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The Comparative Study of the Mouse Osteoblast Response to Two Different Platelet-rich Fibrins with Low Speed Centrifugation Concept

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Abstract

Objectives: To compare the response of mouse osteoblast (MC3T3-E1 cells) to advanced platelet-rich fibrin (APRF) and advanced platelet-rich fibrin plus (APRF+).

Methods: Blood was collected from eight volunteers, 25 - 38 years of age (four males and four females) to prepare platelet concentrates APRF (1,300 rpm, 14 minutes), APRF+ (1,300 rpm, 8 minutes). The exudates were collected from both platelet concentrates at day 1, 3, 7 and 14. The level of TGF-β1 from exudates were quantified using an ELISA. MC3T3-E1 cells were cultured with the exudates. The cultured cells were tested with MTT assays, alkaline phosphatase (ALP) staining and mineralization, which were analyzed on day 7 and again on day 14.

Results: APRF and APRF+ continuously released TGF-β1 during 14-days period. Only exudates collected at day 1 showed significantly difference of ALP staining between APRF and APRF+ group on day 14 of observation. On day 7 of mineralization assays, cells treated with exudates from APRF+ collected at day 14 resulted in the highest level of mineralization within APRF+ group. On day 14 of mineralization assays, cells treated with exudates from APRF collected at day 7 showed the highest level of mineralization within APRF group.

Conclusions: APRF+ released TGF-β1 at day 14 significantly higher than day 1. On day 14 of mineralization, cells treated with exudates from APRF+ collected at day 14 showed significantly higher mineralization than APRF collected at the same time point.

Keywords: advanced platelet-rich fibrin, advanced platelet-rich fibrin plus, low speed centrifugation concept, mineralization, platelet concentrates

Introduction

Periodontal diseases are the consequences of inflammatory process that affect the supporting structures of the teeth. Periodontal diseases initiate by the buildup of dental plaque and microorganisms which begin as gingivitis and establish the local inflammation on gingiva. Periodontitis occurs when untreated gingivitis proceeds the destruction to gingiva, alveolar bone and periodontal ligament that results in a periodontal pocket which finally cause tooth loss. The goal of treatment is effectively control the inflammation, inhibit disease progression and maintain periodontal status in a healthy state. Periodontal regeneration is an absolute goal in the treatment of periodontal disease, aim to restore both hard and soft tissues to their original state or as closely as possible.[1] The major objectives of most biomaterial studies are focused on promoting wound healing and tissue reaction, while supporting the natural healing in defective areas.[2] The study of biomaterials which support wound healing, and its regenerative abilities are based on the principle that closely mimic the natural mechanism. The earlier study using platelet concentrate concept started in 1970[3] with fibrin glue usage. Then the application of platelet rich-plasma (PRP) in the treatment of periodontal disease is becoming widely used.[4] PRP has been utilized by both oral surgeons and periodontists to demonstrate its regenerative properties. The disadvantage of PRP is its containing anticoagulant which can interfere the healing process and increase risk of life-threatening coagulopathies.[5] Platelet rich-fibrin (PRF) is the second generation of platelet concentrates[6], prepared from centrifuged blood. This technique does not require any anticoagulants or other agents, and it is convenience and inexpensive to perform. PRF was derived from the natural polymerization occurred during centrifugation. Slow release of growth factors including transforming growth factor β1 (TGF-β1), platelet derived growth factor AB (PDGF-AB) and vascular endothelial growth factor (VEGF) were observed in PRF investigations. Dohan *et al.,*[7] demonstrated that PRF had a sustainably slow release of key growth factors at least 7 days. PRF has been used in various treatment procedures i.e. maxillary sinus lift[8], regeneration of intrabony periodontal defect[9], treatment of gingival recession.[10] Recently, investigators have modified centrifugation protocol to increase the growth factor of platelet and leukocyte in the PRF-based matrix.[11] The modified preparation was based on a low speed centrifugation concept (LSCC), described by applying a reduction in relative centrifugation force (RCF)[12], resulting in different clot formation and distribution of cells called advanced-PRF (APRF), the study revealed a more porous structure compared to PRF and increase in total leukocyte numbers was observed.[11] Based on LSCC, the modification of centrifugation time would affect the structure and growth factor releasing characteristics, introducing a new PRF-based matrix called advanced-PRF+ (APRF+; 1,300 rpm; 8 minutes).[12,13] Both of APRF and APRF+ showed a gradual but significant release of TGF-β1, platelet derived growth factor AA (PDGF-AA), platelet derived growth factor BB (PDGF-BB), PDGF-AB and VEGF.[12,13] APRF+ displayed the dispersed homogeneously of platelets all over the clot[12] and demonstrated the highest value of TGF-β1 at day 1, 3, 10 of observation when compared to PRF and APRF.[13] Furthermore, various PRF-matrices exhibited good biocompatibility *in vitro* and also shown that they were able to produce a 3-fold increase in collagen synthesis of human fibroblast cells.[13]

