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Efficacy of Human Platelet Rich Fibrin Exudate Vs Fetal Bovine Serum on Proliferation and Differentiation of Dental Pulp Stem Cells

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Abstract

Aims: The primary aim of this study was to compare the effects of human platelet rich fibrin (hPRF) exudate Vs fetal bovine serum (FBS) on the proliferation and osteodifferentiation of human dental pulp stem cells (hDPSCs). The secondary one was to determine the optimum concentration of hPRF exudate inducing hDPSCs proliferation and osteodifferentiation.

Study design: One hundred and forty ml of blood was collected from 8 healthy donors. hPRF exudate was prepared by the direct method. hDPSCs were isolated from impacted mandibular third molars of twelve donors by the outgrowth method. For cell viability and proliferation rate testing, 96 well plates were used and the assay was done in duplicate and the trial repeated four times under the same conditions. Six wells were used to contain 10% FBS, serum free media, 1%, 5%, 10% and 20% concentrations of hPRF exudates, respectively. The proliferation assay was carried out after 1 and 3 days by MTS tetrazolium cell proliferation assay kit and Elisa reader. The study design for osteodifferentiation protocol was exactly as the proliferation one and instead the assay was carried out after 7 days by alizarin red with Elisa reader.

Results: Compared to 10%FBS, 10% hPRF exudate was the optimum concentration for hDPSCs proliferation, while 1% hPRF exudate was the optimum concentration for osteodifferentiation of hDPSCs.

Conclusion: The preparation technique of hPRF exudate is simpler and cheaper than that of FBS. Avoiding the risk of zoonosis which may be occurred with FBS, it is recommended to use 10% hPRF exudate for proliferation and 1% for osteodifferentiation.

Key words: Human platelet rich fibrin exudate, fetal bovine serum, dental pulp stem cells, MTS cell proliferation assay, alizarin red staining assay.

Introduction

Expansion and culture of mesenchymal stem cells (MSCs) in vitro depends on supplementing culture medium with fetal bovine serum (FBS) or fetal calf serum (FCS) that contains numerous growth factors inducing cell attachment, proliferation, and differentiation[(1)]. Protracted expansion times, risk of xenogenic response, exposure to zoonosis and allergic side effects create obstacles for the use of FBS in clinical trials. Therefore, an alternative supplement with proper inherent growth-promoting activities was demanded[(2)].

Human-derived medium additives, that can replace FBS, have been investigated in the past few years and have led to the discovery of efficient alternatives such as autologous plasma-derived from bone marrow (BM), human platelet lysates (hPL), collagen-activated platelet releasates, thrombin-activated platelet releasates, autologous and allogeneic human serum albumin and human serum[(3)]. Although these products are from human origin, these sera show batch-to-batch variability that may affect reproducibility[(4)].

Under optimal conditions of standardization and safety, hPL was produced through platelet lysis and has been increasingly suggested as the future 'gold standard' supplement replacing FBS for the ex vivo propagation of MSCs for cell therapy applications and translational medicine[(5)]. hPL has higher concentration of

grown factors and is effective in maintaining growth and stem cell phenotype[6]. The disadvantage of hPL is the high cost preparation, time consuming, donor to donor variability and recurrent freezing and thawing process causes protein denaturation. The plasma components of hPL necessitate addition of anticoagulants such as heparins to prevent gelatinization of hPL medium, and their concentration must be standardized[7].

The possibility of obtaining soluble platelet-derived factors by platelet activation (releasate; the product obtained following platelet-rich plasma activation) had been demonstrated. While hPL preparations liberate the entire contents of the platelets and contain aggregates of platelet membranes, thrombin activation triggers a burst of growth factor release that mimics what occurs in vivo during wound healing and tissue repair[8]. Platelet-rich plasma releasate was able to prevent cellular chondrogenic capacity loss[9] and can be used for ex vivo expansion of corneal endothelium cells[10]. A disadvantage of platelet activation is the requirement of using exogenous substance, thus increasing the risk of allergic reactions and infection, especially with the use of bovine substance which is immunogenic[11].

