

Ideal Concentration of Advanced-Platelet Rich Fibrin (A-PRF) Conditioned Media for Human Dental Pulp Stem Cells Differentiation

Dini Asrianti Bagio¹, Indah Julianto², Endang Suprastiwi³, Anggraini Margono⁴

¹Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.  0000-0001-7176-724X

²Department of Dermatology, Faculty of Medicine, Universitas Sebelas Maret, Solo Surakarta, Indonesia.  0000-0002-4246-4845

³Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.  0000-0001-8712-1355

⁴Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.  0000-0003-1598-2599

Author to whom correspondence should be addressed: Dini Asrianti, Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia. Jln. Salemba Raya No 4. Jakarta, 13410. Indonesia. Phone: +62 213913413. E-mail: dini.rz@gmail.com.

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Abstract

Objective: To discover the ideal concentration of Advanced Platelet Rich Fibrin (A-PRF) as modification of PRF, for human Dental Pulp Stem Cells (hDPSCs) differentiation. **Material and Methods:** hDPSCs were divided into five experimental groups: Group I (control group) consist of hDPSCs cultured in 10% FBS, Group II consist of hDPSCs cultured in 1% A-PRF, Group III consist of hDPSCs cultured in 5% A-PRF, Group IV consist of hDPSCs cultured in 10% A-PRF and Group V consist of hDPSCs cultured in 25% A-PRF. All group have been observed for 7 and 14 days and each group had three biological replicates (triplo). Formation of the mineralized nodules was detected after 7 days by Alizarin red-based assay and Dentin Sialophosphoprotein (DSPP) expression after 7 and 14 days quantified by ELISA reader. Statistical analysis was proven with Kruskal-Wallis and post hoc Mann-Whitney test. **Results:** The differentiation of hDPSCs in all A-PRF groups was significantly different on day-7 ($p < 0.05$) compare to control group (Group I). There were no significant differences between all groups on day-14 ($p > 0.05$). Significant differences between Group II (1% A-PRF) and Group I (control), Group II (1% A-PRF) and Group III (5% A-PRF), also Group II (1% A-PRF) and Group V (25% A-PRF) was found from post hoc test analysis. **Conclusion:** The ideal conditioned media concentration for differentiation of human dental pulp stem cells was on 1% up to 5% A-PRF group.

Keywords: Platelet-Rich Fibrin; Dental Pulp; Cell Differentiation.

Introduction

The American Association of Endodontists (AAE) in 2013 describes that Regenerative Endodontics Treatment (RET) can be interpreted as a maintenance procedure with the basic principles of biological sciences, that can replace a physiologically damaged tooth structures and regenerate the healthy pulp-dentin complex. The changing paradigm concept of tissue engineering and regeneration in RET has led to the concept of free based cell therapy using secretome as a source of Growth Factor (GF). Even though the development of stem cell-based therapy that using exogenous stem cells in RET has been reported these recent years, but the visibility of using freshly stem cells may found difficulties in the steps of its clinical application. So the successful outcomes of this type of therapy by in-vitro study not directly parallel with the use of this therapy in clinical application. One of the sources of an exogenous cell is human Dental Pulp Stem Cells (hDPSCs) that has been cultured in-vitro [1,2].

The use of exogenous cell, lately changed by the combination use of stem cell itself endogenously (endogenous stem cells) and secretome from outside or namely as free based cell therapy. In RET fields, the endogenous stem cells can be explained as the last source of hDPCs inside the root canal system may contain progenitor or stem cells, and secretome is a conditioned media consist of GF from outside the root canal. One source of growth factors or secretome that are widely used in dentistry is Platelet-Rich Fibrin (PRF). According to several studies, PRF can act as a regulator of the immune system, and stimulate the wound healing process through various GF (such as Platelet-Derived Growth Factor bb (PDGF-bb), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor b-1 (TGFb-1), IL1- β IL6, IL4 and Tumor Necrotic Factor-a (TNF-a)) [3-5].

