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HUMAN BONE MARROW DERIVED STEM CELL MIGRATION AND PROLIFERATION UPON PLATELET RICH FIBRIN CONDITION

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ABSTRACT

Background: Periodontal diseases cause destruction of periodontal tissues and ultimately resulting into tooth loss. Various approaches including including guided tissue regeneration and bone grafts have been employed to accelerate either soft or hard tissue in periodontal repair. Platelet rich fibrin (PRF) is obtained by centrifuging peripheral wholeblood without anticoagulants. PRF has been used in clinical practice as a bioactive material in facilitating tissue regeneration. **Aim of study:** The study aimed to obtain and evaluate PRF effects on human bone marrow stem cells. **Materials & Methods:** PRF was prepared by centrifuging wholeblood without anticoagulants at 2500 rpm for 15 min. Liquid extract of PRF was obtained and evaluation for growth factors secretion by ELISA, including PDGF and TGF-β1. hBMSC proliferation and migration upon PRF condition were assessed by MTT assay and scratch wound healing assay, respectively. **Results:** PRF clots were formed after centrufugation, which exhibited the release of PDGF and TGF-β1 for up to three days of investigation. PRF liquid showed great biocompatibility to hBMSCs. Furthermore, PRF liquid significantly promoted the proliferation and migration in vitro. **Conclusion:** These results confirmed that PRF is completely suitable to be used as bioactive material for periodontal regeneration.

KEYWORDS: Platelet rich fibrin, bone marrow derived stem cells, proliferation, migration, periodontal regeneration.

INTRODUCTION

Periodontal diseases affect the periodontal tissues leading to inflamatory response to increase the microbial biofilm at the gingival margin of teeh.[1] Untreated gingivitis can cause chronic infection, which progress to loss of the gingiva, alveolar bone, ligament and ultimately tooth lost (periodontitis). A number of approaches have been ultilized, including guided tissue regeneration and bone grafts, to accelerate either soft or hard tissue repair. In addition, implantable materials which gradually release healing growth factors has become widely used in medicine due to their improvement in host cell – material interaction and overall regenerative potential. Platelet rich fibrin (PRF), a three-dimensional fibrin structure entrapping a high concentration of platelets, was developed by French researcher Choukroun et al in 2000 and has become widely used in medicine. The fibrin matrix in PRF, obtained by centrifuging peripheral wholeblood without anticoagulants, exhibits a great density, flexibility, and strength that are suited for handling in various forms, such as clots, plugs, membranes, injectable and/or implantable/packable, alone or even mixed with other grafting components. PRF is completely autologous material, therefore eliminates the risk of adverse rejection reaction. Platelet content in PRF is considered a rich growth factors source (eg., platelet-derived growth factor—PDGF-AB, transforming growth factor—TGF)-β, and vascular endothelial growth factor—VEGF) which has been recognized to stimulate hemostasis, direct cell migration, proliferation, and angiogenesis.[2-4]

Human bone marrow stem cells (hBMSC) are multipotent cells that can differentiate into bone, cartilage, fat, tendon, muscle, dental tissues.[5] When the alveolar bone is damaged, similar to others, they will secrete cytokines and chemokines, including Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), etc., recruit hBMSCs for the reconstruction the structure of alveolar bone.[6] Therefore, hBMSC play an important role in the process of healing damaged alveolar bone. In periodontal studies, PRF promotes the proliferation and osteogenic differentiation of human periodontal ligament

cells in vitro.[7] Our previous study showed PRF influences on proliferation and migration of human gingival fibroblasts.[8] In this research, we aimed to evaluate the effects of Fibrin rich platelet (PRF) on human bone marrow stem cells (hBMSC).

