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Platelet-rich Fibrin Formation was delayed in Plastic Tubes

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Authors' contributions

This work was carried out in collaboration between all authors. Author BJ managed the literature searches, performed the experiments, statistical analysis and wrote the first draft of the manuscript. Author SP prepared the blood from participants. Author KS designed the study, wrote the protocol, managed the analyses of the study and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Platelet-rich fibrin (PRF) is used in many regenerative treatments. Preparing PRF in glass tubes requires quick handling and generates biohazard concerns about silica contamination and glass breakage. Using plastic tubes may be an alternative to glass tubes.
Objectives: This study investigated the formation of PRF prepared in polypropylene (PP) and polystyrene (PS) tubes compared with glass tubes.
Methodology: PRF was prepared from human blood (n=20) in PP, PS, and glass tubes. The time required for PRF clot formation and retraction from the tube wall were observed. The PDGF-AB, TGF-β1 levels, and the gross/SEM appearance of the PRF clots were also evaluated.
Results: The PRF clots in PP and PS tubes formed and retracted significantly slower compared with those in glass tubes. The PDGF-AB levels in the PRF from PP, PS, and glass tubes were not significantly different. Although the TGF-β1 levels in the PRF from PP and glass tubes were not different, that from the PRF from PS tubes was significantly higher. The gross structure and SEM appearance of PRF from the three tube types were similar.

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Conclusion: The slow clot formation in PP and PS tubes can extend PRF handling time while retaining PDGF-AB level and fibrin appearance. Owing to this delay in clot formation, we could obtain the plasma without adding any anticoagulant chemical agents. The plasma can be used for accelerating bone regeneration as platelet-rich plasma.

Keywords: Blood clot; fibrin; plasma; platelet; polypropylene; polystyrene.

1. INTRODUCTION

Platelet-rich fibrin (PRF) is a blood-derived fibrin network containing clusters of activated platelets and leukocytes [1]. PRF acts as a scaffold for growth factors, which accelerate wound healing, including platelet derived growth factor (PDGF), transforming growth factor beta1 (TGF-β1), and vascular endothelial growth factor [2-4]. Currently, PRF is used in regenerative oral surgery procedures, such as sinus floor augmentation for dental implant placement [5-6], dental root coverage [7], and the restoration of intra-bony defects [8-9].

Choukroun et al. described a technique for generating PRF by centrifuging autologous blood without an anticoagulant or other chemical agents in glass tubes [1-2,5]. The yellowish fibrin clot forms in the middle of the tube a few minutes after centrifugation. Thus, quick handling and immediate centrifugation after the blood is drawn are necessary to prevent PRF clot formation failure [1]. However, using inappropriate glass tubes could result in glass breakage and biohazard from blood contamination. These concerns have led to the development of plastic blood-collection tubes [2]. The advantages of plastic tubes are higher centrifugation-force tolerance, greater flexibility, and lower cost [10]. Although PRF preparation in glass-coated plastic tubes resulted in similar PRF architecture compared with those prepared in dry glass tubes [11], concerns about silica particle contamination remained [12]. Tunali et al. reported good results when PRF was prepared in titanium tubes [13-14]; however, titanium centrifuge tubes are not available commercially. Thus, using plastic tubes for PRF preparation is an interesting alternative.

Plastic tubes are made from various materials such as polypropylene (PP), polystyrene (PS), low-density polyethylene (LDPE), and polycarbonate (PC). Although the PC and LDPE tubes can resist higher centrifugal forces, the PP and PS tubes are more available, cost less, and can withstand the centrifugal force required for PRF preparation [15]. Interestingly, the time required for blood clot formation was 7–8 times longer in plastic tubes compared with glass tubes [16]. Approximately 1 h after the blood clot forms, it separates from the glass tube wall. The time from centrifugation to a noticeable separation of the blood clot from the tube wall is called the clot retraction time (CRT) [17]. This phenomenon also occurs in PRF, which is a type of blood clot. We hypothesized that preparing PRF using plastic tubes might increase the complete clot formation time (CCFT) and CRT, thus extending the clinical handling time.

