Optimum Concentration of Platelet-Rich Fibrin Lysate for Human Dental Pulp Stem Cells Culture Medium

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Abstract

Platelet Rich Fibrin Lysate (L-PRF) contains platelets, leukocytes and growth factors, all of which contribute to proliferation and healing processes. This allogeneic and autologous material can be used for cell culture supplementation.

Human dental pulp stem cells (hDPSCs) were isolated from the impacted third molars of 10 healthy donors, and then cultured in 3 different supplemented media cultures (10%, 20% and 25% L-PRF). Cell proliferation was analyzed using flow citometry and MTT-Assay.

Compared to cells without supplementation (control group), the L-PRF group showed significant hDPSC proliferation on day 1 ($p < 0.05$), with the highest proliferation rate observed in the 25% L-PRF group. Significant proliferation was also observed between day 1 and 3 and between day 1 and 5 ($p < 0.05$) in the groups with 10%, 20% and 25% L-PRF supplementation. There is no significant proliferation observed between day 1, 3 and 5 in the control group ($p > 0.05$).

The 10%, 20% and 25% L-PRF groups can serve as supplemental culture media for hDPSCs proliferation, with the highest proliferative potential observed in the 25% L-PRF group.

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Introduction

Human dental pulp stem cells (hDPSCs) are a unique type of mesechymal stem cell (MSC) found in the pulp.[1] MSC demonstrate a high proliferation capacity, self-renewal abilities, and capable of differentiating into mesenchymal derived odontoblasts, osteoblasts, adipocytes and chondrocytes depending of the types of growth factor applied.[2,3] This characteristic facilitates ex vivo expansion and enhances the potential for therapeutics and tissue engineering applications in endodontics.

Ex vivo cell culture requires basal medium and supplementation containing growth factor, protein, and enzyme to support cell attachment, growth, and proliferation. Fetal bovine serum (FBS) is the gold standard supplementation for cell culture because it contains many growth factors, cytokines and chemokines that necessary for cell growth.[3] However the use of FBS as a xenogenic material must deal with the safety and ethical issue create obstacles for the use of FBS in clinical trials. Therefore, an alternative supplement with proper growth factor is necessary.

Human-derived medium supplementations that can replace FBS in cell cultures, such as platelet-rich fibrin lysate (L-PRF), have been investigated in the past few years. L-PRF is obtained by centrifuging fresh human blood, followed by freeze/thaw activation without the aid of animal-derived coagulants such as bovine thrombin.[4] L-PRF is a cell-free material that contain low plasma proteins such as albumin, fibrinogen and immunoglobulin.[3] Additionally, L-PRF contains more growth factors than FBS, therefore it is more effective in maintaining stem cell growth and cell phenotype.[5]

Currently, there is no consensus regarding the optimum concentration of L-PRF for MSC culture. Risya et al. stated that the optimum L-PRF

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concentration for MSC proliferation was 25%, whereas the lowest proliferation seen on 12,5% L-PRF.[6] Conversely, Saeed et al. reported the optimum concentration for hDPSCs proliferation is 10% L-PRF.[5] The present study aimed to analyze the potency of L-PRF on hDPSCs proliferation on day 1, 3 and 5.

Materials and methods

This study's protocol was approved by Ethical Committe of Faculty of Dentistry Universitas Indonesia (No. 133/Ethical Approval/FKGUI/XII/2017, No. Protocol: 051331017). The hDPSCs was isolated from 10 healthy donors who had visited the Universitas Indonesia Dental Hospital to undergo odontectomy of fully formed third molar. Informed consent was obtained from all participating adult subjects prior to the study. Immediately after extraction, the molars were stored in phosphate-buffered saline (Gibco). The extracted molars were split open, and the pulp tissues were removed then minced with surgical scissors. The minced pulp was placed in 6-well cell culture plates containing Dubelcco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL *penicillin*-G (Roche, Basel, Switzerland) and 100 mg/mL *streptomycin* (Roche, Basel, Switzerland) in humidified atmosphere of 5% CO2 at 370C until reaching 80% confluence. The serum starvation technique was applied by replacing the cell culture supplement with 1% FBS for 24 hours. To visualize the hDPSCs in pulp cell culture, immunohistochemistry staining for cluster differentiation (CD) 90, CD73 and CD105 was performed and analyzed with flow citometry. Meanwhile the analysis for HLA-DR, CD34 and CD 45 were done by fluorescence-activated cell sorting.

