A proposed protocol for the standardized preparation of PRF membranes for clinical use

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ABSTRACT

Upon clinical application, thick platelet-rich fibrin (PRF) is usually compressed to fit the implantation site. However, it is speculated that the preservation of platelets and plasma content depends on the compression methods used. To accurately evaluate the clinical outcome of PRF, the preparation protocol should be standardized. Freshly prepared PRF clots were compressed into a thin membrane by our novel PRF compression device. The localization of platelets was examined by SEM and immunostaining. Growth factor levels were evaluated by bioassays and cytokine-antibody array techniques. The angiogenic activity was examined by the chick chorioallantoic membrane assay and the scratch assay using HUVEC cultures. Platelets were concentrated on the surface of the region adjacent to the red thrombus and this region was subjected to the experiments. Compared to the PRF membrane compressed by dry gauze (G-PRF), the preservation of the plasma content, 3D-fibrin meshwork, and platelets was more intact in the compressor-prepared PRF membrane (C-PRF). Among the growth factors tested, C-PRF contained PDGF isoforms at higher levels, and significantly stimulated cell proliferation and neo-vascularization. C-PRF may be useful for grafting while minimizing the loss of bioactive factors. This C-PRF preparation protocol is proposed as a standardized protocol for PRF membrane preparation.

1. Introduction

Platelet-rich plasma (PRP) is a platelet-rich fraction of plasma and is clinically available as a source of growth factors to facilitate tissue repair and regeneration. To improve the handling efficiency, the retention at application sites, and the release of growth factors, bovine thrombin and/or calcium have been preferentially added to PRP to directly or indirectly facilitate the conversion from fibrinogen to fibrin. To avoid the use of a xenofactor, bovine thrombin has often been substituted with the autologous human thrombin. We have been using calcium [1] or an alginate hemostatic agent [2,3] to improve the handling of PRP and to take advantage of PRP's beneficial effects in a clinical setting.

Recently, Choukroun and his coworkers developed a simple method to prepare fibrin gels without exogenously added supplements [4,5]. This fibrin gel is designated as platelet-rich fibrin (PRF) and is widely recognized as a new generation of PRP. This preparation protocol is very simple: a blood sample is collected without an anticoagulant in 10-mL tubes and is immediately centrifuged at 3,000 rpm (800 *g*) for 10 min. Even when thrombin or calcium is not added to the blood sample, most platelets can be activated in a few minutes through contact with the tube walls to trigger the intrinsic coagulation cascades. Therefore, another characteristic of Choukroun's PRF is that the resulting fibrin gel is less stiff than that prepared by the addition of thrombin. As reported elsewhere [6,7], the fiber density and the branch-point density of the fibrin networks mainly regulate the stiffness of the fibrin gel, and these parameters are increased by thrombin in a dose-dependent manner. However, PRF prepared without exogenous thrombin stimulation according to Choukroun is becoming more clinically accepted among dentists and oral surgeons.

For utilization in alveolar bone regeneration and plastic surgery, thick tube-like PRF clots should be shaped to fit the size of the implantation site. For compression of the PRF clot to make a PRF

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membrane, moist (or dry) gauze has been conventionally used. However, there has been concern whether this compression may damage the platelets and exude significant quantities of valuable growth factors. In support of this possibility, Su and Burnouf demonstrated that substantial amounts of growth factors, which are thought to be involved in tissue regeneration, are indeed removed by squeezing [8,9]. Therefore, the squeezing process could influence the quality and clinical effectiveness of the PRF preparations as a grafting material.

The growth factor content in the PRF will largely vary with individual blood samples, and one reason for this observation can be attributed to how the PRF preparations are made. We believe that it is necessary to establish a standardized protocol for preparing PRF preparations to satisfy the following criteria: 1) growth factors stored in platelets should be retained to stimulate the cells of the surrounding host tissues; 2) platelets should be preserved in the fibrin meshwork with minimal damage or activation; and 3) the three-dimensional fibrin meshwork construction should be preserved as a scaffold for surrounding host cells. To prepare the most clinically effective PRF membrane, we have developed a compression device for the preparation of a standard PRF membrane, tested the performance of the device in retaining vital growth factors, and proposed a standardized protocol for PRF membrane preparation.