MATERIALS AND METHODS

Preparation of Platelet rich fibrin (PRF) and PRF liquid extraction

5 mL of venous blood was harvested from healthy volunteers. Sterile tubes containing silica beads was used to store the blood samples and prepare PRF. Theses tubes was also free from anticoagulant. After blood collection, the tubes were centrifuged at 2500 rpm for 15 minutes according to our lab's optimized protocol. PRF clots were removed from the tubes using sterile forcepts. Each PRF clot harvested from 5-mL blood sample was incubated in 12.5 mL of serum-free medium (DMEM/F12, Sigma-Aldrich, USA) for 24 hours. After the incubation, the medium was collected and used as liquid extract of PRF for further experiments.

Enzyme-linked immunosorbent assay (ELISA)

Liquid extract of PRF was evaluated for the presence and quantity of growth factors, including Platelet-derived growth factor (PDGF) and Transforming growth factor beta 1 (TGF-β1), and follow the manufacturer guidelines (Sigma, USA). Enzyme-linked immunosorbent assay (ELISA) was carried out with the experimental group (PRF liquid extract) and human plasma.

Human Bone marrow stem cells (hBMSCs) culture

The available hBMSCs at fourth passage were obtained from Laboratory of Tissue engineering and Biomedical materials. hBMSCs were cultured in complete medium as DMEM/F12 (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, USA), 1X Penicilin-Streptomycin (Sigma-Aldrich, USA).

Cytotoxicity testing of PRF

Liquid extract of PRF was prepared by incubating PRF clot in 5 mL of DMEM/F12 for 72 hours as mentioned earlier. hBMSCs were seeded into 96 well-plate with 4×10^3 cell per well, and cultured for 24 hours. All liquid extract samples, including test group (PRF extract), blank group (Culture medium), and positive group (Latex extract) were added into the wells seeded with hBMSCs, and incubated for further 24 hours. MTT assay was carried out to evaluate relative percentage of cell viability in the test group and positive group versus the blank group. Relative percentage of cell viability was represented as Relative growth rate (RGR) which was calculated upon OD575 values according to ISO10993/5 protocol as RGR (%) = (OD test group / OD blank group) × 100%. Cytotoxicity level was determined according to RGR as shown in Table 1 (ISO 10993-5,[9]).

Investigation of hBMSCs proliferation

Liquid extract of PRF was prepared by incubating PRF clot in 5 mL of DMEM/F12 for 72 hours as mentioned earlier. hBMSCs were trypsinized and reseeded into 96-well plate (10^3 cells per well). The cells were cultured overnight by complete medium for adherence and speading on the culture surface. In the next day, complete medium was replaced by PRF liquid extract to access hBMSCs growth upon PRF stimulation. Complete medium and DMEM/F12 were used as positve control and negative control, respectively. hBMSCs proliferation after 1, 3, 5, 7, 9, and 11 days was recorded via MTT assay.

MTT assay

In each day of investigation, PRF liquid extract or medium was discarded; the cells were washed once with 1X PBS. 0.5 mg/ml MTT (Sigma-Aldrich, USA) solution was added into each well and incubated for 4 hours at 37°C for formazan crystal development. Formazan crystals were dissolved in dimethylsulfoxide/Ethanol (DMSO/Ethanol, 1:1) (DMSO, Sigma-Aldrich, USA) (Ethanol, Merck, Germny) and read at 570 nm in The EZ Read 400 Plate reader (Biochrom, United Kingdom).

Scratch wound healing

hBMSCs were trypsinized and reseeded into 6-well plate (10^5 cells per well). The cells were cultured overnight in complete medium to form a monolayer at 80-90% confluency, followed by the next 18-hour starvation in DMEM/F12. In the scratch assay, a pipette tip (100-1000 µL tip) was utilized to vertically glid across cell surface in each well. Excess debris and cell clumps were removed by rinsing the adherent monolayer with 1X PBS. PRF liquid extract was added into each well. Complete medium and DMEM/F12 were used as positve control and negative control, respectively. After 0, 12, and 24 hours, images of cell migration into the scratch area were captured with a Olympus CKX-RCD microscope (Olympus, Japan) equipped with a DP2-BSW microscope digital camera and analyzed by Image J. This experiment was repeated three times.

