Advanced Platelet Rich Fibrin (A-PRF) Supplemented Culture Medium for Human Dental Pulp Stem Cell Proliferation

Illmilda[1], Dini Asrianti[2]*, Anggraini Margono[2], Indah Julianto[3], Mettasari Puspa Wardoyo[1]

1. Post Graduate Student of Conservative Dentistry Specialist, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.
2. Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.
3. Department of Dermatology, Faculty of Medicine, Universitas Sebelas Maret, Solo, Indonesia.

Abstract

Recently, Advanced Platelet Rich Fibrin (A-PRF) has been considered an alternative to fetal bovine serum (FBS) as a culture medium supplement for cells. A-PRF can release the growth factors (GF) that are required for fibroblast proliferation. However, the ability of A-PRF to support proliferation in human Dental Pulp Stem Cells (hDPSCs) has not been reported yet.

We analyzed hDPSC proliferation from cultures in three different concentrations of A-PRF cultured medium (10%, 20%, and 25% A-PRF) using flow cytometry and an MTT assay compared with a control group. Compared with 10% and 20% A-PRF, the proliferation of hDPSCs in 25% A-PRF cultured medium was significantly different on the first day (P<.05). The proliferation rate of hDPSCs between days 1 and 3 and days 1 and 5 was significantly different (P<.05) in the different media. There were no significant differences in the proliferation rate between days 3 and 5 (P>.05). The highest rate of proliferation was observed with 25% A-PRF on day 1 compared with 10% and 20% A-PRF.

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Introduction

In the last few years, there has been a changing paradigm in conventional endodontic into Regenerative Endodontic Treatment (RET) procedure. The American Association of Endodontists (AAE) defines RET as a biologically based procedure designed to replace damaged structures, including dentine and root structures, as well as cells of the pulp-dentin complex. [1] RET is based on the tissue engineering triad of stem cells, growth factors, and scaffolds. [2–8] In the dentistry field, Mesenchymal Stem Cells (MSCs) such as human Dental Pulps Stem Cells (hDPSCs) are adult multipotent stem cells located in the cell-rich zone of the pulp that can differentiate into odontoblasts, osteoblasts, adipocytes, chondrocytes, and other cells depending on the type of regulatory molecules present.[4,9–11]

Corresponding author:
Dini Asrianti
Department of Conservative Dentistry,
Faculty of Dentistry, Universitas Indonesia,
Jakarta, Indonesia.
E-mail: dini.rz@gmail.com

Fetal Bovine Serum (FBS) is the gold standard cell culture medium and has numerous growth factor and low gamma globulins compared with other animal-derived supplements. However, FBS can be contaminated with the bovine virus and cause a xenogeneic reaction in the cells. [12,13] Recently, the use of a human-derived serum, such as Platelet Rich Fibrin (PRF), has been developed to replace FBS as a cell culture supplement. Ghanaati *et al.* conducted a PRF modification study by lengthening the centrifugation from 12 to 14 min and lowering the velocity from 2700 to 1500 rpm, which became known as Advanced Platelet Rich Fibrin (A-PRF). A-PRF has higher platelet counts and neutrophil granulocytes.[14] Another study showed that a low centrifugation speed increased the number of leukocytes in the matrix scaffold and upper layer of A-PRF and could be pressed down into a thin membrane to produce an A-PRF exudate.

According to a study by Kobayashi-Fujioka *et al.*, a PRF matrix test showed the proliferation and migration rate of gingival fibroblasts is significantly higher with 20% A-PRF and 20% A-PRF+ compared with 20% L-PRF on day 5.[15] A study conducted by Diananda *et al.* proved that A-PRF lysate could support fibroblast

proliferation best with 25% A-PRF.[16] However, no research has demonstrated the potency of A-PRF on the proliferation of hDPSCs. Based on a study by Akpinar *et al.*, hDPSCs in the G1 phase of the cell cycle showed the most significant number of cells (78%) compared with the S and G2 phases.[17] Our study was conducted to determine the ability of A-PRF to support hDPSC proliferation *in vitro* on day 1, 3, and 5 after plating with three concentrations.