Therefore, the centrifugal force and time in different preparative protocols may cause a various response of cells, which then affects the wound healing process. At present, there are limited studies which compare the level of growth factors, cell biocompatibility between APRF and APRF+. In addition, it remains unknown the effect of APRF and APRF+ on osteoblast cell.

In theory, less centrifugation time would allow for the increased collection of cells and subsequent growth factors in a fibrin clot. We then evaluated the level of growth factor (TGF-β1) in the APRF+ and APRF groups, comparing the response of MC3T3-E1 cells by determining cell viability, differentiation, and mineralization. Therefore, the aim of this study was to determine *in vitro* effect of APRF, APRF+ on mouse osteoblast (MC3T3-E1 cells), which have been prepared by using a low speed centrifugation concept (LSCC).

Materials and Methods

Ethical approval

This study was approved prior to the data collection by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand (NO.74/2020). The study details were explained to all subjects and

informed consent was obtained before participation.

Platelet concentrates preparation

Sample size was calculated by using G Power program from previous study.[13] Inclusion criteria of blood donors included 1. age 25-40 years 2. healthy 3. denies drug allergy 4. no history of anticoagulant usage. Exclusion criteria of blood donors included 1. smoking 2. pregnancy. The peripheral blood was drawn from eight healthy volunteers (four males and four females) with a mean age of 30.13 years (ranging from 25-38 years of age). Informed consent was obtained from each donor who participated in this study. The venous blood was collected in 10-ml sterile tube (A-PRF, Zhejiang Gongdong Medical Technology Co, Ltd, Zhejiang, China) and centrifuged using centrifugation machine (DUO centrifuge, Process for PRF, Nice, France). The preparation was performed according to following protocols:

APRF: 10 ml; 1300 rpm; 14 minutes

APRF+: 10 ml; 1300 rpm; 8 minutes

The clots were carefully removed and separated from the red blood cell layer. Individual platelet concentrates were collected and placed on separated well on 6-well plates. Five milliliters of α-modified Eagle medium (α-MEM; Gibco, Grant Island, NY, USA) was added to APRF and APRF+, and then incubated at 37°C in 5% CO_2 for 14 days. At each time point (day 1, 3, 7 and 14), all exudates were collected and replaced with 5 ml of the culture media. The exudates were stored at -80°C. Each collected exudate from APRF and APRF+ was defined as 100% exudates. For the cell culture experiments, exudates were diluted with α- MEM to get 20% exudates concentration.

Measurement of TGF-β1 level

To determine the level of TGF-β1 released from APRF and APRF+ at day 1, 3, 7 and 14. At the desired time intervals, TGF-β1 from the exudate was quantified using an ELISA assays according to the manufacturer's instruction (R&D systems, Minneapolis, MN, USA). Absorbance was measured using a microplate reader (Tecan Microplate Reader, Grödig, Austria) at a wavelength of 450 nm and the process were repeated.