Statistical Analysis

The obtained data was processed by the Excel program, and the Least Significant Differentiation (LSD) was calculated at a probability of p = 95% by means of differential analysis (Analysis of Variance - ANOVA) under the Statgraphic Program 7.0, 1997 of the University of Michigan (USA).

RESULTS

Platelet rich fibrin (PRF) preparation and growth factor secretion

PRF was prepared by centrifugation at 2500 rpm for 15 min by a fixed angle centrifugator. After centrifugation, an platelet poor serum was found on the upper layer, which was followed by a PRF clot. The red bood cells (RBCs) fraction was spun down at the botton of the tube

(Fig. 1A). The PRF clot with a RBC tail was collected by a sterile forcept (Fig.1B).

In order to mornitor the TGF-β1 and PDGF secretion, PRF clots were incubated in serum free medium. It was found that PDGF was released consistently after 1, 8, 24, and 72 hours of incubation. There was a significantly high amount of PDGF detected at 72 hour time point (Fig. 1C). TGF-β1 presented the most secretion from PRF clots during the first 1 hour of incubation. On the next 8 and 24 hours, PRF clots slowly released the growth factor (Fig. 1D). There was a small amount of TGF-β1 secreted from PRF clots at 72 hour time point.

PRF cytotoxicity

There was no cell death and abnormal morphology in groups incubated with complete medium as a blank control (Fig. 2A) and PRF liquid extract (Fig. 2B). However, liquid extract of Latex, as a positive control, caused a serious cell detachment and death (Fig. 2C. Black arrows). After the incubation with MTT solution, the formation of insoluble formazan crystals reflects the number of viable cells present. A dense formazan crystals formation was detected in blank control group (Fig. 2D) and PRF liquid extract group (Fig. 2E). In positive control group, there was minor amount of formazan crystal (Fig. 2F). Relative growth rate (RGR - %) of hBMSC incubated with PRF liquid extract was determined as 116 ± 5.568% (level 0, according to Table 2), compared to Latex liquid extract which had low RGR as 28.33 ± 4.509% (level 4, according to Table 2). Taken together, these results demonstrated that PRF liquid extract showed no cytotoxicity for hBMSCs.

The effects of PRF the migration of hBMSCs

PRF extract was prepared by incubating PRF clots in serum free medium for 72 hours. The effect of PRF extract on hBMSC migration was first investigated using scratch wound healing assay for 24 hours (Fig. 3). Complete medium and serum free medium were used as positive and negative control, respectively. At 0 hour, an artificial scratch was generated on cell monolayer, which was indicated as an acellular area (Fig. 3A-C). Control mediums and PRF extract were added into each well and cultured for the next 24 hours. Serum free medium did not promote any cell migration into the acellular area (Fig. 3D). Whereas, the acellular area was mostly covered by hBMSCs due to migration under complete medium (Fig. 3E). Similarly, hBMSCs migrated strongly into the empty space under PRF extract condition (Fig. 3F).

The effects of PRF the proliferation of hBMSCs

To evaluate the effect of PRF extract on the proliferation of hBMSCs, the cells were seeded and cultured for 1 – 11 days under PRF condition. MTT assay was conducted to evaluate cell growth on each investigated day. Complete medium and serum free medium were used as positive and negative control, respectively. In complete medium (Fig. 4, blue line), hBMSCs presented a significantly higher proliferation from day 3, 5 and 7, however, remained stability during day 9 and 11. On the other hand, hBMSCs did not proliferated in serum free medium due to lack of nutrients (Fig. 4, gray line). Under PRF extract incubation (Fig. 4, orange line), the hBMSCs proliferation pattern was observed to be similar to those in complete medium. The cells increased in quantity significantly from day 3, 5, and 7. This result demonstrated PRF liquid extract had a positive effect on hBMSCs proliferation.