The purpose of this study was to investigate the CCFT, CRT, gross and microstructure appearance, and quantity of PDGF-AB and TGF-β1 of PRF in PP and PS plastic tubes, using glass tubes as a control.

2. MATERIALS AND METHODS

2.1 Volunteer Selection

Twenty volunteers (10 male and 10 female) who did not take any medications over the previous 2 weeks and had normal blood screening results (complete blood count, prothrombin time (PT), and partial thromboplastin time (PTT)) participated in the present study. The study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (Study code: HREC-DCU 2011064) and performed in accordance with the Declaration of Helsinki 2008. All of the volunteers were informed about the study and signed consents.

2.2 Sample Collection and PRF Preparation

Eighteen ml of blood without added anticoagulant was obtained from the median cubital vein of each volunteer. Three ml of blood was randomly aliquoted into two tubes of the three types of centrifuge tubes: glass tubes (Pyrex, Corning Inc, MA, USA), PP tubes (Taizhou, Taizhou Runlab Labware Manufacturing Ltd, Zhejiang, China) and PS tubes (SPL, SPL Lifesciences Inc, Gyeonggi-do, South Korea). The 6 tubes per volunteer were immediately centrifuged at 3000

rpm (420 *g*), at 25℃ for 10 min (Dynamica Velocity 14R, Dynamica Pty Ltd, Victoria, Australia) and left at room temperature. The formation of the PRF in the tubes was observed immediately after centrifugation and every minute until an elastic yellow fibrin clot was generated in the middle of the tube. At this time point, the CCFT of each tube was recorded. Subsequently, one PRF clot from each tube type was placed on a sterile gauze to drain its liquid content and obtain the PRF membrane. The membranes were randomly selected to measure the PDGF-AB and TGF-β1 levels. The PRF clots in the other tubes of each group were used to observe the time that the clot began to retract from the tube wall (CRT) up to 4 h after centrifugation.

2.3 Scanning Electron Microscope (SEM) Evaluation

PRF membranes generated from each type of tube were randomly chosen from 3 male and 3 female volunteers for investigating the membrane micro-structure. Each PRF membrane was placed on a glass slide and fixed in 3% glutaraldehyde for 30 min then rinsed with phosphate buffered saline for electron microscope analysis. The samples were dehydrated in a graded ethyl alcohol series (30, 50, 70, 90, and 100%) for 2 min each and immersed in hexamethyldisilazane for 5 min and air dried at room temperature for 2–3 days. The specimens were attached to studs and sputter-coated with 20 nm gold using a Fine Coater (JFC-1200, JEOL Inc, Tokyo, Japan) and examined with a SEM (JSM-5410LV JEOL Inc, Tokyo, Japan). The photographs were taken at 15 kilovolt at 3,500X.

2.4 Sample Preparation for Enzyme-linked Immunosorbent Assay (ELISA) Quantification

PRF membranes generated from each tube type were randomly chosen from 3 male and 3 female volunteers for measuring the amount of PDGF-AB and TGF-β1 in the membrane. Each membrane was weighed and placed into a microcentrifuge tube with 1 ml of sterile Dulbecco's modified eagle's medium. Each sample was centrifuged at 10,000 *g* at 25℃ for 15 min (Spectrafuge 16 M microcentrifuge, Labnet International Inc, NJ, USA). The membrane supernatant was diluted at a 1:10 ratio and the amount of PDGF-AB and TGF-β1 was measured using ELISA kits (Quantikine, R&D systems, Minneapolis, MN, USA).The tests were performed in duplicate. The absorbance was read using an ELISA microplate reader (Zenyth 200 rt, AnthosLabtec, Wals/Salzburg, Austria). The amount of PDGF-AB and TGF-β1 were calculated per 1 g of PRF membrane.

2.5 Statistical Analysis

One-way ANOVA was used to analyze the mean CCFT, CRT, and amount of PDGF-AB and TGF-β1 of the PRF from the PP, PS, and glass tube groups. Statistical significance was determined at $P < .05$.

3. RESULTS

3.1 Complete Clot Formation Time (CCFT)

The subjects' blood screening tests were in the normal range (average platelet count 216,900/ml, PT 15.33 sec, and PTT 42.59 sec). The means CCFT of the PRF in the PP tube and PS tube groups were 60.55±6.26 and 56±5.17 min, respectively (Fig. 1A), which were not significantly different ($P = .84$). However, they were significantly longer than that in the glass tube group (0.28±1.43 min) ($P < .001$).