Materials and methods

The Ethical Committee of the Faculty of Dentistry, Universitas Indonesia approved this study (No. 136/Ethical Approval/FKGUI/XII/2017, No. Protocol: 051281017). Six human third molars were collected from 19-35-year-old healthy donors, with no history of alcohol or smoking at RSGM FKG UI, following informed consent. The teeth were transferred to the cell culture laboratory where stem cells from the pulp tissue were isolated according to a previously published method.[16] The tooth was cut around the cementoenamel junction. Then, the pulp tissue was gently removed from the chambers and sliced with scissors into 0.5 to 1 mm sections. Then, the samples were diluted with an enzyme solution that consisted of 3 mg/ml collagenase type I and 4 mg/ml dispase (Sigma-Aldrich, St. Louis, MO, USA) for 30-60 min at 37 °C. The cells were cultured in 3 ml DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin-G, and 100 mg/ml streptomycin (Roche, Basel, Switzerland), and were incubated in an atmosphere of 5% CO_2 at 37 °C.

The cells used in this study were at the second passage. hDPSCs were divided into three different exprerimental culture media groups: 10%, 20%, and 25% A-PRF groups. Also one control group, hDPSCs were exposed 24 h starvation in DMEM supplemented with FBS 1% (control medium). Each group had six biological replicates. The serum starvation procedures were according to a previously published method.[16]

A-PRF preparation

A certified laboratory assistant collected 10 ml of cubitus vein blood from four healthy donors. Less than 2 min after collection, they were centrifuged at 1500 rpm for 14 min and the A-PRF gel layer was separated from the red blood cells. Then, the A-PRF was incubated for 24 h at 4 °C to collect the supernatant layer (Fig. 1). This supernatant layer was used as a culture medium supplement and diluted to a concentration of 10%, 20%, and 25% A-PRF. The supplement was added separately to the culture medium containing 1 × 10^3 hDPSCs per well in a 96-well plate.

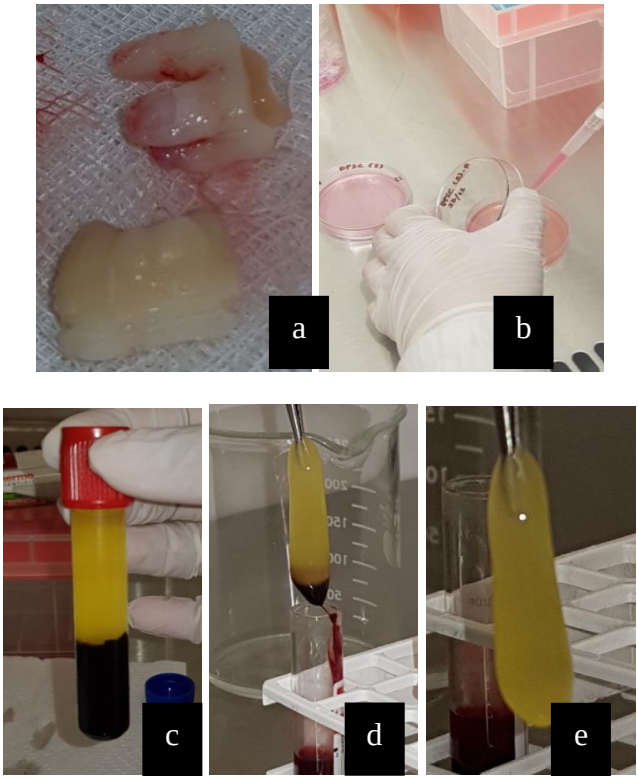

Figure 1. Preparation of the pulp tissue and advanced platelet-rich fibrin (A-PRF). (a) The tooth sample was cut horizontally on the cementoenamel junction to obtain the pulp tissue. (b) Seeding of the pulp tissue. (c) A-PRF after centrifugation at 1500 rpm for 14 min. (d) A-PRF gel. (e) A-PRF gel separated from the red blood cells.

Immunophenotypic analysis of hDPSCs

FACSVerse (BD Biosciences) was used for the flow cytometry analysis, and FlowJo software was used to analyze the data. An MSC positive cocktail (CD73, CD90, and CD105) and negative cocktail for hDPSCs (CD45, CD34, CD14, CD19, and HLA-DR) were evaluated.

During days 1, 3, and 5 hDPSCs proliferation was examined using 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium

bromide (MTT) in accordance with manufacturer's instructions. The data analysis for the potential ability of each group was analyzed using a one-way analysis of variance (ANOVA) with post hoc Tamhane and Kruskal Wallis. Differences between days 1, 3, and 5 were analyzed using the Friedman test, and comparisons between groups were examined using the Wilcoxon test.