Table 1: Cytotoxicity level according to Relative growth rate (RGR %) (ISO 10993-5,[9]).

RGR (%)	Cytotoxicity
≥ 100	0
75 - 99	1
50 - 74	2
24 - 49	3
1 - 24	4
0	5

FIGURES

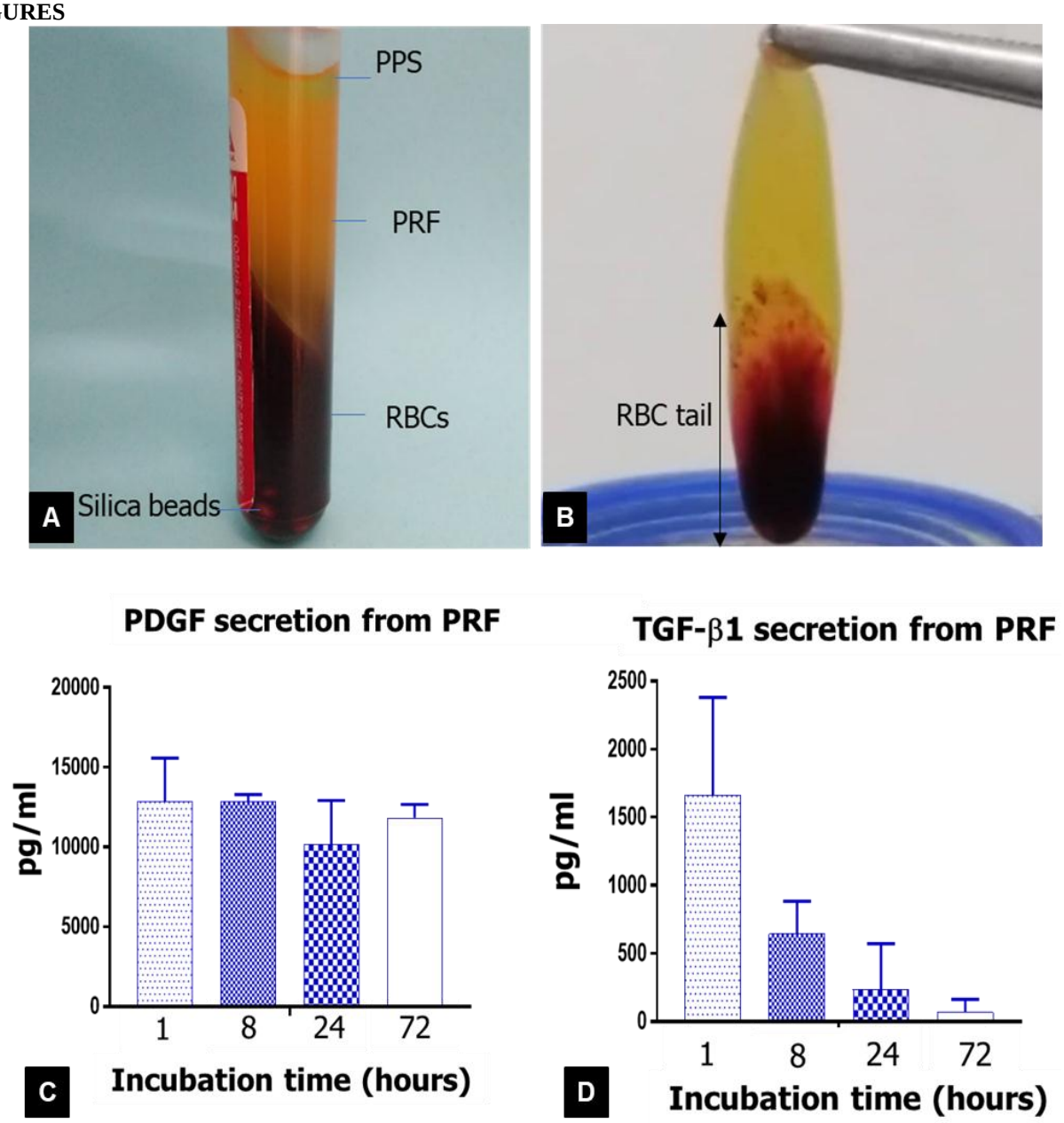

Figure 1: Prepartion of Platelet rich fibrin. (A) The blood sample after centrifugation, showing the red blood cell (RBCs) fraction, the PRF clot, and the platelet poor serum (PPS). (B) PRF clot with a RBC tail. (C-D) ELISA results for PDGF and TGF-β1 secretion from PRF.