Human Dental Pulp Cells (hDPCs) is one of Mesenchymal Stem Cells (MSCs) or Adult Stem Cells (ASC) or progenitor cells originating from Dental Pulp. The specific characteristics of hDPCs are the ability to differentiate into several other cell forms through limited lineages or multipotent. In the case of pulp irritation due to caries, mechanical/thermal irritants, fractures or dental procedures, the odontoblast layer or pulp cell progenitor (hDPCs) may damage, pulp cells can proliferate and migrate to the area of damage to be activated by protein molecules that induce signal pathway differentiation of pulp cells into odontoblasts like cells and forming reparative dentine. This process is a mechanism that initiates the formation of the dentinogenesis process and regeneration of the pulp dentinal complex [6,7].

It has been demonstrated that PRF can increase the proliferation and differentiation of Dental Pulp Cells (DPCs) by upregulating of Osteoprotegerin (OPG) and Alkaline Phosphatase (ALP) [8]. Another study reported, modification [of the speed and time of centrifugation of original PRF (Choukroun's PRF) from 2700 rpm for 12 minutes to 1500 rpm for 14 minutes, can create a new type of PRF namely Advanced PRF (A-PRF) [9]. This change causes an increasing number of granulocytes that have the ability to release growth factors on the distal gel PRF. According to previous authors, an increase in the proliferation of fibroblast cells can be induced by the application

of 25% A-PRF that start from the first 1 hour to its peak in the first 24 hours and continuing to decrease on the third and fifth days. This study was conducted by comparing three concentrations of A-PRF 12.5%, 25%, 50% [9].

A-PRF and Concentrate Growth Factor (CGF) contained higher GFs like TGF- β 1, PDGF-BB, VEGF, and cytokine pro-inflammatory IL-1 β levels than Platelet Rich Plasma (PRP). A-PRF produces the highest GF compared to CGF and PRP, and the lowest is Platelet Rich Growth Factor (PRGF) group [10]. Other researchers have shown that 10% PRF exudate has the ability to replace Fetal Bovine Serum (FBS) in the process of proliferation of Dental Pulp Stem Cell (DPSC), the highest potential on day 1 with a decrease on day 3 to day 5, evaluation using MTS tetrazolium cell proliferation assay kit and ELISA reader. The ability of osteogenic differentiation of DPSCs was best on the 7th day in 1% PRF exudate group. Even though PRF has proven has the potential ability in fibroblast cells and hDPCs proliferation, but other studies revealed that PRF did not have a strong stimulatory effect on cell viability and proliferation on Human Gingival Fibroblast cells [10-15].

Until now the use of PRF has been proven to be able to induce the process of fibroblast proliferation and differentiation of hDPCs in many modification forms such as Platelet Rich Growth Factor (PRGF), PRF exudate, and Advanced (A-PRF) with various concentrations, but the ideal concentration of A-PRF that can use especially in hDPCs differentiation not been reported yet. The authors of this study attempt to seek the ideal concentration of A-PRF that can ideally induce the differentiation of hDPCs.

Material and Methods

Ethical Aspects

This study was approved by the Ethical Committee of the Faculty of Dentistry, Universitas Indonesia (No. 35/Ethical Approval/FKG UI/V/2018; Protocol No. 090250218). hDPSCs that used in this study was given from the previous research (with letter of approval from the Ethical Committee of Faculty of Dentistry Universitas Indonesia No. 135/Ethical Approval/FKG UI/XII/2017; Protocol No. 051331017).