For each L-PRF sample, 5 ml of blood was collected from four volunteers who did not smoke or drink and were not receiving anticoagulant treatment. Briefly, the blood samples were obtained without anticoagulants in 5-ml vacuum blood collection tubes and immediately centrifuged at 2700 rpm for 12 min. A white clot of PRF was formed in the middle of the tubes between plasma and RBCs. The PRF clot was taken by sterile forceps and placed in empty tube for 24 hours incubation at 40C to produce L-PRF, according to the previously described method. After the residual fibrin attached to the bottom of the tubes, the supernatant was aspirated and transferred to 2 ml Eppendorf tubes and stored at -200C. The supernatant diluted with DMEM to obtain L-PRF 10%, 20% and 25%.

After serum starvation and flow cytometry, the hDPSCs were placed in 96 wellplate at the beginning of passage 2 (P2). Each well contained 1x103 cells and was cultured in DMEM with 100 μl of one of the three different concentrations of L-PRF. The well that did not contain any L-PRF were used as control. hDPSCs proliferation was assessed on day 1, 3 and 5 using MTT-Assay and ELISA reader at 596 nm wave length. Duplicate experiments were performed to ensure reproducibility. Statistical analysis was performed using one way ANOVA and Kruskal-Wallis to analyze the differences between groups. The friedman test was used to analize difference in proliferation rate between days.

Results

Stem cell surface marker expression was analyzed by flow cytometry to characterize the hDPSCs. The results for positive cocktail of hDPSCs were showed MSC characterization based on the evaluation of hDPSCs markers CD73 (98,5%), CD90 (98,3%) and CD105 (71%). Meanwhile, the results of hematopoietic molecules of CD45 and CD34 showed negative result (2,5%). This characterization was held before administering the treatments in passage 2.

The hDPSCs morphology was observed via microcroscopy with a magnification od 10x. As shown in Figure 1, wells without any supplementation showed a lower cell density than wells with L-PRF supplementation. The highest cell density was observed in the wells with L-PRF 25%.

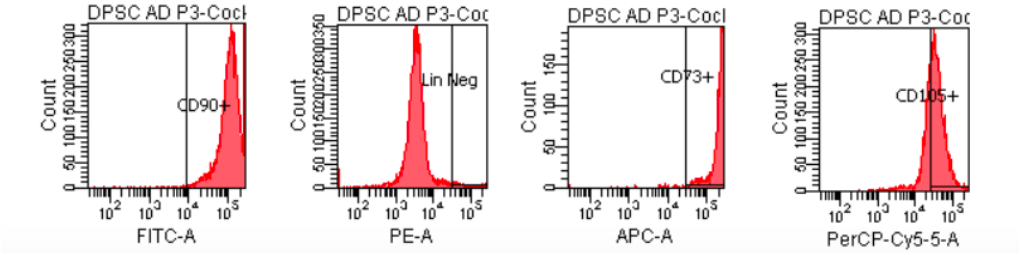

Figure 1. Flowcitometry analysis using CD73, CD90 and CD105

The effects of L-PRF on the proliferation of hDPSCs that were seeded in cell culture plates were evaluated on day 1, 3, and 5 using MTT assay. Absorbance data obtained from ELISA reader was converted into viability cell percentage. Day 1 data for each group were analyzed using one-way ANOVA. Compared to cell without supplementation, application of 10%, 20% and 25% L-PRF groups increased hDPSCs proliferation on day 1 ($p < 0,05$) with the highest proliferation rate observed in the 25% L-PRF group (Table 1). However, there was no significant difference between the proliferation rate without supplementation and cell treated with 10%, 20%, 25% L-PRF ($p>0.05$) on day 3 and 5 based on Kruskal-Wallis analysis (Table 2).