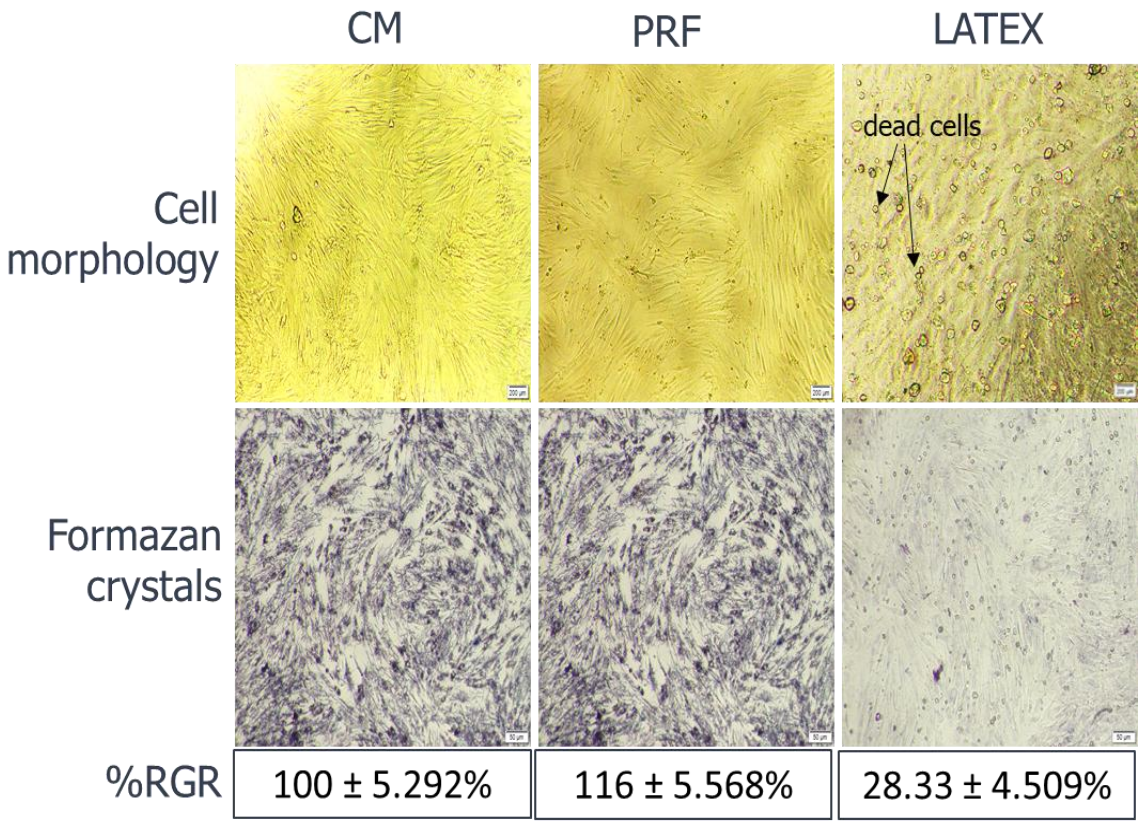

Figure 2: In vitro cytotoxicity testing of PRF on human bone marrow derived stem cells (hBMSCs). (A-C) Morphology of hBMSCs in different treatment (complete medium, PRF liquid extract, and Latex liquid extract). (D-E) Formazan crystals formation in MTT assay. Black arrows indicate dead cells. Numbers in the boxes indicate Relative growth rate (RGR - %) of hBMSCs in different treatments.