3.2 Clot Retraction Time (CRT)

Over time, the PRF clots in the tubes began to separate from the tube walls. The means CRT of the PRF in the PP and PS tube groups were 178.60±13.65 and 202.10±10.60 min, respectively (Fig. 1B), which were not significantly different ($P = .45$). However, they were significantly longer compared with that in the glass tube group (68.45±5.69 min) ($P < .001$). A number of PRF clots did not retract from the PP and PS tube walls after 4 h and their CRTs were recorded as 240 min.

3.3 PRF Clot and PRF Membrane Appearance

The PRF clots and PRF membranes from the glass, PP, and PS tube groups were similar in appearance (Fig. 2). Each tube type generated three separate phases of blood components: (1) a lower phase containing red blood corpuscles, (2) an intermediate yellow gel phase (the PRF clot), and (3) an upper phase of clear yellow serum, the acellular plasma or platelet-poor plasma (PPP) [1]. After the liquid content in the PRF clot was released, the PRF membrane was a thin elastic yellowish white sheet with minimal red corpuscles attached at the bottom.

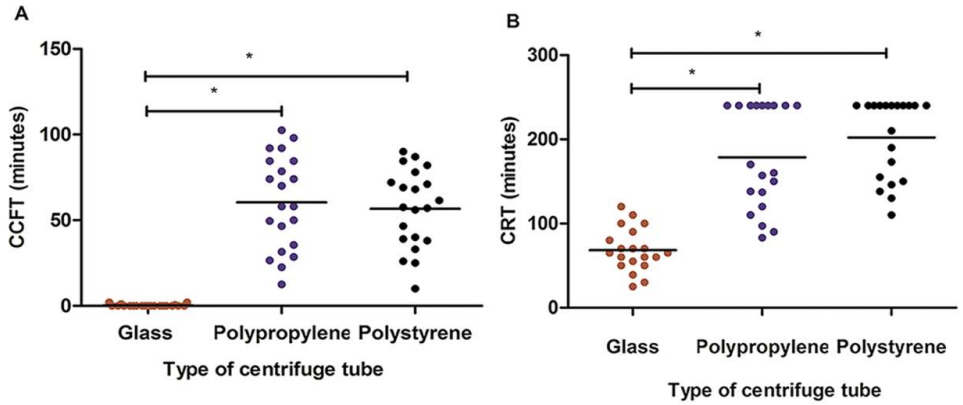

Fig. 1. The CCFT and CRT of the PRF clots in the three tube types

The horizontal lines represent the mean PRF CCFT and CRT. ***(A)*** *The PRF CCFT in both plastic tube groups were significantly longer compared with the glass tube group (* P < .001).* ***(B)*** *Many PRF clots in the PP (Polypropylene) and PS (Polystyrene) tube groups did not retract by 4 h and their CRT was recorded as 240 min. The PRF clots in the glass tube group retracted significantly faster than those in the PP and PS tube groups (* P < .001)*