Platelet rich fibrin (PRF) can be prepared solely through the activation of an endogenous coagulation process without the aid of animal-derived coagulants such as bovine thrombin Freshly prepared PRF clots were compressed into a thin membrane by PRF compression device to get rid of PRF exudate[12]. PRF exudate is superior to platelet rich plasma (PRP), from the aspects of expression of alkaline phosphatase and induction of mineralization[13]. Currently, little information is available on the effect of human PRF (hPRF) exudate on the efficacy of human dental pulp stem cells (hDPSCs) in normal and osteogenic culture media (CM). This study aimed to evaluate the biological influence of various concentrations of hPRF exudate on the proliferation and osteodifferentiation of hDPSCs. The research null hypothesis was that no effect for hPRF exudate on hDPSCs proliferation and osteodifferentiation.

Materials and methods

Preparation of hPRF exudate

In accordance with our institutional ethics committee, hPRF exudate was prepared from 8 healthy donors who had visited the outpatient clinic at Faculty of Dentistry, Delta University for Science and Technology, Egypt. They were nonsmokers and non-alcoholic, 4 of them were males and the others were females with age range (20 to 30 years) after obtaining their informed consent. One hundred and forty millimeter of blood was collected. The blood samples were centrifuged in centrifugation machine (Sigma Aldrich) at 2700 rpm for 12min. 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 scissor. The clot was placed on the grid of endobox and compressed by endobox cover. After 1min, PRF clot was converted into PRF membrane and the exudate was collected in the tray of the endobox. PRF exudate was centrifuged at 1800 rpm for 5min to obtain exudate only without RBCs which were precipitated. The exudate was filtered by 0.22 µm sterile syringe filter unit (Millipore Corporation, Bedford, MA 01730). PRF exudate was stored in eppendorf tubes at -200C immediately after preparation. The final PRF exudate concentration (1%, 5%, 10% and 20%) was calculated on the basis of the volume of PRF exudate that was added to the total volume of the CM.

Isolation of dental pulp stem cells

Twelve impacted third molars from 12 donors were extracted and used for DPSCs isolation with written permission. The ethics committee of our institution approved the experimental protocol. The inclusion criteria for selection were 1) patients free of any systemic or local diseases affecting dental tissues, 2) mean age of 19-22 years, 3) vertically impacted third molar and 4) sound non-carious teeth. Surgeries were carried out at Oral and Maxillofacial Surgery Department, Faculty of Dentistry, Delta University for Science and Technology. Surgeries were carried out under meticulous sterile conditions.

The extracted teeth were irrigated immediately and consecutively for 15s after

extraction with saline, 70% ethyl chloride, and phosphate buffer saline (PBS, Gibco). After irrigation, the teeth were preserved in sterile falcon tube containing pasteurized milk. The extracted teeth were transported to Nile Experimental Private Center for Researches under careful aseptic conditions. All the following steps were done in the laminar flow hood under meticulous aseptic conditions. The teeth with the pasteurized milk were put in sterile culture dish. All soft tissue attached to the extracted teeth was removed using sterile curette, then the teeth were irrigated with chlorhexidene (Kenara mouth wash; Macro Group Pharmaceuticals) followed by PBS. The pulp of each tooth was harvested using Hand Held Pulp Isolator device (designed by Ahmed Shetewy, a student at Faculty of Dentistry, Mansoura University under supervision of Prof/ Yousry EL-Hawary, Oral Biologist at Oral Biology Department). Briefly, the tooth was sunken in PBS and rested on its most widest and regular surface in the device. The tooth was splinted in two parts using the device handle. The two parts were put in sterile dish. The pulp was dissected from the surrounding hard tissue using the blunted side of small excavator. Each pulp tissue was cut horizontally into ten fragments using two scalpels moved in a cross manner.

The method of DPSCs isolation was carried out using direct outgrowth of stem cells from pulp tissue explants. Briefly, pulp pieces were cultured in 25 cm2 flask (Greiner) with 10 ml Dulbecco's Modified Eagle Media- Ham's F12 (DMEM/F12; lonza) supplemented with 10% FBS (Gibco) and 1% streptomycin and penicillin (Gibco), then the cultured pieces were incubated at 370C and 5% CO2 atmosphere. Examination by inverted microscope (Olympus) was carried out daily and the CM was changed every three days. When the cell colony formation units reached 80% confluence, the cells were transferred to 75 cm2 flask and this was passage one. Feeding was continued with the protocol of cell culturing and cells at passage six were used for this study. During passaging, cells were transported from flask to another one with trypsenization process aiming to de-adhering cells from the flask.