2. Materials and methods

2.1. Preparation of PRF membranes with a compression device

The stainless steel PRF compression device developed for PRF membrane preparation is composed of two spoon shaped parts as illustrated in Fig. 1. The stage where the PRF clot was placed included many pinholes for securing the PRF clot and for draining excess fluid from the serum when the clot was compressed. The clearance of both spoon parts was adjusted to be 1 mm. Thus, when the PRF clots were compressed with this device, a standard 1-mm thick PRF membrane was consistently prepared.

Immediately after venous stasis was induced (∼1 min), blood was collected from 5 healthy and non-smoking volunteers aged 26–32 years (2 females and 3 males) using butterfly needles (21G × 3/4″; NIPRO, Osaka, Japan) and Vacutainer™ tubes (Japan Becton, Dickinson and Company, Tokyo, Japan). To prepare the PRF, the blood samples were immediately (within approximately 2 min after blood collection) centrifuged by a Medifuge centrifugation system (Silfradent S. r. l., Santa Sofia, Italy) using a program with the following characteristics: 30″-acceleration, 2′-2700 rpm (600 *g*), 4′-2400 rpm (500 *g*), 3′-3000 rpm (800 *g*), and 36″-deceleration and stop [10]. The resulting PRF preparations were picked up with forceps, and the red thrombus (the fraction of red blood cells) was eliminated with scissors along the border between this fraction and the PRF. The borders were recognized visually, and the PRF preparations were not damaged at a macroscopic level. Then, the PRF was compressed by the PRF compression device (test) or dry gauze (control). In this preparation process, the wet weight of each PRF sample was measured with an electronic balance (PL303 Precision Balance, Mettler-Toledo K.K., Tokyo, Japan).

The study design and consent forms for all procedures performed with the study subjects were approved by the ethical committee for human subject use at Niigata University Medical and Dental Hospital in accordance with the Helsinki Declaration of 1975 and as revised in 2000.

2.2. Scanning electron microscopy (SEM) observation

The PRF membrane samples, which were compressed by the PRF compressor or dry gauze, were fixed, dehydrated with a series of ethanol and *t*-butanol washes, freeze-dried, and then examined with a scanning electron microscope (TM-1000, Hitachi, Tokyo, Japan) with an accelerating voltage of 15 kV, as described previously [11].

2.3. Immunocytochemical examination

In addition to SEM observations, the localization of platelets in the PRF membranes was also examined by an immunocytochemical method. Freshly prepared PRF clots were compressed as described above, fixed in 10% neutralized formalin, dehydrated in a series of ethanol washes, and embedded in paraffin, as described previously [12]. The blocks were sectioned in round slices at a thickness of 6 μm. As described previously [11], deparaffinized sections were
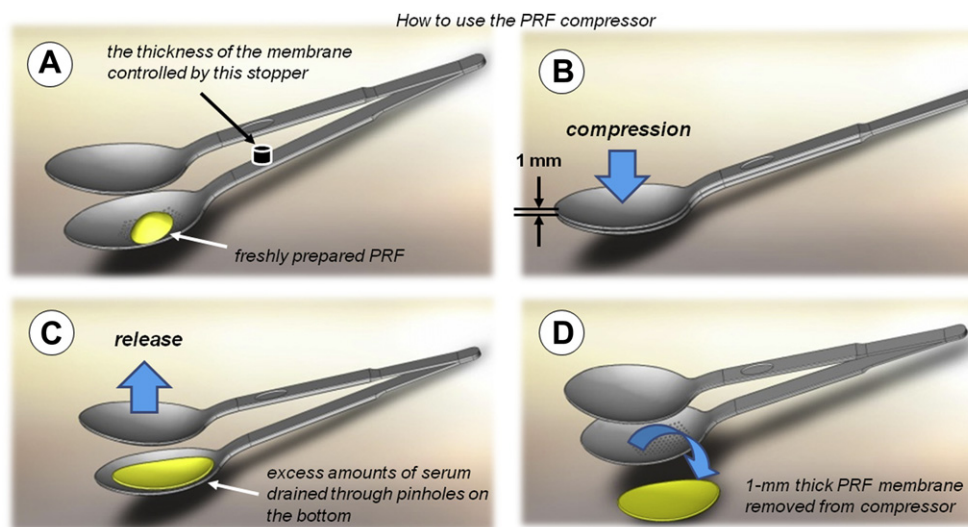