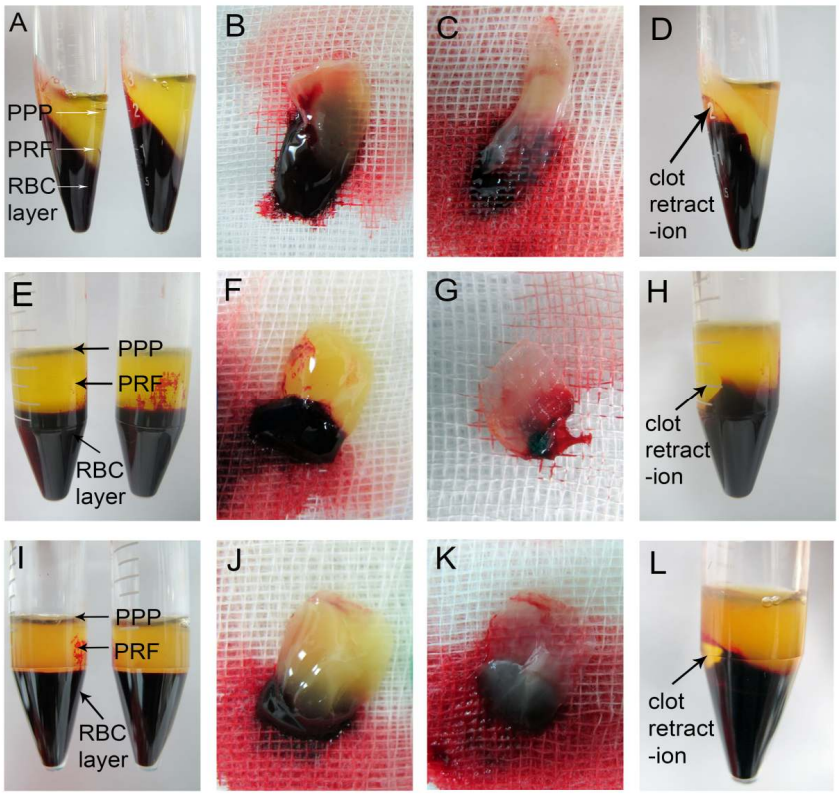

Fig. 2. Gross appearance of the PRF generated from the three tube types

The PRF generated from ***(A)*** *glass,* ***(E)*** *polypropylene, and* ***(I)*** *polystyrene tubes. The PRF clots from each tube type appeared as a yellow gel between the upper acellular plasma phase (PPP) and the lower red blood corpuscle phase (RBC layer).* ***(B-C, F-G, and J-K)*** *After removing the clots from the tubes and placing them on gauze, the liquid content inside the clots drained out. The PRF membranes then appeared as an elastic yellow-white sheet with minimal red blood corpuscles attached at the bottom.* ***(D, H, and L)*** *The PRF clots retracted from their respective tube's surface*

The SEM analysis revealed that the PRF membrane microstructure from the three tube types had a similar appearance (Fig. 3). The membranes consisted of clusters of platelets and leukocytes on a dense matrix fibrin network with various sizes of fibrin in each membrane. However, erythrocytes were rarely found in this network. Moreover, the connections of the fibrin network showed characteristic tri-molecular or equilateral junctions.

3.4 PDGF-AB and TGF-β1 Quantification

The mean amounts of PDGF-AB released from the PRF membranes in the PP, PS, and glass tube groups were 11.91±0.48, 8.56±0.76, and 10.64±0.24 ng/ml, respectively (Fig. 4A). These amounts in the PP and PS tube groups were significantly different from each other (P < .001) but not significantly different from that in the glass tube group (P = .13 and .14, respectively).

The mean amounts of TGF-β1 released from the PRF membranes in the PP, PS, and glass tube groups were 319.52±7.46, 555.37±25.23, and 306.97±24.82 ng/ml, respectively (Fig. 4B). These amounts in the PP and glass tube groups were not significantly different (P = .95). However, they were significantly lower than that in the PS tube group (P < .001).