Direct staining method for surface marker characterization and flow cytometry

Two million of DPSCs were trypsinized and harvested. They were washed and resuspended in PBS supplemented with 3% FBS that contained saturating concentrations (1:100 dilution) of the following fluorescein isothiocynate-conjugated anti-human monoclonal antibodies: anti CD34-PE which distinguish hematopoietic stem cells, progenitor cells and endothelial cells, anti-leukocyte common antigen CD45 and anti-CD44-phycoerythrin (PE) cell surface glycoprotein. The cells were incubated against isotype controls in dark place at room temperature for 30min. Then, the cells were washed with 2 ml of PBS, centrifuged at 1500 rpm for 5min and the resulting supernatant was discarded. The cells were suspended in 0.2 ml of 0.5% paraformaldehyde in PBS. The analysis was done by fluorescein activated cell sorting {(FACS) Canto, BD, USA)} to acquire data. The data were analyzed with BD Cell Quest TM Pro software (version 6.0). All these steps were carried out at Genetics Department, Children Hospital, Mansoura University according to the manufacture instructions.

Proliferation assay

According to the PRF exudate concentrations, six wells of 96-well plate were used. The assay was done in duplicate and the trial repeated four times under the same conditions. Each well contained 2×103 cells with 200 µl of complete media. They were incubated at 370C and 5% CO2 atmosphere overnight till cells became adhered. After cells adhesion, they were incubated for 24h with serum free media (SFM) at 370C and 5% CO2 atmosphere. As proliferation was measured after 1st and 3rd day, 2 of 96 well plates were used. In the first well, the complete media was supplemented with 10% FBS and considered as positive control. While the second one, contained cells with SFM only and considered as negative control. In the 3rd, 4th, 5th and 6th wells, the complete media were supplemented with 1%, 5%, 10%, and 20% PRF exudate, respectively. 100 µl of CM were removed from each well and 100 µl of 3-(4, 5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H

tetra zolium inner salt (MTS; promega) were added using pipette tip. The plate was incubated at 370C and humidified at 5% CO2 atmosphere for 4h. The absorbance was recorded at 490 nm using 96 well plate readers "Elisa reader."

Osteogenic differentiation assay

The osteogenic differentiation media was consisted of nutritional media (DMEM-F12), 50 µg/ml of L-ascorbic acid 2-phosphate, 10 mM of β-glycerophosphate sodium salt, 10 µM of dexamethasone and antibiotics (100 mg/ml streptomycin and 100 u/ml penicillin-G). For osteogenic differentiation four 6-well plates were used. 1×105 cells/well in the 6 well plates were cultured with complete media till reach nearly 70% confluences and then incubated with SFM at 370C and 5% CO2 for 24h. The cells were maintained in osteogenic differentiation media with the different concentrations of PRF exudate. Each well in the 6-well plate contains specific concentration as in the following 10% FBS, media only, 1%, 5%, 10% and 20% of PRF exudate. Media was changed every 3 days. Formation of the mineralized nodules was detected and quantified after 7 days by Alizarin red-based assay. This assay was carried out according to the protocol of Carl A. Gregory et al. [14]. Briefly, the cells in the 6-well plates were washed with PBS and fixed for 15min in 10 % (v/v) formaldehyde at room temperature. The cells were then washed twice with an excess of dH2O before the addition of 250 µl of 40 mM Alizarin Red S (pH 4.1) per well. The plates were incubated at room temperature for 20min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 1ml dH2O during a 5min shaking. The stained monolayers were visualized by phase inverted microscope (Olympus Optical Co., Ltd., Japan). For the quantification of staining, 200 µl 10% (v/v) acetic acid was added to each well, and the plate was incubated at room temperature for 30min with shaking. The monolayer was then scraped from the plate with a cell scraper and transferred with 10% (v/v) acetic acid to an eppendorf tubes. After the slurry was vortexed for 30s, it was overlaid with 125 µl mineral oil (Sigma-Aldrich), heated to exactly 850C for 10min, and transferred to ice for 5min. The slurry was then centrifuged at 14799 rpm for 15min, and 125 µ of the supernatant was transferred to a new eppendorf tubes. Then, 50 µl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (150 µl) of the supernatant were read at 405 nm in 96-well plate. All these steps were repeated at least three times.

