has been emerged as a possible alternative substitute for FBS due to its ability to maintain MSC population, immunophenotypic characteristics and differentiation potential. Previously, Abuarqoub et al[11] studied the PL effect on DPSCs and SCAP and suggested that PL significantly improved the stem cell growth compared to FBS. However, to the best of our knowledge, there are no studies to evaluate the effect of PL on proliferation and differentiation ability of PDLSCs and SHEDs. In this study, we aimed to study and compare the proliferation and differentiation potential of PDLSCs and SHEDs in the presence of PL and FBS.

MATERIALS AND METHODS

Isolation of DPSCs and SHEDs

SHEDs were isolated and cultured according to the established protocols with few modifications. This work was approved by institutional ethical committee board, Osmania University and informed consent were taken from all the participants. The dental pulp cavity of the crown was opened using drills in aseptic conditions and the pulp was extracted with a broach followed by suspension of in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, USA) Later, pulp was washed two times with phosphate-buffered saline (PBS) and cut into small pieces (≤0.5 mm) in 10-cm culture dishes. Isolated cells were cultured in DMEM (Gibco) maintenance medium supplemented 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco) and 100 U/ml streptomycin (Gibco) at 37˚C and 5% CO2 humidified incubator. The culture medium was changed every three days. After reaching 90% confluence, the cells were digested with TrypLE (Gibco) and passaged. Cells at passages 5-6 were used in the subsequent experiments. PDLSCs were collected from deciduous teeth of 6 years boy. PDLSCs isolation and culture procedure was carried out according to previous protocol[12]. For cell growth analysis, platelet lysate (PLTMax® Human Platelet Lysate (hPL), Sigma Aldrich, USA) was used at different concentrations. 2×10^4 cells per well were seeded in 6 well plate and cultured with different concentrations i.e. 2.5%, 5% and 10% at different time intervals (24hr,48hr and 72hr). 10% FBS was used as a positive control whereas serum free medium was used as negative control. Cell was incubated at 37ºC

in 5% CO2.Cell count was assessed by hemocytometer. All experiments were carried out in triplicate.

Colony-forming unit-fibroblast assay

To achieve single-cell suspensions, PDLSCs, and SHEDs cells were trypsinized with Trypsin-EDTA solution (Thermo Fisher Scientific, USA) and filtered through 70µm cell strainers (Sigma-Aldrich, USA). Later, cells were seeded in six-well culture plates (480 cells/ well) and incubated for 10-12 days. Cultures were then fixed with 10% buffered formalin (Sigma) for 1h, and stained with 0.3% crystal violet (BD Biosciences) for 5-8 min. The number of colonies containing more than 50 cells was counted by using light microscope.

Characterization by flow cytometry

For flow cytometry analysis, single cell suspension was achieved by trypsinizing the PDLSCs and SHEDs monolayer cells (at 4th to 6th passage) and filtered through 40 µm cell strainers. Later, cells were washed with ice cold PBS supplemented with 0.5% BSA (Sigma-Aldrich, USA). Cell density at 5×10^4 cells were incubated with mouse monoclonal anti-human antibodies (FITC-conjugated CD146, PE-conjugated CD90, PE-conjugated CD105, PE-conjugated CD34, PE-conjugatedCD45. Cell surface marker assessment was carried out using FACS Calibur flow cytometer and analyzed with FCSExpress V3 software.

Osteogenic and neurogenic differentiation

Osteogenic and neurogenic differentiation was carried out according to the previous protocol with some modifications[18]. For the induction of osteogenic differentiation, cells were seeded at a density of 3×10^3 cells/cm2 in cell culture media composed with 10 nM dexamethasone, 50 µg/ml ascorbic acid 2-phosphate and 10 mMβ-glycerophosphate. After completion of the cultivation time, cells were fixed with 4% paraformaldehyde for 15 minutes followed by alizarin red staining for 15 minutes at room temperature and carried out the mineralized bone matrix examination.

Neural differentiation was achieved by seeding the cells at a density of 5000 cell/cm^2 on poly-L-lysine (Sigma, P4707)-coated glass coverslips and cultured in neurobasal medium composed with 1% ITS (Invitrogen, USA), and cytokines including 100 ng/ml basic

fibroblast growth factor (bFGF, Sigma) for five days followed by 100 ng/ml bFGF, 10 ng/ml FGF8 (Sigma), and 100 ng/ml sonic hedgehog (SHH, Sigma) for an additional five days.

Expression analysis of selected genes using Quantitative Real-Time PCR (qRT-PCR).