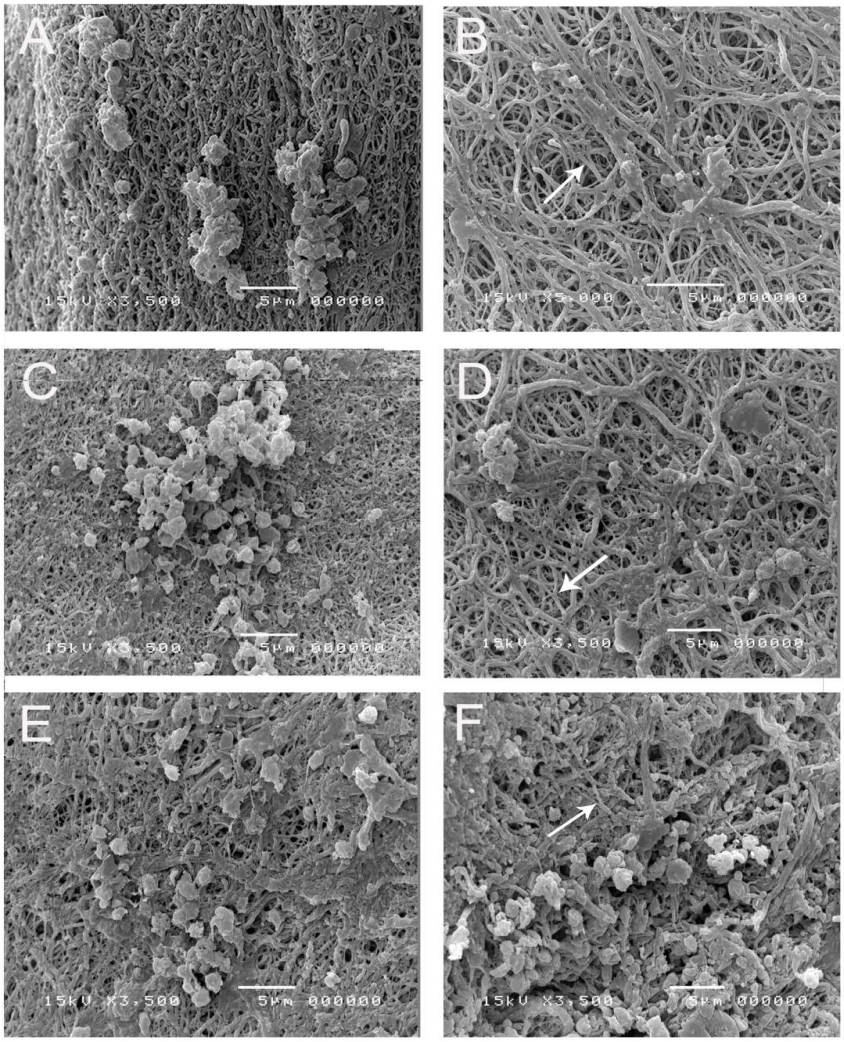

Fig. 3. Scanning electron micrographs of the PRF membranes from the three tube types

*The PRF membranes from the **(A)** glass, **(C)** PP, and **(E)** PS tubes appeared as a dense matrix fibrin networks with clusters of aggregated platelets and leukocytes, with erythrocytes rarely found. The connected tri-molecular or equilateral junctions of the fibrin network were observed in the PRF membranes from **(B)** glass, **(D)** PP, and **(F)** PS tubes (white arrows) (Original magnification: A-F, x3,500, Bar = 5 µm)*

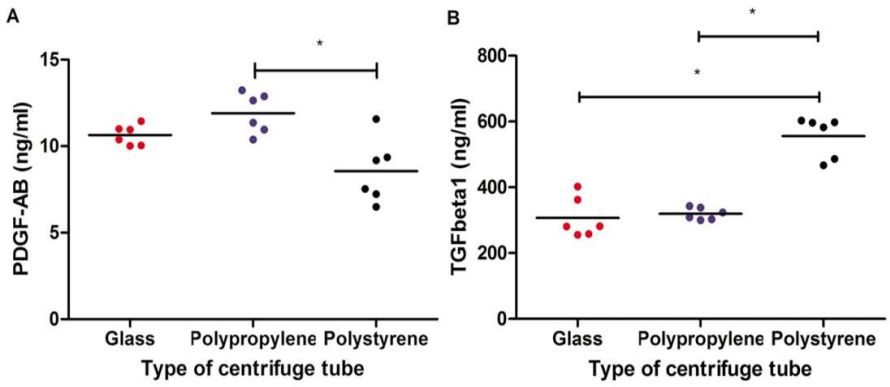

Fig. 4. The levels of PDGF-AB and TGF-β1 released from the PRF membranes

*The horizontal lines represent the mean amounts of PDGF-AB and TGF-β1. **(A)** PDGF-AB levels in the PRF membranes from the plastic tube groups were not significantly different compared with the glass tube group. However, the membrane from the PS tube group had significantly lower PDGF-AB level than that from the PP tube group (* P < .001). **(B)** The TGF-β1 level in the PRF membrane from the PS tube group was significantly higher than that of the other groups (* P < .001). The membrane from the PP and glass tube groups had similar TGF-β1 level*

3.5 Plasma Obtained before PRF Clot Formation

Before the formation of a PRF clot in the PP and PS tubes, we did a trial by drawing the plasma and mixed it with bone graft particles. After a few minutes, the plasma and bone particles formed a gelatinous clump (Fig. 5). We could carry all the bone particles as a single clump.