Statistical analysis

Statistical analyses were performed using SPSS software (version 20). The effects of the different concentrations of hPRF exudate on hDPSCs viability (proliferation) were analyzed using two way–ANOVA statistical test while the data of osteodifferentiation were analyzed using one way-ANOVA. Factors for the two way-ANOVA were 1) different concentrations 2) different time periods while the factor for one way-ANOVA was the different concentrations.

Cell viability ratio was calculated as follow:

1) First, normalization of each well containing different concentration was done by subtracting the absorbance of the blank wells (medium without cells) from the absorbance of all wells (SFM, 10% FBS, 1%PRF, 5% PRF, 10% PRF and 20% PRF exudate.(

2) Second, the mean absorbance of 10% FBS served as reference value for cell viability. The cell viability was calculated using the following equation:

$$\frac{\text{Absorbance of each concentration X 100}}{\text{Absorbance of 10\% FBS}}$$

Data are expressed as mean ± SD. For all tests, statistical significances were accepted for P values of < 0.05.

Results:

Characters of hPRF exudate

The PRF exudate obtained was yellowish clear fluid. Each 50 ml blood sample produced 4.5 ml PRF exudate.

Morphology of the cultured hDPSCs

At the 3rd day, few numbers of rounded cells were adhered to the flask while at the 6th day, cell adherence to the flask was increased and some rounded cells were converted to star shaped cells with the majority of them were rounded in shape. At 11th day, 3 colonies of cells were notified. At the 19thth day, cell confluence was notified. At this stage all

adhered cells were spindle shaped cells.

Flow cytometry characterization

The results of flow cytometric analysis revealed that hDPSCs were negative to CD34 (6.8%) and CD45 (6.8%) while their clusters were positive to CD44 (62%) (**Figs. 1A & 1B**).

Proliferation results

Two way-ANOVA statistical test for the continuous data revealed a significant difference between different groups (F ratio = 28.952 & P = 0.001), non-significant difference between different time periods (F ratio= 3.120 & P = 0.081) and a significant interaction between the time periods and the groups (F ratio= 6.223 & P = 0.001) (**Table 1**). The highest mean values for the cell viability was for 10% PRF exudate at 1 day (109.48±3.19) while the lowest one was for 1% PRF (41.36±1.41). The cell viabilities mean values at 1 day for 10% FBS, SFM, 5% PRF and 20% PRF were 100.00±0.00, 62.73±1.27, 66.56±2.80 and 65.86±7.77, respectively. Furthermore, he cell viabilities mean values at 3 day for 10% FBS, SFM, 1%PRF, 5% PRF, 10% PRF and 20% PRF were 100.00±0.00, 58.60±1.26, 58.49±4.81, 62.65±0.88, 71.76±3.92 and 63.51±3.61, respectively (**Table 1, Figs. 2 & 3**). There were significant differences between 10% FBS & SFM, 10% FBS & 1% PRF, 10% FBS & 5% PRF, 10% FBS & 20% PRF, SFM & 1% PRF, SFM & 10% PRF, 1% PRF & 5% PRF, 1% PRF & 10% PRF, 1% PRF & 20% PRF, 5% PRF & 10% PRF, 10% PRF & 20% PRF. Meanwhile, non-significant differences were found between 10% FBS & 10% PRF, SFM & 5% PRF, SFM & 20% PRF, 5% PRF & 20% PRF (**Table 1, Figs. 2 & 3**).