Osteogenic and Odontogenic Differentiation Assay

The hDPCs were at the third to four passages. hDPCs were also exposed to 24 h starvation conditions in high-glucose Dulbecco's modified Eagle's medium (DMEM-Gibco) (Thermo Fisher Scientific Inc., Massachusetts, USA) supplemented 1% with Fetal Bovine Serum (1% FBS) as control medium before cultured in the osteogenic differentiation media. The serum starvation procedures were according to a previously published method [11-13]. The osteogenic differentiation media consisted of nutritional media (DMEM), 50 μ g/ml of L-ascorbic acid 2-phosphate, 10 mM of β -glycerophosphate sodium salt, 10 μ M of dexamethasone and antibiotics (100 mg/ml streptomycin and 100 μ /ml penicillin). For osteogenic differentiation 6-well plates were used 1×10^5 cells/well in the 6 well plates were cultured with complete media till reach nearly 70% confluence and then

incubated at 37°C and 5% CO₂ for 24 h. The cells were maintained in osteogenic differentiation media with the different concentrations of conditioned media. There were five experimental groups in this study: Group I (control group) consist of hDPSCs cultured in 10% FBS, Group II consists of hDPSCs cultured in 1% A-PRF, Group III consist of hDPSCs cultured in 5% A-PRF, Group IV consist of hDPSCs cultured in 10% A-PRF and Group V consist of hDPSCs cultured in 25% A-PRF. All group has been observed for 7 and 14 days and each group had three biological replicates (triple).

Conditioned media of five experimental groups in this study; 10% FBS (control), 1%, 5%, 10%, and 25% A-PRF was changed every 3 days. The formation of the mineralized nodules was detected after 7 days by Alizarin red-based assay. The cells in the 6-well plates were washed twice with PBS and fixed for 30 minutes in formaldehyde (PFA 4%) at room temperature. Before the addition of 250 µl of 40 mM Alizarin Red S (pH 4.1) per well, the cells washed twice again with PBS. The plates were incubated at room temperature for 20 min with gentle shaking. After the aspiration of the excess dye, the wells were washed twice with 1ml dH₂O during a 5 min shaking. The stained monolayers were visualized by phase-inverted microscope (Olympus Optical Co., Ltd., Japan).

Quantitative evaluation of odontogenic differentiation from Dentin Sialophosphoprotein Protein (DSPP) expression for ELISA (Abexxa Ltd, Cambridge, UK) was used to analyze the odontogenic activity on day 7 and day 14 following the manufacturer's protocol. The values were measured using a microplate reader under a wavelength of 405 nm.

A-PRF Preparation

Blood collection inclusion criteria were 19-35 year-old healthy donors, with no smoking and no alcohol consumption habit. Blood collected from 3 donors and 10 ml of cubitus vein blood collected by a certified laboratory assistant. After collection, in less than 2 min 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 24h at 4°C to collect the supernatant layer. This supernatant layer was used as a culture medium supplement and diluted to a concentration of 1%, 5%, 10%, and 25% A-PRF (Figure 1).

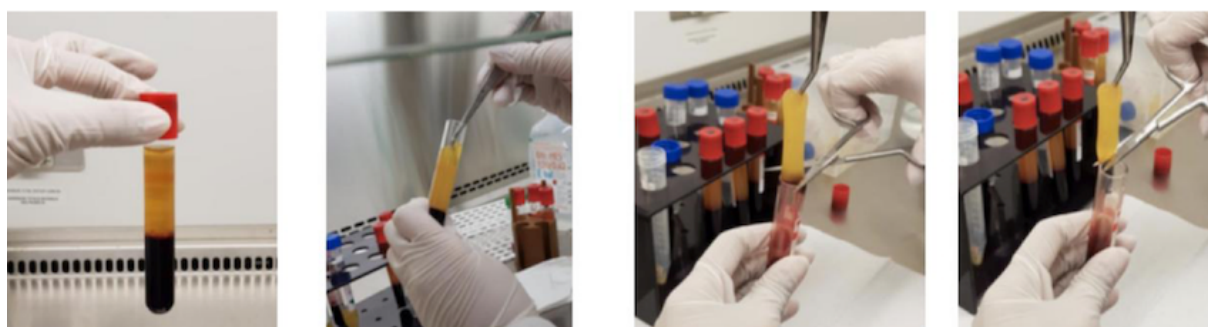


Figure 1. Preparation of A-PRF, the A-PRF gel layer was separated from the red blood cells using scissor and after that was incubated for 24 h at 4 °C to collect the supernatant layer.