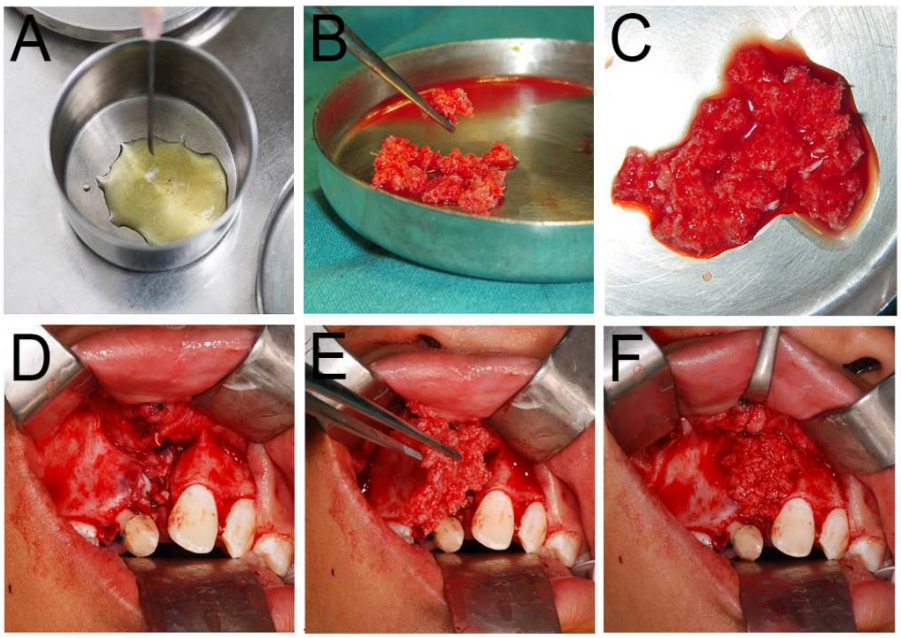

Fig. 5. Clinical application of the plasma obtained before PRF clot formation

***(A)** The plasma drawn before the formation of a PRF clot in the PP and PS tubes. **(B)** Cancellous bone particles harvested from the anterior iliac crest. **(C)** After mixing with the plasma, the bone particles were held together in the plasma gel. **(D)** The alveolar cleft site was prepared for bone grafting. **(E)** The gelatinous bone particles were easily transferred to the alveolar cleft site. **(F)** The alveolar defect was filled with the bone-plasma gel*

4. DISCUSSION

Our study evaluated the characteristics of platelet-rich fibrin (PRF) generated in PP and PS tubes compared with those in glass tubes. We found that the CCFT and CRT in the PP and PS tube groups were significantly longer compared with the glass tube group. The gross and SEM appearance of the PRFs formed in each group were similar. We also found that the levels of the growth factors PDGF-AB and TGF-β1 were relatively similar between the groups.

A PRF clot forms in the same manner as a whole blood clot, except that the red blood cells are separated from the PRF clot by centrifugation. Platelets are activated by contacting the tube surface and release factors initiating the coagulation cascade [18]. In our study, the PRF in the PS and PP plastic tube groups formed clots much slower compared with the clots in the glass tube group. This result is consistent with that of Stone et al., where the whole-blood clotting time in plastic containers was longer compared with glass tubes [16].

The different PRF CCFTs may be due to the different blood-surface contact interactions of each material. Glass tubes are typically made from borosilicate glass that has silicon oxide ions (SiO^-) on their surface. This negative charge is hydrophilic, resulting in high adsorption when exposed to a protein solution such as blood [19]. Furthermore, glass surface contact activates platelets, generates hydrophilic interactions due to hydrogen bonds, and has an electronegative surface that acts similar to exposed collagen in the subendothelium of blood vessels. Platelet aggregation and the activation of the factor XII intrinsic pathway promote rapid blood clot formation on a glass surface similar to what occurs in damaged vessels during primary hemostasis [20]. Although the PP and PS surfaces are negatively charged due to their hydrocarbon polymer (-C-H-) structure, these plastic surfaces are hydrophobic and have low plasma protein adsorption [18]. The different adhesion properties make blood clot formation times on these polymeric surfaces longer compared with glass surfaces. However, the slow polymerization of PRF clots in plastic tubes could allow for increased cytokine enrichment that may result in increased cellular migration into the clot.