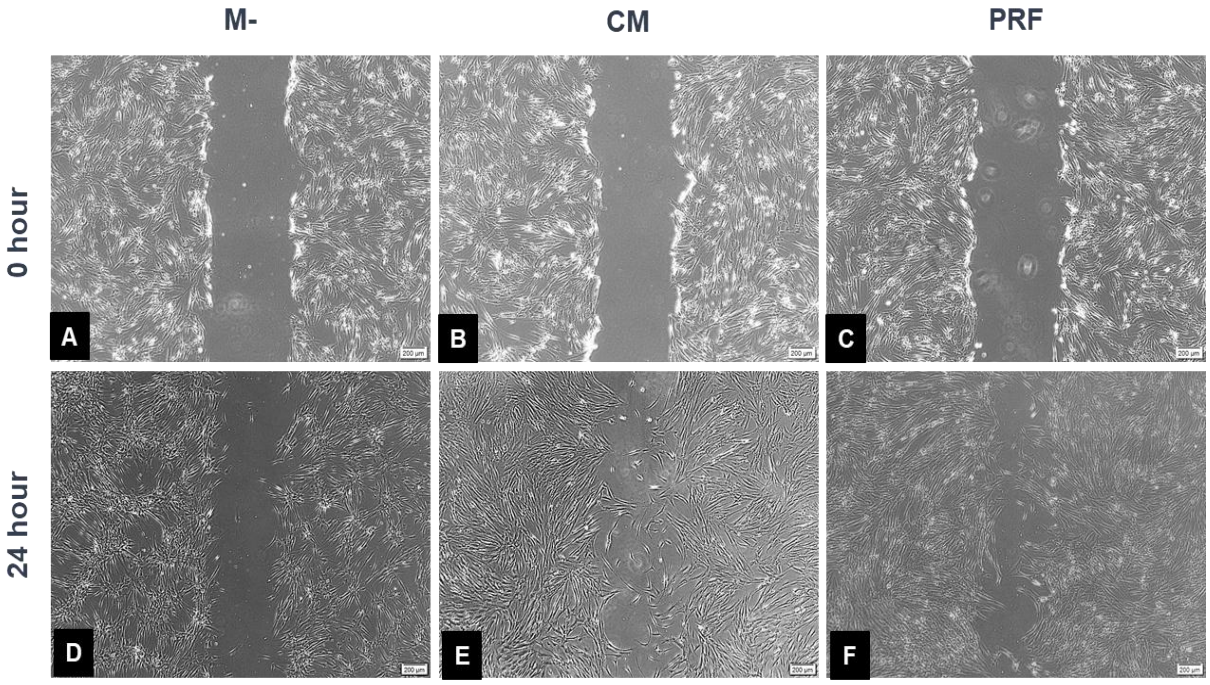

Figure 3: In vitro migration of hBMSC after 0 and 24 hours in different treatment. (A, D) Serum free DMEM/F12 medium (M-). (B, E) Complete medium (CM), and PRF liquid extract (PRF).