Total RNA was isolated using TRIzol reagent (Thermo Fisher, USA). The quality and quantity of RNA was measured by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) followed by cDNA conversion using High capacity cDNA conversion kit (Thermo Fisher). The expression levels of βIII Tribulin, GAD, NeuN, DSP, Runx2, and OCN were analysed by Taqman gene expression assays (Thermo Fisher, USA) The assay IDs of selected genes as follow, DSP; Hs00950591_m1, Runx2; Hs01047973_m1, OCN; Hs01587814_g1, Tubulin 3 beta; Hs00801390_s1, GAD; Hs01065893_m1; NeuN; Hs01370654_m1. All experiments were performed in triplicate in three independent experiments using ABI PRISM 7500 (Applied Biosystems, USA). The housekeeping gene GAPDH was used as an internal control to normalize the expression levels. The data for gene expression was analyzed with the ΔΔCt method.

2.5. Statistical analysis

Statistical analyses were performed using SPSS software (version 20.0). The data were presented as mean ± standard deviation (SD) for all quantitative measurements and experiments were carried out in triplicate. Paired t-test and One-way analysis of variance (ANOVA) were used to analyze the gene expression between groups. All statistical analyses were performed at the significance level $p < 0.05$.

RESULTS

Colony-forming unit-fibroblast assay

Both PDLSCs and SHEDs showed colony-forming ability. The number of colony-forming unit-fibroplasts (CFU-Fs) per 480 cells in PDLSCs (37.3–13.3) was slightly lower than that in SHEDs (44.4–18.2), However, the difference was not statistically significant($p>0.13$).

Flow cytometry analysis

Flow cytometry analysis was revealed that nearly two types of the cells expressed positivity for CD90 and CD105 (>96.9%). PDLSCs expressed 91.2% positivity for CD146 whereas

SHEDs were expressed 93.8% positivity. However, the expressions of CD31 (an endothelial stem cell marker) was found to be 8% and CD45 positivity (a hematopoietic cell marker) was observed to be <3.5% (see Fig 1).

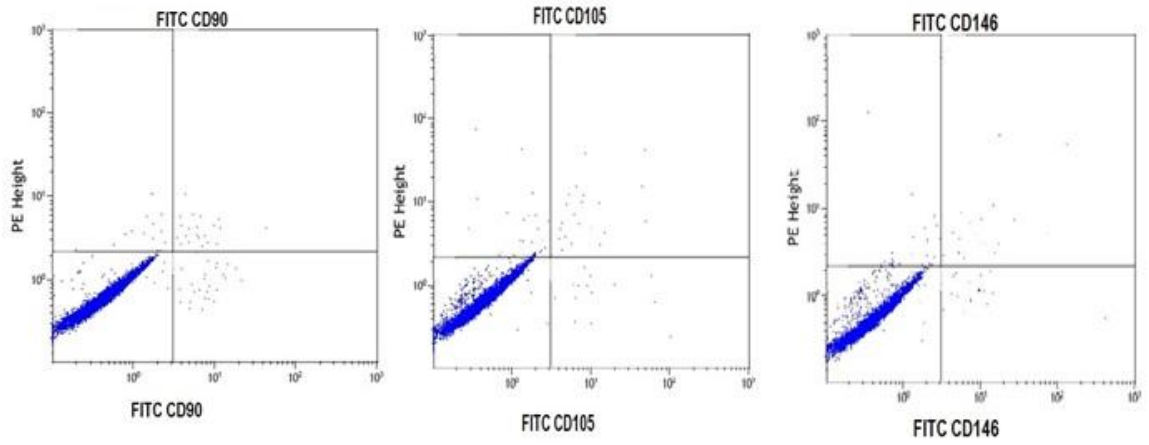

Figure 1 Flow cytometry analysis of stem cell surface markers

Effect of platelet lysate on proliferation of PDLSCs and SHEDs

We observed significant differences with respect to cell proliferation at different concentrations of PL for different time intervals. After 24 hours of incubation with three different concentrations i.e. 2.5%, 55 and 10% PL, there was no significant difference in PDLSCs cell proliferation rate, however, slightly increased proliferation rate was observed in SHEDs compared to 10% FBS. Over 48 hours incubation, both cells showed a significant increase in proliferation rate with 5% and 10% PL concentration. After completion of 72 hours incubation, SHEDs at 5% PL showed increased cell proliferation rate compared to PDLSCs. Moreover, we observed that both cells with 2.5% and 10% PL concentration showed more or less similar proliferation rate.

Effect of PL on osteogenic and neurogenic differentiation of PDLSCs and SHEDs

We evaluated the *in vitro* functional effect of PL on the osteogenic and neurogenic differentiation of PDLSCs and SHEDs at different concentrations of PL for different time intervals. After 3 weeks of cultures with osteogenic differentiation medium, both cells were showed increased aggregation and high density of clusters compared to cell cultured in 10% FBS. Further, mineralized matrix staining was found to be more in cells cultured with 5% and 10% PL compared to 10% FBS. In neural differentiation medium, SHEDs began to acquired

neural cell like morphology on day 15 at 5% PL concentration. However, PDLSCs showed neural cell like morphology on day 19. Moreover, we observed that SHEDs have more complex projections and pyramidal appearance than PDLSCs (see Fig 2 and 3).