Osteodifferentiation

The mineralized nodules were clearly formed and stained with alizarin red within all wells (**Fig. 4**). One way-ANOVA statistical test for the continuous data revealed a total significant difference among different groups (F ratio =42.26 & P=0.001) (**Table 2**). The highest mean values for the optical density (absorbance) was for 1% PRF exudate (0.1913±0.006) while the lowest one was for 10% FBS (0.1095±0 004). The mean values for SFM, 5% PRF, 10% PRF and 20% FBS were 0.1273±0.006, 0.1273±0.006, 0.1290±0.017 and 0.1280±0.002, respectively (**Table 2 & Fig. 5**). There were significant differences between 10% FBS & SFM, 10% FBS & 1% PRF, 10% FBS & 5% PRF, 10% FBS & 10% PRF, 10% FBS & 20% PRF, SFM & 1% PRF, 1 % PRF & 5% PRF, 1% PRF & 10% PRF, 1% PRF & 20% PRF. Meanwhile, non-significant differences were found between SFM & 5% PRF, SFM & 10% PRF, SFM & 20% PRF, 5% PRF & 10% PRF, 5% PRF & 20% PRF, 10% PRF & 20% PRF (**Table 2 & Fig. 5**).

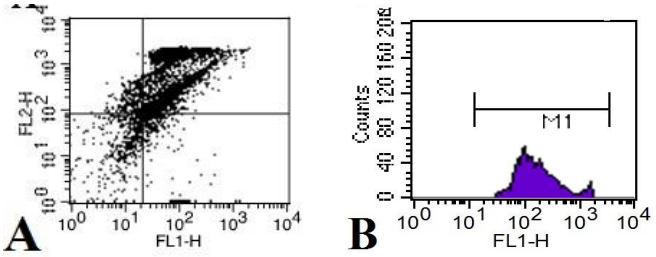

Figure 1: Flow cytometry chart showing double stain for CD34 (FL1-H) & CD45 (FL2-H) (**A**) and single parameter histogram for CD44 (**B**).

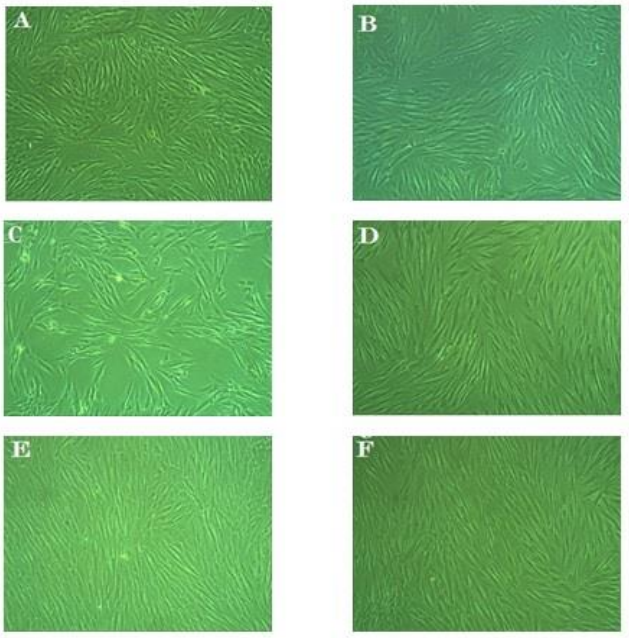

Figure 2: Inverted microscopy photographs show proliferation after 3 days with 10% FBS (100.00±0.00; **A**), SFM (62.73±1.27, **B**), with 1% PRF exudate (41.36±1.41; **C**), with 5% PRF exudate (66.56±2.80; **D**), with 10% PRF exudate (109.48±3.19; **E**) and 20% PRF exudate (65.86±7.77; **F**).

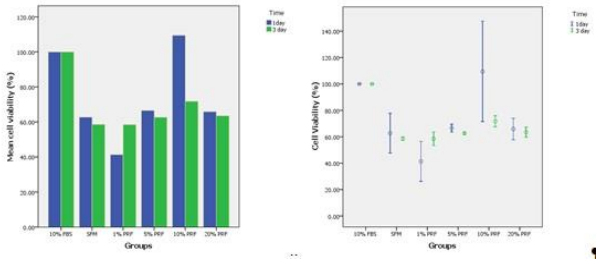

Figure 3: Bar chart (**left**) and error bars (**right**) for the effects of FBS, SFM and the PRF different concentrations on DPSCs viability at the different time periods.