Cell culture

MC3T3-E1 cells were cultured with α-MEM containing 10% fetal bovine serum (Invitrogen, Waltham, MA, USA) and 1% penicillin-streptomycin (Invitrogen, Waltham, MA, USA) at 37°C in 5% CO_2. For osteogenic experiment, the media used containing 5 mM of β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) and 25 mg/ml of ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). MC3T3-E1 cells were seeded at a density of $2x10^3$ cells/well in 96-well plates for MTT assays and mineralization assays, and at a density of $1.2x10^4$ cells/well in 24-well plates for analysis of alkaline phosphatase (ALP) staining. Each experiment was performed on day 7 and day 14 of the cell culture procedure.

Cell viability

Cell viability was determined using the 3 - (4, 5 - Dimethylthiazol - 2 - yl) - 2, 5 -Diphenyltetrazolium Bromide (MTT) (BioChemica, Darmstadt, Germany) assay. Ten microliters of 5 mg/ml MTT was added to the cell cultures. The plates were incubated at 37°C in 5% CO_2 for 4 hours. Then the media was removed and 100 µl of dimethyl sulfoxide was added to dissolve the formazan crystals for 15 minutes. Absorbance was measured by using a microplate reader at 595 nm wavelength. The mean values of the triplicated experiments were determined.

Measurement of alkaline phosphatase staining

The ALP staining was performed by fixing experimented cells for 30 minutes with a 4% paraformaldehyde then 500 µl of CHAP buffer (100 nM Tris pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$ in distilled water) was added and incubated in the dark room at room temperature for 30 minutes. Then the CHAP buffer was removed and cells were stained using a 0.5 ml BCIP/NBT solution (ROCHE, Basel, Switzerland) in distilled water at room temperature for 30 minutes in the dark room. Each well was washed with PBS twice. The ratio of positive surface area for each well was captured using stereoscopic microscope and calculated using ImageJ program (Figure 1).

Measurement of mineralization

The mineralization of the MC3T3-E1 cells was determined by fixing treated cells for 30 minutes with a 4% paraformaldehyde. Then the cells were stained with

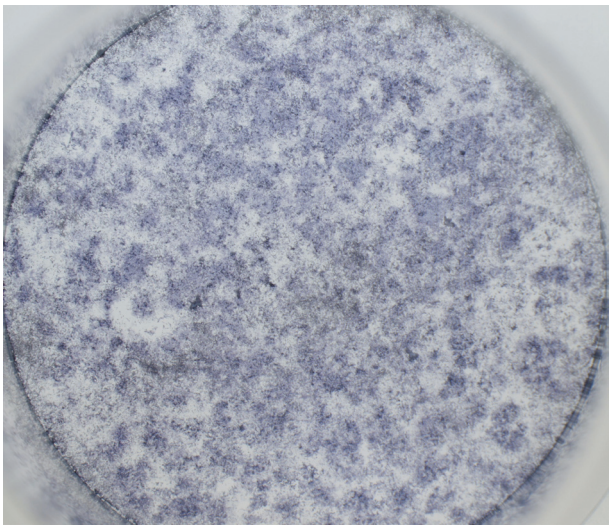

Figure 1: ALP staining on 24-well plate which captured by stereoscopic microscope

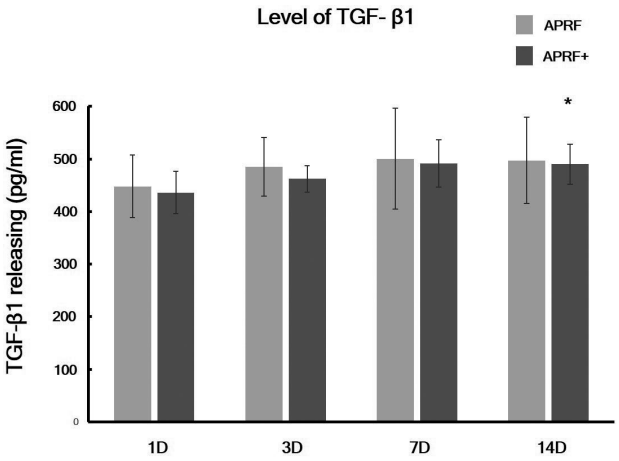

Figure 2: The levels of TGF-β1 release from APRF, APRF+ during each experiment time. Exudates collected at day 1 (1D), day 3 (3D), day 7 (7D), day 14 (14D). Statistical analysis of *p<0.05, compared with APRF+ at day 1