Interestingly, we could draw the plasma from the PP and PS tubes before the clot formed. The obtained plasma from our study looked like that when preparing platelet-rich plasma [21]. However, no anticoagulant agent was needed to maintain the liquidity of the blood in our study. Moreover, the plasma formed a gel without adding thrombin and could glue bone graft particles together. Instead of carrying the particulate bone piece by piece, we could easily transfer the gelatinous bone particles to the recipient site as a single clump. Recently, this bone gelling technique has become popular in performing bone grafts for dental implant placement.

After blood clot formation, clot retraction occurs as a normal physiologic mechanism [17], and PRF clots behave similarly. They retracted from the glass tube surfaces approximately 1 h after the PRF clot completely formed. In contrast, the PRF clots in the PP and PS plastic tubes developed much slower compared with the glass tubes. The longer PRF CCFT in the plastic tubes may be a factor contributing to their longer CRT. Normally, a whole blood clot lyses after remaining in a glass tube for 24 h [22], however, we did not observe the lysis of PRF clots after 4 h in the present study, in any of the groups. The length of time before PRF clot lysis needs to be clarified for better clinical application.

The PRF clots formed in the glass, PP, and PS centrifuge tubes had the same appearance as described in previous studies [1-5]. In the present study, the PRF clots in the glass tubes inclined 45º to the tube surface because the clots formed immediately after centrifugation, where the tubes are at a 45° angle. In contrast, the PRF clots in both types of plastic tubes were horizontal because the clots formed while the plastic tubes were in a rack after centrifugation. However, the gross appearance of the PRF membranes from the PP and PS tubes was similar to those formed in the glass tubes.

The SEM images revealed that the microstructure of the PRF membranes generated from the glass, PP, and PS tubes were similar to those found in other studies [11,23]. The membranes were composed of clusters of platelets and leukocytes in a dense fibrin matrix network that had various sizes of fibrin, with few erythrocytes. Furthermore, the connected tri-molecular or equilateral junctions of the fibrin network, a specific characteristic of PRF, were found in the PRF membranes generated from the three tube types. This feature could increase the elasticity and strength of mature fibrin to support growth factor enrichment and cellular migration.

The amount of PDGF-AB released from the PRF membranes in the PP and PS tubes was similar compared with the glass tubes. In contrast, although the TGF-β1 levels released from the PRF membranes from the PP and glass tubes were similar, the TGF-β1 level from the PS tubes was significantly higher. This result implies that PRF preparation in PP and PS plastic tubes preserves the levels of growth factors, promoting wound healing similar to PRF prepared in glass tubes. We could not compare the levels of growth factors in our study with those of other studies due to the use of different protocols. Su et al. [4] measured the amount of several growth factors, including PDGF-AB and TGF-β1, released from PRF membranes that were prepared from 10 ml of blood and serum. In their study, the growth factors released from the PRF membrane increased over 300 min, while the growth factors in serum did not. To avoid drawing excess blood from the volunteers in our study, we measured growth factors released from PRF membranes prepared from 3 ml of blood. Moreover, the results of Su et al. suggested that the PRF membrane should be used within the first hour to allow it to release growth factors into the wound. However, the proper amount of growth factors in PRF membranes for enhancing tissue regeneration has not been determined and should be further investigated.

5. CONCLUSION

Although PRF clot formation and retraction in the PP and PS plastic tubes occurred much slower compared with glass tubes, the appearance of the PRF membranes were similar. PDGF-AB and TGF-β1 which promote wound healing were retained in the membranes from both types of plastic tube. Therefore, PP and PS plastic tubes could be used as an alternative for PRF preparation with the benefit of increased handling time in their clinical use. Moreover, using plastic tubes can reduce costs and biohazard from accidental glass breakage. However, the effectiveness of PRF generated from PP and PS plastic tubes in patients should be further investigated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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