Results

The flow cytometry analysis of the hDPSCs revealed MSC characteristics from the positive cocktail with CD 90 (98.3%), CD 73 (98.5%), and CD 105 (71.0%) and negative cocktail with CD45, CD34, CD14, CD19, and HLA-DR (2.5%) (Fig. 2). Therefore, it can be concluded that the cells are hDPSCs.

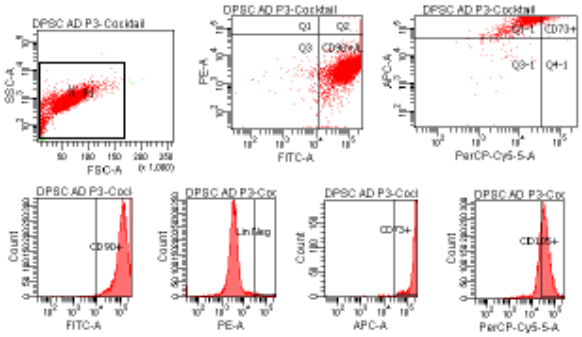

Figure 2. Flow cytometry was used to test MCS positive and negative cocktail for hDPSCs.

There were significant differences amongst the treatment groups (Table 1). The highest mean value of hDPSCs proliferation was 25% A-PRF (881,083 cells/ml), followed by 20% A-PRF (587,764 cells/ml), and 10% A-PRF (279,305 cells/ml). hDPSCs proliferation in A-PRF medium showed a higher cell density and more homogenous size and shape than other culture media (Fig. 3).

Culture Medium	N	Mean (%)	SD	*P*-value
Control	6	35,823	2,506	.000*
10% A-PRF	6	279,305	14,962	
20% A-PRF	6	587,764	37,145	
25% A-PRF	6	881,083	67,279	

*One Way ANOVA, *P*<.05

Table 1. The mean analysis of hDPSCs proliferation between the control medium (starvation), 10%, 20%, and 25% A-PRF.

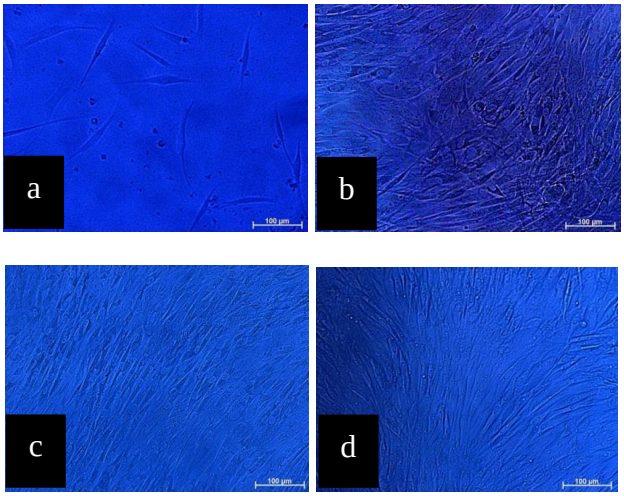

Figure 3. Images of hDPSCs proliferation in A-PRF medium showed a higher cell density and more homogenous size and shape than other culture media on the fifth days of treatment. (a) hDPSCs in starvation media, (b) 10% A-PRF, (c) 20% A-PRF, (d) and 25% A-PRF, which showed the highest cell density.

The proliferation rate of hDPSCs was significantly different amongst the control (starvation) and experimental media. Proliferation rate of hDPSCs on the first day of treatment was significantly different between 10% A-PRF compared with 20% A-PRF and 25% A-PRF; also between 20% A-PRF compared with 25% A-PRF. (Table 2).

Culture Medium	*P*-value
Control vs 10% A-PRF	.000*
Control vs 20% A-PRF	.000*
Control vs 25% A-PRF	.000*
10% A-PRF vs 20% A-PRF	.000*
10% A-PRF vs 25% A-PRF	.000*
20% A-PRF vs 25% A-PRF	.000*

*Post hoc Tamhane, One Way ANOVA, *P<.05*

Table 2. hDPSCs proliferation between the control medium (starvation), 10%, 20%, and 25% A-PRF.

There were no significant differences in the hDPSCs proliferation rate between the control medium and A-PRF on days 3 and 5 (Table 3). Nevertheless, the mean value of hDPSC proliferation on day 1 showed that 25% A-PRF has the potential to initiate the highest hDPSCs proliferation rate compared with 10% and 20% A-PRF. There were also significant differences in proliferation between treatment groups on each day of evaluation. A post hoc Wilcoxon test was performed to see the

differences between A-PRF medium on days 1, 3, and 5. There were significant differences in proliferation with all concentrations of A-PRF between days 1 and 3 and between days 1 and 5.