100 µl of 2% alizarin red solution (Sigma-Aldrich, St. Louis, MO, USA), pH 4.1-4.3 at room temperature for 45 minutes, and reaction was stopped with 500 µl of PBS. For quantification of mineralization, 10% cetylpyridinium chloride was then added and incubated at 37°C in 5% CO_2 for 1 hour. The absorbance was measured at a 550 nm wavelength. The mean values of the triplicated experiments were determined.

Statistical analysis

SPSS program version 25 (IBM, Chicago, IL, USA) was used. The mean and standard deviation was analyzed for levels of TGF- β1, cell viability, ratio of positive surface area for ALP staining and mineralization of each interested time point using pair t-test, one-way ANOVA and one-way repeated ANOVA (α=0.05). For all analyses, statistical significances were accepted at p values of <0.05.

Results

Level of TGF-β1 in APRF and APRF+ at each time points

There were no statistical significant differences comparing TGF-β1 levels between APRF and APRF+ at each time interval. In the APRF+ group, TGF-β1 level at day 14 were significantly higher than day 1 (p<0.05). Conversely, there were no statistically significant differences shown in APRF group at each time interval. (Figure 2)

Cell viability

MTT assay was performed to test for cell viability. On day 7, cells that were treated with exudates collected at day 7 and day 14 of APRF and APRF+ exhibited statistically significant differences in cell viability (p<0.05). Cells that were treated with exudates from APRF+ collected at day 1 resulted in higher cell viability than that treated with exudates from APRF (p<0.05). Cells that were treated with exudates from APRF and APRF+ collected at day 3, 7, 14 showed statistically significant differences when compared to the control group (p<0.5). For MC3T3-E1 cells that were cultured for 14 days, only exudates from APRF and APRF+ collected at day 1 showed statistically significant difference (p<0.05). Moreover, cells that were treated with exudates from APRF collected at day 1, 3, 14 and APRF+ at day 1, 3 showed statistically significant differences in cell viability when compare to the control group (p<0.05). (Figure 3)

Alkaline phosphatase staining

ALP staining of cells were performed after cells were cultured for 7 days. There were no statistically significant differences of ALP staining in cell cultured from exudates of both APRF and APRF+ at all tested time intervals. Following 14 days of mineralization, only cell treated with exudate collected from APRF+ at day 1 showed significantly higher expression of ALP staining than that cultured with exudate from APRF collected at the same day (p<0.05). (Figure 4)