Data Analysis

Statistical analysis was performed with Kruskal-Wallis and post hoc Mann-Whitney tests. The level of significance was 5%. All data was analysed using IBM SPSS Statistics Software, version 20 (IBM Corp., Armonk, NY, USA).

Results

The mineralized nodules were clearly formed and stained with Alizarin Red within all wells. There were obvious differences in the amounts of mineralization among the different groups. In particular, the mineralized nodules progressively increased with 25% A-PRF exudate at day-7 compare to all groups. The control group (10% FBS) also performed a good amount of mineralization compare to other groups (Figure 2).

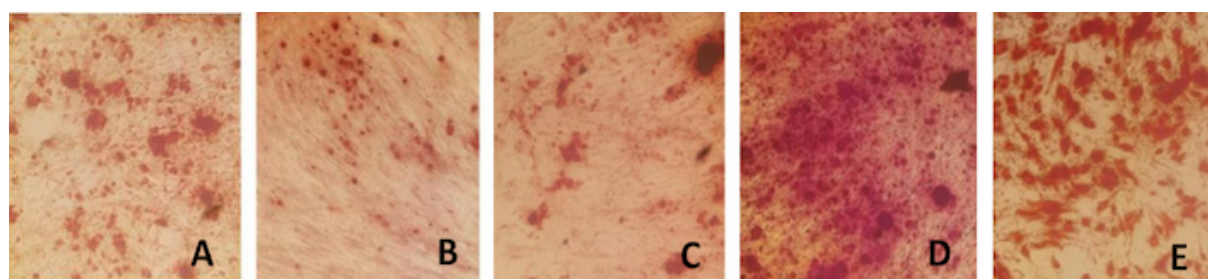


Figure 2. Qualitative evaluation of hDPSCs cultured in osteogenic differentiation media that supplemented with 1% A-PRF (A), 5% A-PRF (B), 10% A-PRF (C), 25% A-PRF (D), and 10% FBS (control) (E). Formation of the mineralized nodules was detected and quantified after 7 days by Alizarin red-based assay.

Odontogenic differentiation hDPSCs were analyzed using DSPP expression, Kruskal-Wallis statistical test revealed significant differences between all groups on day-7 and no significant differences among all groups on day-14 (Table 1). Non-parametric test was chosen due to the normality of the data. The highest mean values for DSPP expression was in 1% A-PRF group (0.955 ± 0.005) while the lowest mean was for 25% A-PRF group (0.843 ± 0.015) (Figure 3).

Table 1. Odontogenic differentiation analysis by DSPP expression of hDPSCs cultured in 10% FBS (control), 1%, 5%, 10%, and 25% A-PRF on day-7 and day-14 observation.

Experimental Groups	Time		p-value
	Day-7	Day-14	
FBS 10% (Control)	0.890 (0.000-0.925)	0.806 (0.000-0.918)	<0.001*
1% APRF	0.978 (0.949-0.958)	0.806 (0.000-0.860)	<0.001*
5% APRF	0.944 (0.930-0.950)	0.871 (0.870-0.875)	<0.001*
10% APRF	0.928 (0.879-0.935)	0.795 (0.780-0.808)	<0.001*
25% APRF	0.836 (0.832-0.861)	0.818 (0.646-0.830)	<0.001*
p-value	0.026*	0.397	

*Significantly differences in $p < 0.05$; Kruskal-Wallis and Friedman tests.