Fig. 1. Appearance of the PRF compressor and the protocol for operation. The PRF clot is placed on the lower spoon, which possesses many pinholes (A), and is compressed by pushing the handles together (B). In this step, excess amounts of exudates would be removed. The compression force is then released (C), and the resulting 1-mm thick PRF membrane is removed (D).

antigen-retrieved and blocked with 2.5% normal horse serum (Vector Labs, Burlingame, CA) and subsequently probed with a rabbit monoclonal anti-CD41 antibody (1:100) (Epitomics, Inc., Burlingame, CA) overnight at 4 °C, followed by incubation in the ImmPRESS® anti-rabbit IgG (Vector). Immunoreactive proteins were visualized by a DAB substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

2.4. Preparation of crude PRF exudates (homogenates)

A PRF membrane compressed by the PRF compressor (C-PRF) was inserted into 1.5-mL sample tubes and centrifuged to determine the volume of the extracts. The same volume of serum-free DMEM was added to the sample tube containing the PRF membrane compressed by dry gauze (G-PRF). C-PRF and G-PRF were prepared from the same blood samples. The platelet-rich regions (Region 1) of both PRF membranes, which were cut to similar sizes, were homogenized on ice for 30 s with a micro-multimixer (Ieda Trading, Corp., Tokyo, Japan). Because the G-PRF lost more plasma exudates than the C-PRF, a similar volume ($\sim$180 μL) of each corresponding medium was added as a substitute for the exudates prior to homogenization. The resulting exudates (crude homogenates) were directly used for the bioassay and were alternatively used for the microarray assay after the cell-lysis treatment described below.

2.5. Growth factor antibody microarray

Growth factor production was semi-quantitated with the RayBio® Human Growth Factor Antibody Array 1 (Ray Biotech, Norcross, GA) in accordance with the manufacturer's instructions. The samples prepared as described above were then treated with the cell-lysis buffer provided with the kit. The resulting array spots were visualized with Light Capture (Atto, Tokyo, Japan) for image analysis using Total Lab (TL100; Nonlinear Dynamics, Newcastle upon Tyne, UK) [12].

2.6. Growth assay using human gingival fibroblasts

Human gingival fibroblastic Gin-1 cells (ATCC, CRL 1292) were obtained from DS Pharma (Osaka, Japan) and maintained in DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA) in a CO_2 incubator at 37 °C [13]. The cells (passage 5–8) were seeded into 96-well plates at a density of 2×10^3 cells and treated with 5% exudates (crude homogenates) of the PRF membranes in 1% FBS-containing medium for 3 days. At the end of the treatment, the cell numbers were determined by the WST-8 cell proliferation assay kit (Dojin, Kumomoto, Japan).

2.7. The ex vivo chorioallantoic membrane (CAM) model

As described previously [14], fertilized chicken eggs were incubated in a hatching incubator equipped with an automatic rotator (KingSURO20; Belbird, Siki, Japan) at 37 °C with a relative air humidity of 65%. On embryo developmental day 11, a hole of approximately 16 mm in diameter was opened in the eggshell, and C-PRF and G-PRF (5×5 mm) were placed on the central area of the CAMs. After the holes were sealed, the eggs were incubated for an additional 3 d. CAM vasculature was macroscopically photographed at the initial time point (day 0) and the end point (day 3). The number of vessels in the center circle (φ 10 mm) was determined using image analysis software (WinROOF, Mitani Corp., Fukui, Japan). In brief, after RGB separation and manually adjusting the threshold, the images were binarized [1], and the vessels were counted manually.

2.8. Culture of endothelial cells and the scratch assay

Human umbilical vein endothelial cells (HUVECs) were obtained from AllCells, LLC (Emeryville, CA) and maintained in an endothelial cell medium that was fully supplemented with growth factors (HuMedia-EB2; KURABO, Osaka, Japan). For the scratch assay, the HUVECs were seeded into 6-well plates at a density of 1×10^5 cells/well and cultured in a CO_2 incubator until they reached confluence. Then, the medium was replaced with growth factor-reduced (1:5) HuMedia-EB2, and the monolayer was scratched using a scraper with a 1-mm blade. The HUVECs were further incubated for up to 24 h with 5% (v/v) exudates of the PRF membranes. The wound areas were photographed at specific time points, and the width of the scratched gap was determined using Image J (National Institute of Health, Bethesda, MD).