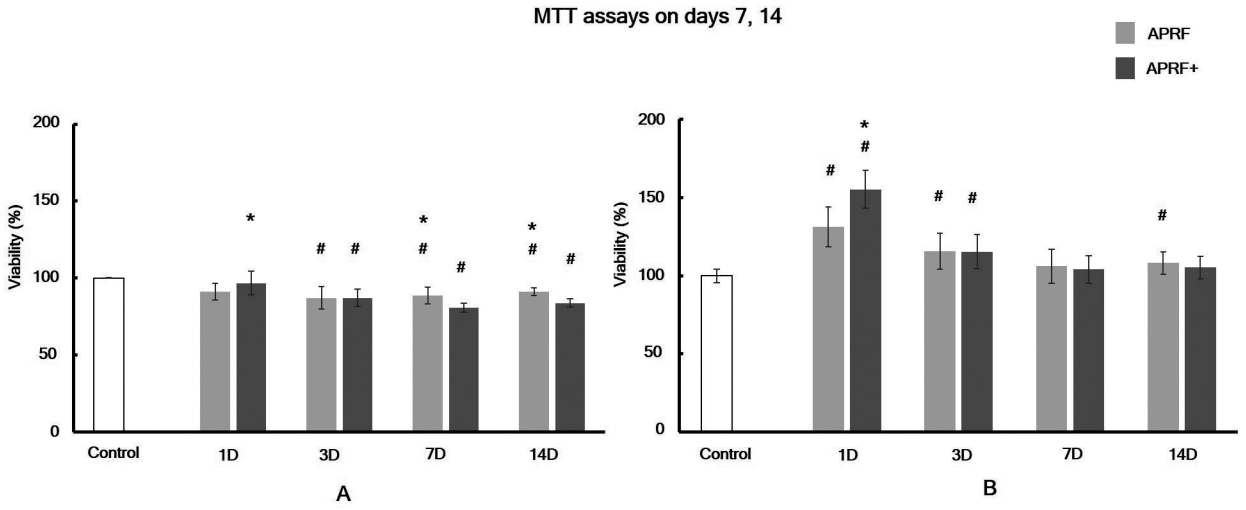

Figure 3: MC3T3-E1 cells viability, incubated with exudates of APRF or APRF+ collected at different time intervals (from day1- day 14). (A) MTT assays on day 7. (B) MTT assays on day 14. Exudates collected at day 1 (1D), day 3 (3D), day 7 (7D), day 14 (14D). Statistical analysis of *p<0.05, compared between APRF and APRF+ group. #p<0.05, compared with control group

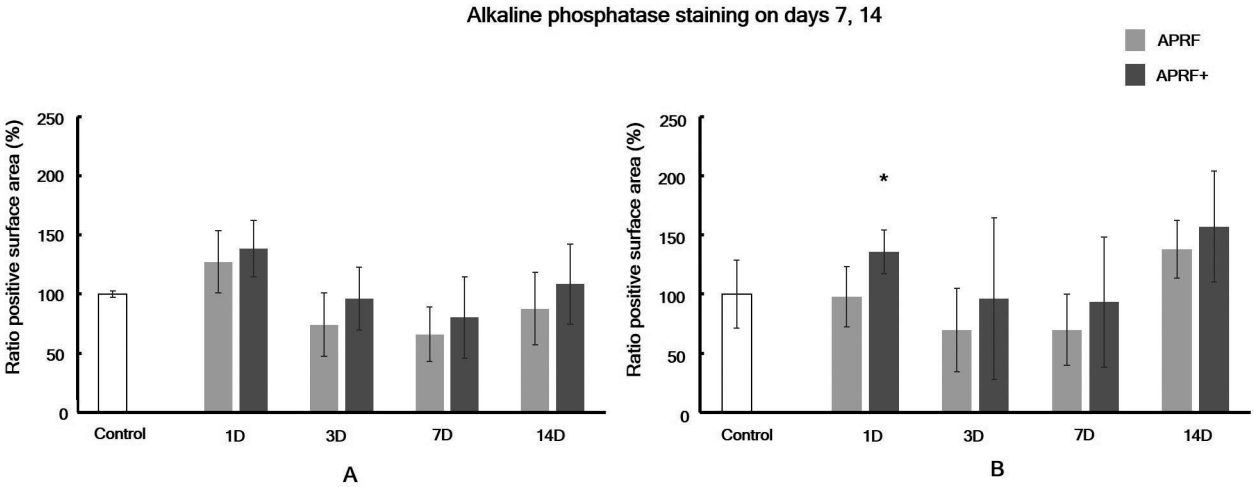

Figure 4: ALP staining of MC3T3-E1 cells, incubated with exudates of APRF or APRF+ collected at different time intervals (from day1- day 14). (A) Ratio of the positive surface area on day 7. (B) Ratio of the positive surface area on day 14. Exudates collected at day 1 (1D), day 3 (3D), day 7 (7D), day 14 (14D). Statistical analysis of *p<0.05, compared between APRF and APRF+ group

Mineralization

The effects of APRF and APRF+ on mineralization of MC3T3-E1 cells were shown. (Figure 5) On day 7 of mineralization assay, cells treated with exudate from APRF+ collected at day 14 showed the highest mineralization when compared to those treated with exudates from other time intervals in APRF+ group (p<0.05). Cells treated with exudate from APRF+ collected at day 7 reached significantly higher mineralization than cell treated with exudate from APRF+ collected at day 1 (p<0.05). When comparing between APRF and APRF+ groups, only cells cultured with exudate from APRF collected at day 1 resulted in a higher mineralization than that cultured with exudate from APRF+ (p<0.05).