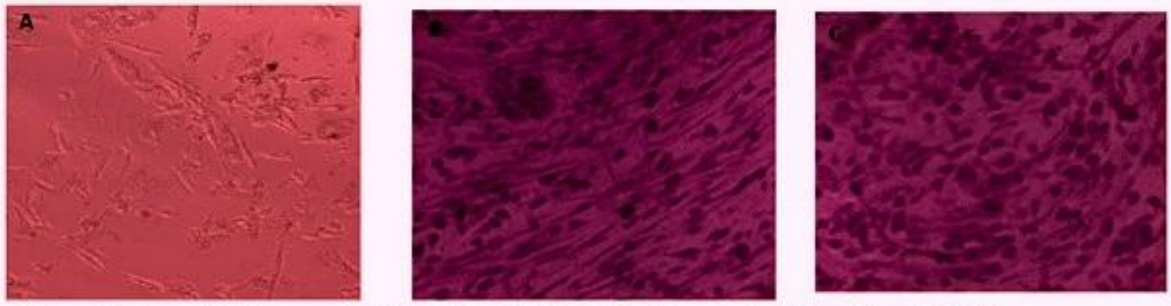

Figure 2 A. Control B. Neurogenic differentiation in PDLSCs C. Neurogenic differentiation in SHEDs

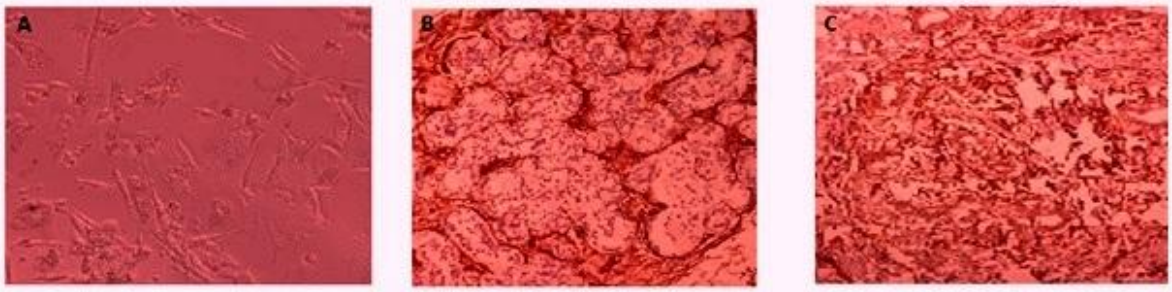

Figure 3 A. Control B. Osteogenic differentiation in PDLSCs C. Osteogenic differentiation in SHEDs

Gene expression analysis of osteogenic and neurogenic markers of PDLSCs and SHEDs

We assessed the mRNA expression levels of different osteogenic and neurogenic markers. Expression of osteogenic markers like OCN, DSP and RUNX2 was elevated when cell cultured in osteogenic differentiation medium. Increased expression of these markers observed in cells cultured at 5% PL compared to 10% FBS (DSP; $p<0.04$, Runx2;$p<0.06$;OCN;$p<0.05$). Further, we observed that PDLSCs showed increased osteogenic maker expression compared to SHEDs irrespective of cell culture conditions and medium. Cells in neurogenic differentiation medium with 5% PL showed increased neurogenic marker expression compared to cell cultured in 10% FBS. (βIII Tubulin; $p<0.03$, GAD;$p<0.06$, NeuN; $p<0.02$. Moreover, we observed that SHEDs showed elevated neurogenic marker expression compared to PDLSCs at 5% PL as compared to 10% FBS (see Fig 4 and 5).