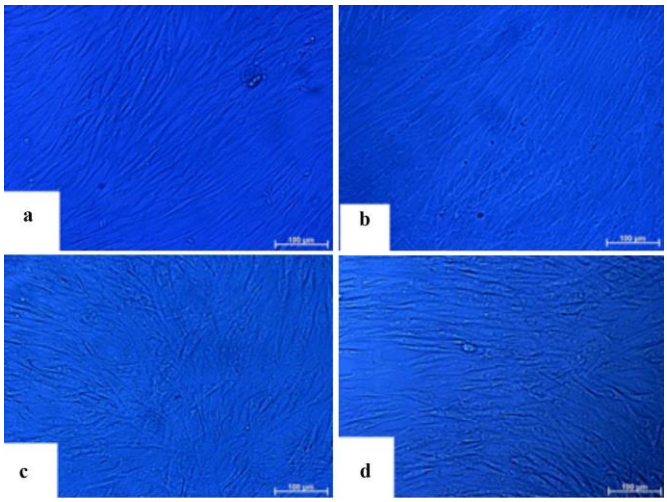

Figure 2. The hDPSCs under microscope (a) cell without supplementation as control group, (b) hDPSCs with 10% L-PRF, (c) hDPSCs with 20% L-PRF, (d) hDPSCs with 25% L-PRF

The proliferation rate was significantly different between day 1 and 3, and between day 1 and 5 ($p < 0.05$) in media with 10%, 20% and 25% L-PRF (Table 3). However, there was no significant difference in proliferation rates on day 1, 3, and 5 in cells without supplementation (control group) ($p>0,05$). Proliferation rate between groups also significantly different on day 1 and day 3 (Table 4).

Discussion

The imperative role of hDPSCs in dental pulp engineering requires an in-vitro expansion wich must address the safety and ethical problems associated with the use of FBS as cell culture supplementation. The FBS may contain endotoxins, viral contaminants, mycoplasma or prion proteins.5 Therefore, our study aimed to investigate L-PRF as an alternative to FBS for promoting proliferation of hDPSCs in vitro.

Methologically, hDPSCs must first undergo the serum starvation process. This process aims to standardize the cell condition so that the cells can develop simultaneously according to the cell cycle and divide in relatively the same time.[7] Flow cytometry was used to test for CD73, CD90 and CD105 in the cells. The CD is a cell surface receptor that is used to identify cell types and differentiation stage via antibodies. In our study, the cells were positive for CD73 (98,5%), CD90 (98,3%) dan CD105 (71%). Another characteristic of hDPSCs is their ability to attach to plastic surfaces.[8]

The L-PRF in the present study was obtained from fresh human blood without anticoagulants, thus making it more efficient in cell migration and proliferation.[9] The blood samples were then centrifuged in 2700 rpm for 12 minutes to produce a better splitting of red blood cell from PRF gel. The L-PRF also can be obtained from apheresis.[10,11,3] Lysate from PRF is advantageous over lysate from platelet rich plasma (**PRP**) because it contains more growth factors and releases them over a longer period.[12,13,14] PRF releases the following growth factors: platelet derived growth factor (**PDGF**),

transforming growth factor β1 (**TGFβ1**), insulin-like growth factor (**IGF**) and vascular endothelial growth factor (**VEGF**).[12,15] PRF also contains several neutrophils that may play a role in angiogenesis by expressing matrix metalloproteinase 9 (**MMP 9**).[13]

L-PRF is a refinement of standard PRF that is obtained by incubating the PRF gel at 40C for 24 hours so that the gel deflats and leaves the supernatant which contains many growth factor on top.[16] This supernatant is called L-PRF, which is immediately stored at -200C to avoid protein denaturation thereby allowing the growth factors to last for 5 months.[5,17] The use of platelet derivatives in the form of lysate also reduces the amount of immunoglobulin and albumin, thereby reducing the risk of immune rejection when applied allogenically.[17] In our study, the growth factors contained in L-PRF were activated by freeze/thaw. This activation method is the most effective than methods involving CaCl2 coagulation, ADP activation, fibrillar collagen type I, thrombin or zeolite.[18]

The microscopic observations demonstrated different cell densities between wells. Wells with 25% L-PRF supplementation showed the highest density followed by 20% L-PRF, 10% L-PRF and well without suplementation. The corresponding result were seen in the statistical test results in Table 1 which shows that 25% and 20% L-PRF had the highest proliferative potential of hDPSCs on the first day. This is in accordance with a study by Risya et al. who reported that the proliverative potential of L-PRF application was highest in the 25% L-PRF group.[6] Similarly, Barsotti et al (2013) stated that the optimum platelet lysate concentration for human umbilical vein endhotelial cell was 20%.[19] In contrast, Saeed et al. noted that optimum L-PRF concentration for hDPSCs proliferation was 10%. These varying conclussion may resulted from the different centrifugation method used. Conversely, Saeed et al. performed 2 centrifugation treatmenta prior to freeze/thaw to obtain lysates and separate the red blood cells from PRF membrane. Rauch et al. stated that delaying the freeze phase can degrade the growth factors.[17]