On day 14, comparing cells treated within APRF and APRF+ group, cells treated with exudate from APRF collected at day 7 showed highest mineralization than those treated with exudates from other time interval in APRF group (p<0.05), cells treated with exudate from APRF+ collected at day 14 resulted in higher mineralization than those treated with exudates collected from day 1 and 3 (p<0.05). Cells treated with exudate from APRF collected at day 7 showed significantly higher mineralization than that treated with APRF+ at the same time point

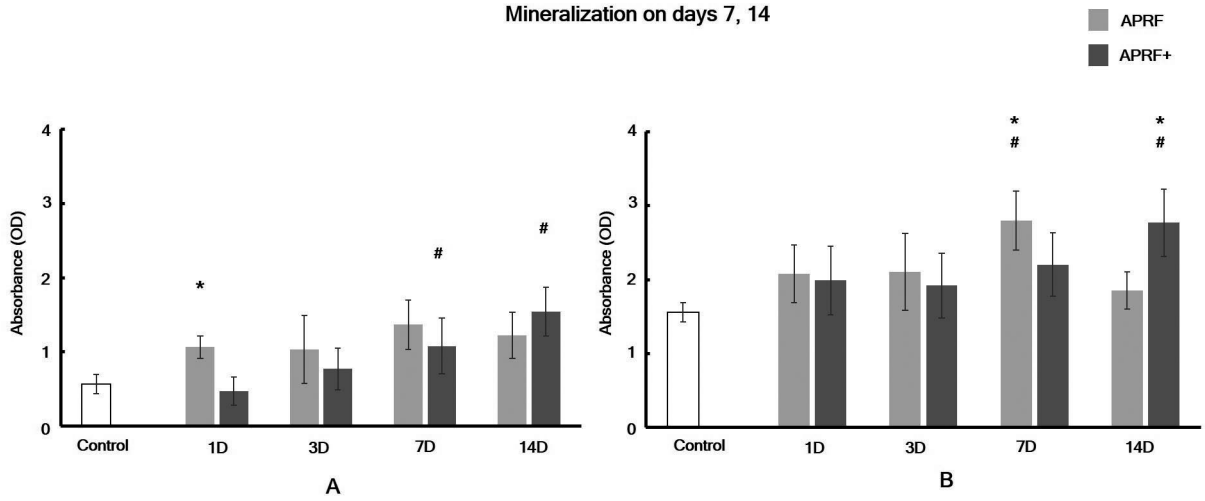

Figure 5: Mineralization of MC3T3-E1 cells, incubated with exudates of APRF or APRF+ collected at different time points (from day 1- day 14). (A) Mineralization on day 7. (B) Mineralization on day 14. Exudates collected at day 1 (1D), day 3 (3D), day 7 (7D), day 14 (14D). Statistical analysis of *p<0.05, compared between APRF and APRF+ group. #p<0.05, compared within APRF and APRF+ group

(p<0.05). Conversely, cells treated with exudates collected at day 14, APRF+ group resulted in more mineralization than APRF group (p<0.05).