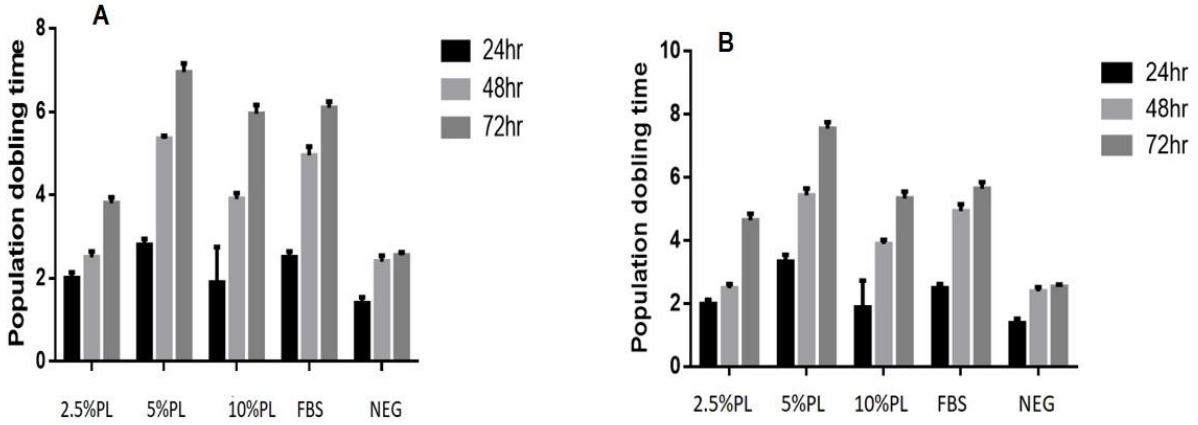

Figure 4. Effect of PL on proliferation of PDLSCs (A) and SHEDs (B)

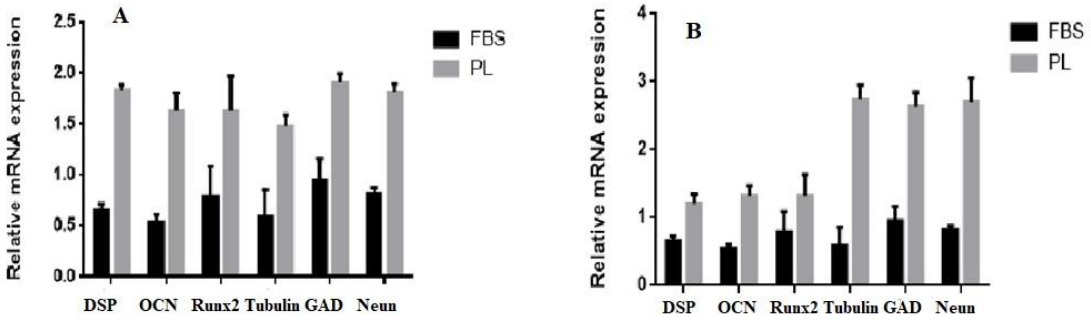

Figure 5 Effect of PL on expression of osteo/neurogenic markers in PDLSCs (A) and SHEDs (B)

DISCUSSION:

Using PDLSCs and SHEDs from deciduous teeth is now becoming a potential cell based therapeutic procedure as they have excellent proliferation rate and multipotential differentiation capacity. Previously, several investigators demonstrated the role of PDLSCs and SHEDs in mineralized tissue regeneration thereby considered their clinical relevance as a valuable stem cell source[19,20,21,22.] However, in order to mediate the translation of dental stem cells from basic science to clinical applications, establishment of proper cell culture protocols are fundamental requirement. Stem cells cultured in FBS (animal serum), poses risk of transmission of animal viruses and bacteria. Autologous human serum suggested to be a reliable alternative source to expand MSCs for clinical therapies. Lee et al.[13] studied the effect of human platelet lysate on proliferation rate and differentiation ability of human dental stem cells.

In our study, we observed a significant varied proliferation rate when PDLSCs and SHEDs cultured with PL as compared to FBS. Among three different concentrations of PL used, the proliferation rate was high at 5% PL. Previously, several researchers reported the optimum concentration of PL for cell proliferation. Abuarqoub et al [11]suggested that DPSCs and SCAP were showed increased proliferation rate when cultured with 5% PL. In another study, 0.5 – 1% PL concentration suggested to be an optimum concentration for induction of cell proliferation and mineralization Soffer et al [14]. However, few inconsistent results have been reported with respect to optimum PL concentrations. Ferreira et al [15] reported that 50% PL concentration required for osteoblast proliferation whereas 10% PL concentration suggested by Lucarelli et al.[16]andCastegnaro et al. [17].

We observed, increased neurogenic differentiation of PDLSCs and SHEDs at 5% PL compared to 10% FBS on day 15. However, cell cultured with 10% FBS showed neurogenic differentiation after day 21 suggesting that, PL significantly enhanced the neurogenic differentiation potential in short period compared to FBS. Similar results were observed in previous studies with respect to osteogenic differentiation whereas formation of mineralized matrix found to be increased at 5% PL compared to FBS. D. Abuarquoub et al 2015 reported that cells with 5% PL showed elevated OPN and ALP activity in short period of time.

CONCLUSION

Our finding suggests that platelet lysate (PL) can be considered as an alternative substitute for FBS as PDLSCs and SHEDs showed significant proliferation rate and differentiation potential. Moreover, SHEDs may have increased neurogenic differentiation capacity compared to PDLSCs at 5% PL concentration.

ACKNOWLEDGEMENT

We would like to thank all participants who agreed to give dental pulp tissues to conduct research work.

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