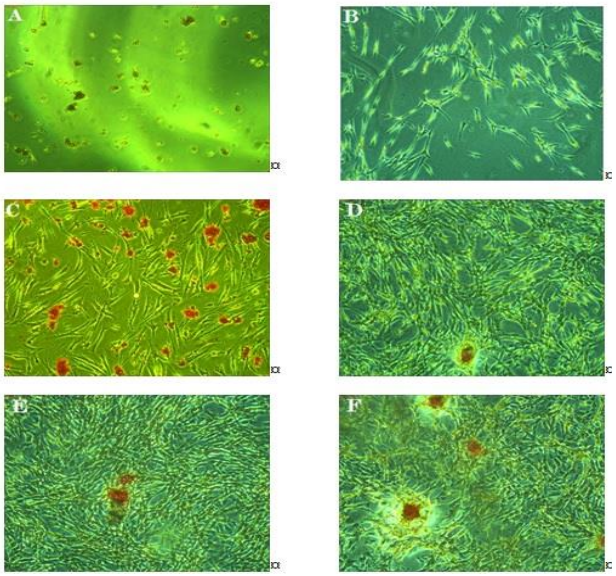

Figure 4: Inverted microscope photographs show mineralized nodules formation with 10% FBS (0.004±0.1095; **A**), osteodifferentiation media only (0.006±0.1273; **B**), 1% PRF exudate (0.006±0.1913; **C**), 5% PRF exudate (0.1273±0.006; **D**), 10% PRF exudate (0.1290±0.017; **E**) and 20% PRF exudate (0.1280±0.002; **F**).

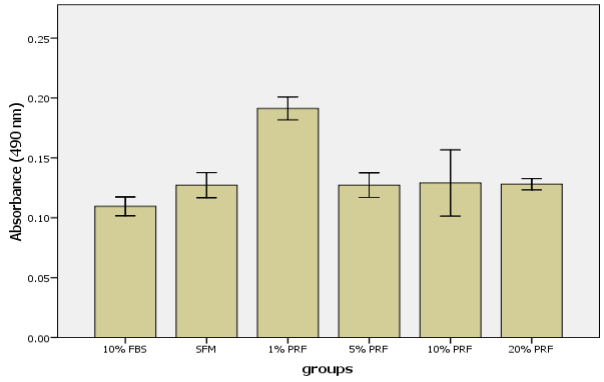

Figure 5: Bar chart for the effects of FBS, SFM and the PRF different concentrations on hDPSCs at the absorbance of 490 nm.

Discussion

In a previous study, hPRF exudate was prepared by indirect method through adding 5 ml of alpha modified Eagle medium (α-MEM) to PRF and they were incubated at 37^0C and 5% CO_2. All collected exudate was stored at -80^0C before use. The α-MEM enriched with exudate is considered as the 100 % exudate[13]. In the present study, hPRF exudate was prepared by direct, easy, cheap and fast method through collecting the blood samples quickly and without exposure to air avoiding blood coagulation before centrifugation. All collected exudate was stored in -20^0C immediately to avoid denaturation of its proteins.

In the previous studies[14,15], the surfaces of the extracted teeth were cleaned using sterile instruments and brought in αMEM supplemented with 300 UI/mL penicillin, 300 µg/mL streptomycin and 0, 75 µg/mL amphotericin B. Meanwhile, in our study the extracted teeth were irrigated with normal saline to remove blood from its external surfaces. In addition, irrigation with 70% ethyl chloride was carried out providing some sort of protection against microbial infection and this step was followed by irrigation with PBS to remove any remnants of ethyl chloride which might affect pulp vitality. The extracted teeth were brought to the laboratory in commercial pasteurized whole milk. The commercial pasteurized whole milk is considered by the International Association of Dental Traumatology and the American Academy of Pediatric Dentistry as the best interim transporting medium for teeth. It is isotonic liquid with a physiologically compatible pH and osmolality[16].

To the best of our knowledge, this is the first study used the Hand Handle Held Pulp Isolator device for pulp tissue harvesting. The idea of using that device was for achieving tooth splitting without heat generation that might affect pulp vitality and liability for microbial infection. The DPSCs were isolated by the outgrowth expansion technique. Each pulp was cut into 10 fragments horizontally by 2 scalpels in cross manner to minimize tension and tearing of the pulp tissue. This was done in consistent with the method described by Batouli et al[17].