Discussion

PRF was prepared without addition of thrombin, many investigations showed that PRF slowly release the growth factors for at least 7 days.[7] The development of the low speed centrifugation concept has been initiated to improve the capability of PRF.[11-13] In this concept, less centrifugation time increases cell numbers and growth factors in the PRF matrix. TGF-β1 plays a key role during bone formation, contributes to the chemotaxis and mitogenesis of the osteoblast. This growth factor is crucial in the osteoblast's deposition of mineralized tissue.[14] In this study, APRF+ showed a gradual increase of TGF-β1, while a steady release of character was shown in APRF group (Figure 2). When comparing APRF and APRF+, no statistically significant differences of TGF-β1 levels were found in every experimental time interval. The similar result was observed in the study by Bagdadi *et al*.,[12] that reported no difference between TGF-β1 release from APRF+ and APRF. Notably, only VEGF showed a higher release on day 7, and higher level in the accumulated release on day 10 for APRF+ compared with APRF and PRF. On contrary Kobayashi *et al*.,[13] revealed that APRF+ released the highest value of TGF-β1 at day 1, 3 and 10 when compared to PRF and APRF. Kobayashi *et al*.,[14] placed the sample of platelet concentrates into a shaking incubator at 37°C to allow the growth factor release into the culture media that was different than the methods done by Bagdadi *et al*.,[12] and this study. This difference may be affect the outcome in the release of TGF-β1. Thus, the method that was employed to detect growth factor was very specific in each study. In addition, the mechanism of growth factor release in PRF depends on leukocyte, platelet, and its structure.[15] Dohan *et al*.,[16] revealed approximately 97% of the platelets and 50% of the leukocytes were entrapped in the PRF clot. From this study showed that the level of TGF-β1 in APRF+ group collected at day 14 statistically significant difference from day 1, this result may cause by the reducing centrifugation time that collected percentage of cells within the APRF+ clot more than APRF clot. The histologic features, compositions and quantity of cells in APRF and APRF+ should be investigated in further study. Additionally, the unique profile of PRF matrix may be influenced by the individual growth factor binding affinity to the fibrin matrix. VEGF had a high affinity binding to the fibrin, so this growth factor was found to be sustainably released during the study period.[17] From this point, VEGF released from Bagdadi *et al*.,[12] showed a significant increase on day 7. Conversely, EGF reached its highest level on day 1, due to low binding affinity of EGF to the fibrin.[18] Further studies of growth factors that play a role in bone and soft tissue regeneration, such as EGF, PDGF[19] and VEGF[20] should be inspected.

The MC3T3-E1 cells showed a reduced viability on day 7 and day 14 when incubated with the exudates of APRF+ and APRF from day 3, 7 and 14. This finding is similar to the result of Kermani *et al*.,[21] The study was done using mouse dental pulp stem cells (DPSCs) that showed a reduction in cell viability during the differentiation of cells in osteoblastic induction state. Suggesting that the MC3T3-E1 cells decrease the ability to proliferate during differentiation state.

From the result of cell viability on day 14, exudates from both APRF and APRF+ collected at day 1 (Figure 3B) showed statistically significant differences from the control group. We hypothesized that the exudates collected at earlier time point (1D) might have a greater effect on cell proliferation than cell differentiation. During differentiation of osteoblast cells, cells always express and produce ALP.[22] MC3T3-E1 cells incubated with exudates from APRF and APRF+ did not show any statistically significant differences in ALP production when compared to the control group. The outcome was similar to the study by He *et al*.,[23] that showed no significant difference in the level of ALP of rat osteoblasts when cultured with PRP and PRF. Therefore, the future study should include the cell proliferation assays and ALP activity of osteoblast to elucidate the effect of APRF, APRF+.

Finally, the different mineralization patterns (Figure 5B) probably caused by the clot of APRF+ providing additional protection of growth factors and living cells from proteolytic degradation.[24] From the results of our study, the use of a low speed centrifugation concept that modified the centrifugation time affect the release of growth factor, viability and mineralization of MC3T3-E1 cells. The clinical applications from this study should be limited because of the study was an *in vitro* system. Future research comparing APRF and APRF+ in clinical scenarios should be established.

Conclusions

APRF and APRF+ having been prepared by using a low speed centrifugation concept were found to released TGF-β1 during 14 days of the experiment period. APRF+ demonstrated release TGF-β1 gradually and statistically significant difference from day 1 to day 14. On day 14 of mineralization, MC3T3-E1 cells treated with exudates from APRF collected at day 7 showed the highest mineralization among APRF group. Besides, exudates from APRF+ collected at day 14 showed significantly higher mineralization than APRF.

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Conflicts of interest

The authors declare no conflicts of interest.

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