Treatment	N	*Mean* (SD)	*p-value*
Control	6	35.82 (2.50)	0.000*
10% L-PRF	6	311.38 (45.05)	
20% L-PRF	6	669.63 (102.44)	
25% L-PRF	6	824.74 (56.20)	

**One-way ANOVA test, p < 0.05*

Table 1. Analysis of hDPSC proliferation in cultured media without supplementation (control group) and with 10%, 20%, and 25% L-PRF supplementation.

Treatment	P value of cell number percentage
Control vs 10% L-PRF	0.000*
Control vs 20% L-PRF	0.000*
Control vs 25% L-PRF	0.000*
10% L-PRF vs 20% L-PRF	0.001*
10% L-PRF vs 25% L-PRF	0.000*
20% L-PRF vs 25% L-PRF	0.071

**ANOVA test, p < 0.05*

Table 2. Comparison of hDPSC proliferation in cultured media without supplementation (control group) and with 10%, 20%, and 25% L-PRF supplementation.

Table 3 shows no difference in cell proliferation between day 3 and 5 in each group, indicating that the proliferation rate remained stable between these days; however, the proliferation rate significantly differed between day 1 and 3 also between day 1 and 5. The cell cycle may be responsible for this change. The cell cycle begins with phase G0, during which have zero growth occurs. In this phase, cells need growth factors to enter the cell cycle which begins with the first period of growth (G1). During G1, cell develop in shape and size to prepare DNA synthesis phase (S). Cells will also determine their continued development in this

phase. If there are sufficient growth factor available, cells will enter the S phase. The G0, G1 and S phase determine cell proliferation, which occurred at the highest rate until day 3. In these phases, growth factors serve as inducers and play a regulatory role.[20] The greater amount of growth factors in L-PRF caused the hDPSCs treated with L-PRF to proliferate faster than the hDPSCs without supplementation.

After the S phase, the cell proliferation rate gradually decreases, and the cells enter G2 and the M phase, the latter of which involves differentiation.[21, 22] These phases are responsible for the stable proliferation rate displayed between days 3 and 5.

Media supplementation	The hDPSCs Proliferation Rate in 3 different observation time			*p-value*
	Day 1 Mean (SD)	Day 3 Median (range)	Day 5 Median (range)	
Control	35.82 (2.50)	15.07 (14.88–82.48)	15.77 (5.01–123.30)	0.513
10% L-PRF	311.38 (45.05)	29.45 (28.86–55.80)	18.27 (15.37–161.08)	0.009*
20% L-PRF	669.63 (102.44)	49.28 (45.51–290.84)	32.14 (28.81–337.22)	0.009*
25% L-PRF	824.74 (56.20)	58.82 (57.97–368.19)	40.19 (31.49–347.73)	0.009*
p-value	0.000*	0.029*	0.121	

One-way ANOVA test; Kruskal–Wallis test, p < 0.05; Friedman test, p < 0.05

Table 3. hDPSC proliferation in cultured media without supplementation (control) and with 10%, 20%, and 25% L-PRF supplementation on days 1, 3, and 5

The PRF centrifugation process plays an important role in determining the amount of growth factors in the lysate. A higher centrifugation speed results in lesser amount of growth factors in the lysate, which will produce more membranes; consequently, more growth factors are attached to the membranes than those that are present in the lysate.[14,23] The hDPSCs in the present study utilized DMEM, which contains 4 times as many amino acids as α-MEM medium, thereby allowing greater cell proliferation. However, the cells displayed lower resistance compared to cells grown in α-MEM medium.[17]

Conclusions

The hDPSCs cultured in 25% L-PRF supplement media has better and faster proliferation rate until the first day of seeding in comparison to cells that lacked of supplementation (control group). The L-PRF is a promising therapeutic approach for regenerating dental pulp. However, more comprehensive studies are necessary.

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Declaration of Interest

The authors report no conflicts of interest.

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