Table 1: Two way-ANOVA and LSD post-hoc statistical test for the effects of FBS, SFM and PRF different concentrations on hDPSCs viability at the different time periods.

Two way-ANOVA (F ratio and *P* value)			LSD post hoc test						
Concentrations	Time	Interaction	Periods	10% FBS	SFM	1% PRF	5% PRF	10% PRF	20% PRF
(28.952, 0.001)	(3.120, 0.081)	(6.223, 0.001)	1day	100.00†±0.00	62.73‡±1.27	41.36≠±1.41	66.56‡±2.80	109.48†±3.19	65.86‡±7.77
			3 day	100.00†±0.00	58.60‡±1.26	58.49≠±4.81	62.65‡±0.88	71.76†±3.92	63.51±‡3.61

Same symbols in the same raw indicate non-significant difference at 0.05 level.
Same symbols in the same column indicate non-significant difference at 0.05 level.

Table 2: One way-ANOVA and LSD post-hoc statistical test for the effects of FBS, SFM and PRF different concentrations on hDPSCs osteodifferentiation.

One way-ANOVA (F ratio and *P* value)	LSD post hoc test	
One way ANOVA	Concentration	(mean ± SD)
(42.262, 0.001)	10% FBS	0.1095±0.004
	SFM	0.1273†±0.006
	1% PRF	0.1913±0.006
	5% PRF	0.1273†‡±0.006
	10% PRF	0.1290†‡¤±0.017
	20% PRF	0.1280†‡¤±0.002
	Total	0.1354±0.027

Same symbols in the raw indicate non-significant difference at 0.05 level.

This was simple, easy, low cost and fast method. In the previous studies[18, 19], the enzymatic digestion method was the most commonly used procedure for DPSCs isolation. However it is expensive, time consuming and allows different cell types to develop. Nakashima[18] reported three different morphologies of DPSCs while Huang et al.[19] showed compact and loose colony types after enzymatic digestion.

The DMEM-F12 is an extremely rich and complex medium that supports growth of wide range of cell types in both serum and serum-free formulation[17]. Contrary, the nutritional media used in previous studies was α-MEM[20, 21]. This type of media should be supplemented with serum to be suitable for a wide range of mammalian cells. DMEM-F12 has twice the concentration of amino acids and four times the amount of vitamins compared to α-MEM.

In this study the cells proliferation rate was measured by MTS assay. MTT protocol includes a liquid handling step to solubilize formazan precipitates during the assay, thus making the protocols less convenient[22, 23]. In this study the proliferation rate was superior using 10% PRF exudate, compared with 10% FBS. In the previous studies, the optimum concentration of PRP varied from 50% to 10%[24, 25] to less than 1%[26]. Soffer et al[26] considered 0.5 -1% PRP as the optimum concentration for cellular proliferation and mineralization rates. However, Ferreira et al[24] found that 50% PRP was the optimum concentration for osteoblast proliferation. On the other hand, 10% PRP was sufficient to induce a marked cell proliferation of MSC, derived from adipose tissue[27]. In addition, 5% PL considered as the optimum concentration for MSC and DPSCs proliferation and osteogenesis[28].

To the best of our knowledge and in the present study 1% PRF exudate induced the osteogenic differentiation of DPSCs within 8 days. This was a very short period needed for osteogenic differentiation; therefore it may be of beneficial role for bone healing using cell therapy technique. Higher concentrations of PRF exudates 5%, 10%, 20% promoted the induction of DPSCs osteodifferentiation after 7 days but with lower formation of calcium deposits. The previous studies showed that the optimum concentration for the formation of calcium deposits was 2% PRP for DPSCs and human periodontal ligament stem cells, while 1% PRP resulted in the highest calcium deposits in human exfoliated deciduous teeth showed that 5% PL was able to induce the osteogenic differentiation with in15 days instead of 21 days[29].

Conclusions

On the base of our study results; 10% PRF exudate was the optimum concentration for hDPSCs proliferation, while 1% PRF exudate was the optimum concentration for osteodifferentiation of DPSCs and mineralized

nodules formation.nerve injury minimizes the immediate functional loss.

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