Culture Medium	Proliferation of hDPSCs Day 1 Mean (SD)	Day 3 Median (Min-Max)	Day 5 Median (Min-Max)	*P-value*
10% A-PRF	279,305 (14,962)	34,605 (29,320-189,490)	18,8405 (17,930-150,000)	.009*
20% A-PRF	587,764 (37,145)	51,466 (48,120-336,660)	25,774 (23,839-300,308)	.009*
25% A-PRF	881,083 (67,279)	75,593 (66,240-425,880)	39,039 (36,770-382,390)	.009*
P-value	.000*	.083	.083	

*One Way ANOVA, *P*<.05; *Kruskal–Wallis, *P*<.05; * Friedman, *P*<.05

Table 3. The analysis of proliferation with 10%, 20%, and 25% A-PRF on days 1, 3, and 5.

Discussion

The expansion and culture of hDPSCs *in vitro* is dependent on the culture supplements in the medium. In addition, the substrate area, cell density during seeding, and physical and chemical environment (O_2 and CO_2 concentrations, temperature, pH, osmolality, and buffer systems) can also affect cell proliferation rates. FBS is an animal serum supplement that is commonly used in culture media but can induce a xenogeneic reaction and takes a long time to expand.[12,13,18,19] The composition of FBS is not fully known and has variations in every batch that can affect reproducibility. However, FBS is still widely used in cell culture.

hDPSCs were first isolated from teeth by Gronthos *et al.* in 2000 and were the primary source of dental MSCs. Freshly isolated MSCs are the best option for therapeutic application because they have better stability, anti-inflammatory properties, and neuroprotective effects. This study aimed at analyzing hDPSCs proliferation with three concentrations of A-PRF.

DPSCs do not have specific markers. Therefore, MSC markers such as STRO-1, CD146, and CD44 are commonly used for the identification of DMSCs.[4,20] In this study, flow cytometry tests revealed that hDPSCs were positive for CD90 (98.3%), CD73 (98.5%), and CD105 (71.0%) and negative for CD45, CD34, CD14, CD19, and HLA-DR (2.5%). These results are in accordance with the Committee of Mesenchymal Stem Cells and Tissues of the International Society for Cellular Therapy. Therefore, it could be concluded that the cells in this study were hDPSCs.

A-PRF can be used as a culture medium supplement that activates the endogenous coagulation processes without additional anticoagulants derived from animals, such as bovine thrombin. There was a significant difference in hDPSCs proliferation with 10%, 20%, and 25% A-PRF supplemented cultured medium between days 1 and 3 and days 1 and 5. This outcome is similar to a study by Diananda *et al.* that proved that A-PRF lysate supported the proliferation in fibroblasts and showed the highest proliferation rate with 25% A-PRF.[16] This result is also consistent with a study by Kobayashi-Fujioka *et al.*, which showed the rate of gingival fibroblast proliferation and migration was significantly higher with 20% A-PRF and 20% A-PRF + compared with 20% L-PRF in a PRF matrix test.[15]

According to the study by Kobayashi-Fujioka *et al.*, a high centrifugal force has a direct impact on the release of growth factors in PRF scaffolds.[15] The application of a low centrifugal force and longer time increases the number of leukocytes in the matrix scaffold or on the top layer of the PRF matrix and positively impact tissue engineering and healing processes.[14,15,21] The proliferation phase begins in the G0 and G1 cycles with the highest amount of proliferation in G0. Due to cell mitosis, the proliferation rate decreases after the G1 phase. This theory is consistent with the result that the highest rate of proliferation in the hDPSCs was on day 1 and decreased on days 3 and 5. In addition, this is also in line with a study by Akpinar *et al.* which stated that hDPSCs in the G1 phase showed the most significant number of cells (78%) compared to the S and G2 phases of the cell cycle. The G1 phase has more mRNAs and higher protein synthesis compared with the mitotic phase. [17]

Conclusions

A-PRF has superior potential for supporting hDPSCs proliferation. The highest proliferation rate was with 25% A-PRF, and the lowest was with 10% A-PRF on the first day of

evaluation. The hDPSC proliferation means did not significantly differ with 10%, 20% or 25% A-PRF on days 3 or 5.

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Declaration of Interest

The authors report no conflict of interest.

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