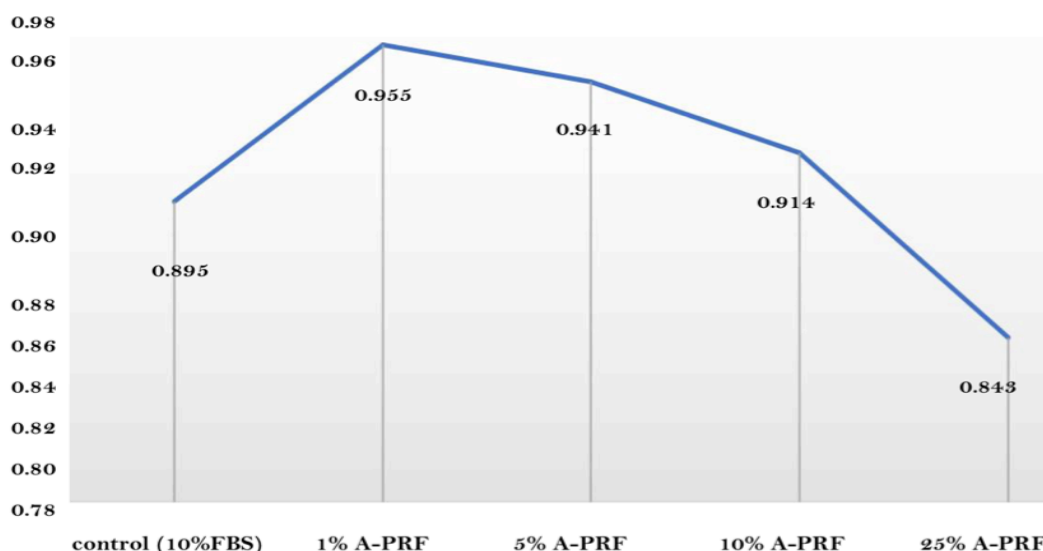


Figure 3. Mean value of DSPP expression of hDPSCs cultured in 10% FBS (control), 1%, 5%, 10%, and 25% APRF. The highest mean values for DSPP expression was in 1% A-PRF group followed with 5% A-PRF, 10% A-PRF, control (10% FBS) and the lowest mean was for 25% A-PRF group.

Friedman statistical test was used to compare the differences after 2 periods of time observation between all groups, it is showed that there are significant differences between day-7 and day-14 for all groups. Post hoc analysis using Mann-Whitney statistical test, it is revealed that 1% A-PRF was significantly different compared to 10% A-PRF and 25% A-PRF group, and there are no significant differences between 1% A-PRF and 5% A-PRF (Table 2).

Table 2. Post Hoc analysis of DSPP expression of hDPSCs between each group after day-7 and day-14 observation.

Experimental Groups	Time	
	Day-7	Day-14
FBS 10% (Control) vs 1% APRF	0.046*	0.513
FBS 10% (Control) vs 5% APRF	0.275	0.050
FBS 10% (Control) vs 10% APRF	0.050	0.827
FBS 10% (Control) vs 25% APRF	0.513	0.827
1% APRF vs 10% APRF	0.046*	0.513
1% APRF vs 25% APRF	0.046*	0.275
5% APRF vs 10% APRF	0.127	0.050
5% APRF vs 25% APRF	0.050	0.050
10% APRF vs 25% APRF	0.050	0.513

*Significantly differences $p < 0.05$, post hoc; Mann-Whitney test.

Discussion

The changing paradigm concept of tissue engineering and regeneration in Regenerative Endodontic Treatment (RET) has now led to the concept of free based cell therapy using secretome as a source of Growth Factors (GFs). It is the use of combination stem cell itself endogenously (endogenous stem cells) and secretome from outside. In RET fields, the endogenous stem cells are the last source of hDPSCs inside the root canal system, and the secretome is a conditioned media from outside the root canal. One of the secretome sources that use in RET is PRF. PRF can increase the

proliferation and differentiation of human Dental Pulp Cells (hDPCs) upregulating by Osteoprotegerin (OPG) and Alkaline Phosphatase (ALP) [3,8].