2.9. Statistical analysis

The statistical significance of the differences between the groups was analyzed by Student's *t*-test or a one-way analysis of variance (ANOVA). Comparisons between the individual groups were determined using Tukey's multiple comparison tests. *P* values <0.05 were considered significant.

3. Results

3.1. Localization of platelets in PRF membranes

In comparing the two compression methods, it was observed that the PRF compressor reduced the wet weight of the PRF clots (the original PRF: 2.180 ± 0.545 g, $n = 3$) by 84% (0.352 ± 0.030 g), while the dry gauze method reduced the clot wet weight by 98% (0.040 ± 0.013 g) (Fig. 2). The C-PRF was divided into three regions, which were equal in length (Fig. 3A), and the presence of platelets was observed by SEM in each region (Fig. 3B–D). In Region 1 (Fig. 3B), numerous platelets aggregated on the membrane surface, and some lymphocytes and/or other white blood cells were observed. The number of platelets decreased with increasing distance from the red thrombus. In Region 2 (Fig. 3C), fibrin fibers could be observed. The number of platelets was further decreased and the fibrin meshwork was clearly observed in Region 3 (Fig. 3D).

The distribution of platelets in the C-PRF and G-PRF was then compared in Region 1 by SEM and immunocytochemistry (Fig. 4).
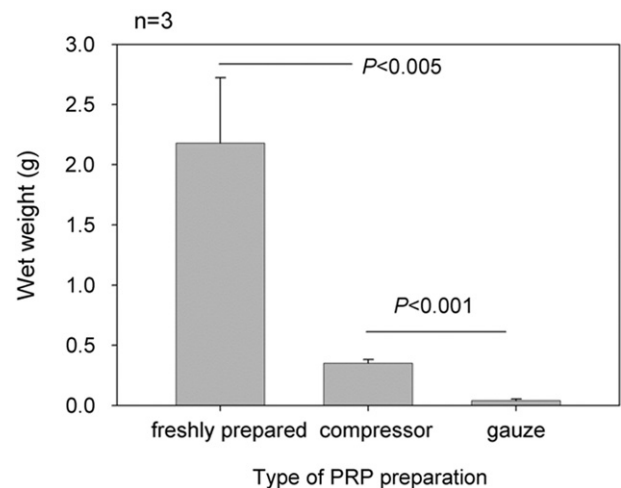