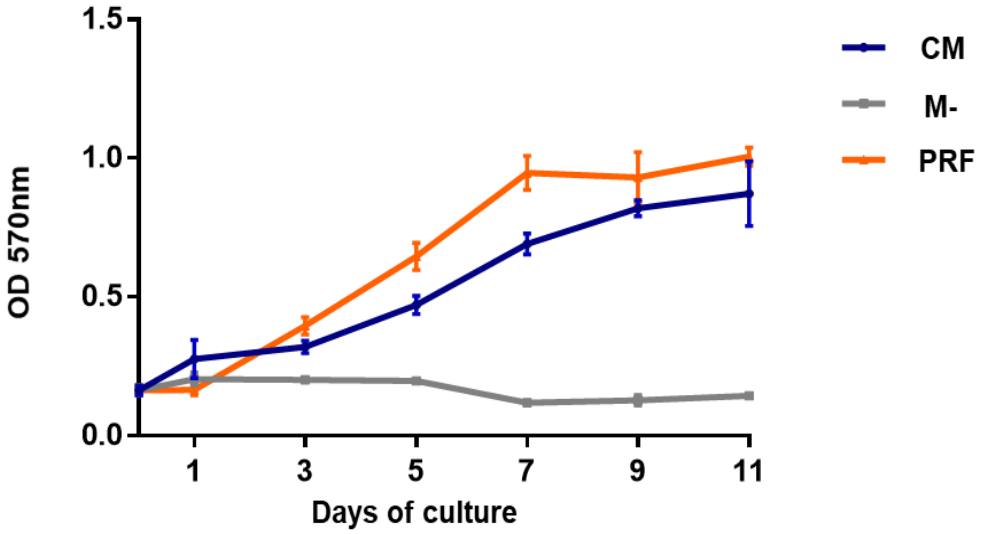

Figure 4: In vitro proliferation of hBMSC in different treatments, including complete medium (CM, blue line), serum free DMEM/F12 medium (M-, gray line), and PRF liquid extract (PRF, orange line).

DISCUSSION

PRF has been described as a second‐generation platelet concentrate, since it is produced naturally without using an anticoagulant. The PRF clot forms an active fibrin matrix with a complex three‐dimensional architecture, in which most of the platelets are trapped. Platelets retained in the fibrin network will be activated and release large growth factors. PRF preparation is considered a simple and inexpensive protocol in comparision with PRP (Platelet rich plasme, the first version of platelet concentrated technology). There are at least two step centrifugation for harvesting PRP, while PRF can be quickly obtained by one step centrifugation. PRF protocol dose not require any specific anticoagulant addition, therefore, allows an open-access method for establishment under different handling conditions in laboratories and clinical practise. In this study, PRF was prepared by our optimized protocol using a fixed angle rotor centrifuge at 2500 rpm for 15 minutes. Centrifuge duration in our protocol was comparable with other publication (within 10 – 15 minutes).[10-12] The 2500 rpm is considered high centrifugal force which was shown to result in a larger clots with a dense fibrin network.[13,14]

PRF liquid extracts was obtained for growth factors release and assessment in vitro effect on the migration and proliferation of hBMSCs. ELISA result presented the secretion of PDGF with high concentration, as well as TGF-β1 upon 72 hours of investigated. Both PDGF and TGF-β1 can be detected over the investigated time, from 1 hour to 72 hours after incubation. In particular, PDGF secretion was found to be stable with high concentration at 72 hours. Overall, the total amounts of PDGF and TGF-β1 after 72-hour secretion ranged aproximately 4000 pg/m/ and 2000 pg/ml, respectively, which was similar with present studies.[10-12] PRF demonstrated no cytotoxic impact on hBMSCs, according to their morphology and a dense formazan crystals formation in MTT assay. With high concentration of growth factors, the liquid extract from PRF clot had shown positive effects on the proliferation and migration of human bone marrow derived stem cells. With a simple centrifugation process, this study has successfully obtained PRF clot which can be applied in clinical for periodontal repair. This is an initial study for the development of research studies on the scientific basis of the impact of PRF on the biological properties of cells related to periodontal treatment to promote the application of PRF materials to healing support in clinical treatment in Vietnam.

CONCLUSION

In this study, Platelet rich fibrin was prepared using one-step centrifugation. The results showed that PRF secreted growth factors, including PDGF and TGF-β1. PRF extract could enhance hBMSC migration and proliferation, therefore provided potential benefits for periodontal tissue regeneration.

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