The use of modification PRF, namely A-PRF is developing in recent years, this is due to the easier preparation of the PRF compare to PRP and the great result of GF releases itself. From the methodological point of view in the present study, A-PRF was prepared by the direct, easy, cheap and fast method through collecting the blood samples quickly and without exposure to air avoiding blood coagulation before centrifugation, also without any continued step of thawing process [10,11]. The result of this study also correlated with the previous research that modification of the speed and time of centrifugation from 2700 rpm for 10 minutes to 1500 rpm for 14 minutes can increase the release of granulocytes of A-PRF and then induce GFs of A-PRF. It is also proved that there is an increase in the proliferation of fibroblast cells induced by the application of 25% A-PRF with an increase starting from the first 1 hour to its peak in the first 24 hours [9,10].

The control group of this study was using a 10% Fetal Bovine Serum (FBS). This study chose FBS as control media because according to the previous study FBS was the gold standard conditioned media that used for cell culture medium. The use of 10% FBS as control was also used previously [11,12].

This study proved that the ideal concentration of A-PRF as odontogenic media was in 1% A-PRF, it is showed from the Dentin Sialophosphoprotein (DSPP) expression the highest mean value was in 1% A-PRF after 7 days of observation. This result correlates with the previous study that concluded that the best supplemented conditioned media for osteogenic differentiation of hDPSCs was in 1% PRF exudate, but other studies revealed that PRF was not suitable for Human Gingival Fibroblast cells proliferation. On the other hand, another study revealed that 25% A-PRF superior to CMWJ (Conditioned Media Wharton's Jelly) in fibroblast cells proliferation and 25% Lysate PRF has the great potential ability as hDPSCs proliferation culture media. So, it is can be assumed that PRF and its modification (in this study A-PRF) might be ideal for hDPCs proliferation and also hPSCs differentiation in percentage variation [11,12,14,15].

The 1% PRF exudate was prepared by two preparation steps. The first one centrifugation with 2700 rpm for 12 min. A white PRF clot was formed between the acellular plasma and RBCs. The PRF clot was held by sterile forceps and separated from RBCs by scissors. The clot was placed on the grid of the endo box and compressed by the endo box cover. After 1 min, the PRF clot was converted into the PRF membrane and the exudate was collected in the tray of the endo box. PRF exudate was centrifuged at 1800 rpm for 5 min to obtain exudate only without RBCs, which were precipitated. In this study preparation of A-PRF only one step centrifugation 1500 rpm for 14 minutes and without any step of compressed PRF using endo box cover [9,11].

The result of qualitative evaluation of osteogenic differentiation of hDPCs did not correlate with the quantitative result of DSPP expression of hDPCs differentiation using ELISA reader. The formation of the mineralized nodules that were detected after 7 days by Alizarin red-based assay showed that the best result was in 25% A-PRF group and 10% FBS group, it is showed by the

amount of the mineralized nodules that appear with a red dot. On the contrary, the higher DSPP expression of hDPCs differentiation using ELISA reader showed from the lower percentage of A-PRF.

The highest mean value was in 1% A-PRF. From both results, we assumed that in Alizarin red analysis the formation mineralized nodules are not specific minerals that form lir-odontoblast, but in DSPP expression the quantitative result only shows the odontogenic differentiation (odontoblast like cells formation) specific in dentinogenesis process. It is showed that the specific characteristics of hDPCs are their ability to differentiate into several other cell forms through limited lineages or multipotent, not only odontoblast but may form to osteoblast cells [6,7].

Conclusion

The ideal concentration of A-PRF which have great potential ability of hDPCs odontogenic differentiation is at 1% to 5% A-PRF. This result can be a standard protocol in using A-PRF for dentin pulp regeneration in regenerative endodontics treatment.

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Conflict of Interest: The authors declare no conflicts of interest.

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