Fig. 2. The decrease in the wet weight of the PRF clot during compression. Whole PRF clots were compressed and weighed ($n = 3$).
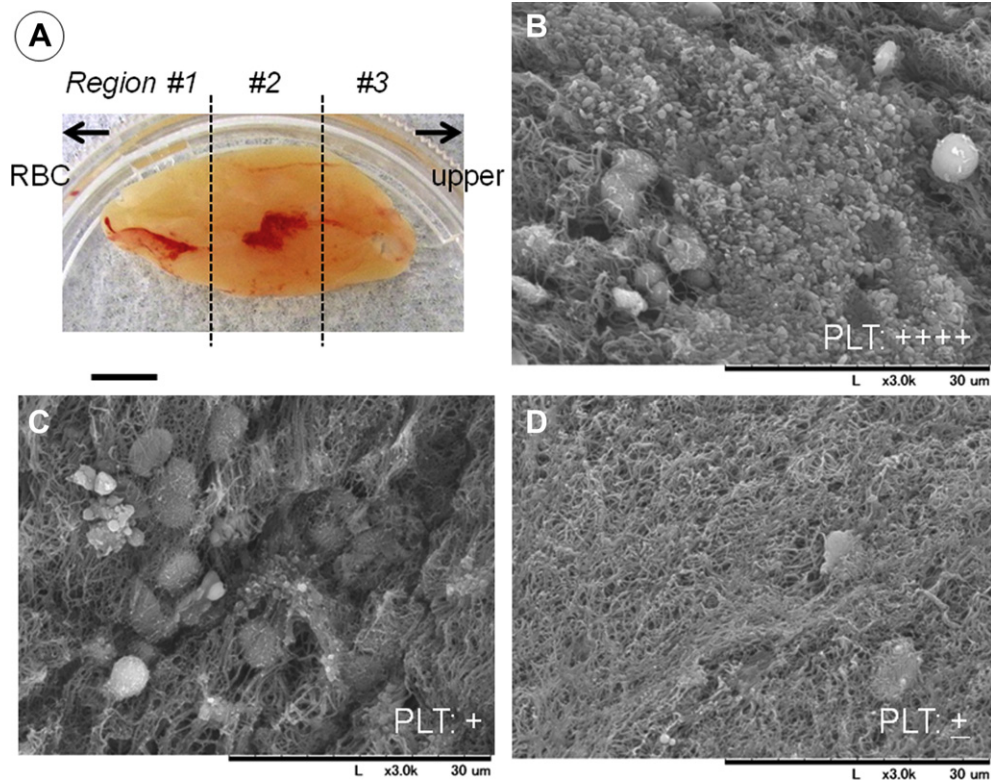
Fig. 3. The three regions of the C-PRF and SEM observations of the platelets on the membrane surface. (A) C-PRF was divided into three regions: Region 1 was adjacent to the red blood cell fraction (RBC), Region 2 was the middle portion, and Region 3 was the distal portion from the red thrombus. Bar = 0.5 mm. (B–D) platelet localization was observed in Region 1 (B), Region 2 (C), and Region 3 (D) by SEM. The platelets were most concentrated in Region 1 and least concentrated in Region 3. Bar = 30 μm.
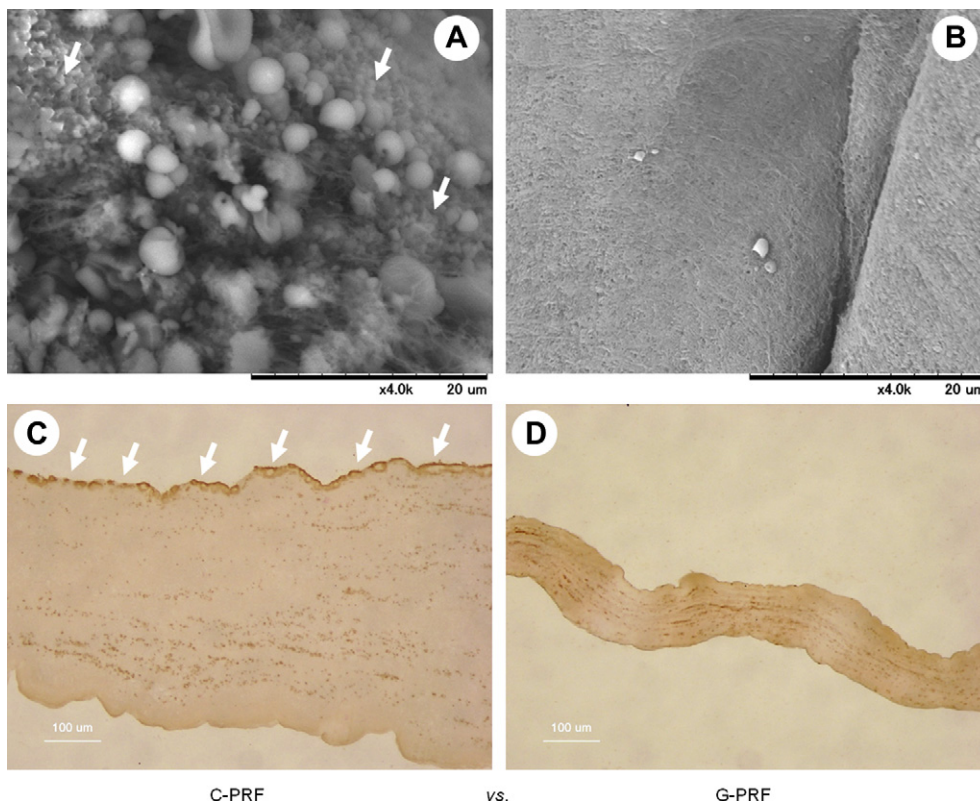

Fig. 4. The distribution of platelets in C-PRF and G-PRF (Region 1). Platelets and fibrin meshwork were observed in C-PRF (A) and G-PRF (B). Arrows indicate platelet aggregates. Bar = 20 μm. Alternatively, platelets (stained dark-brown) were detected by immunocytochemistry using an anti-CD41 antibody and visualized by DAB. Arrows indicate platelet aggregates on the surface of the C-PRF (C); however, these aggregates were not or minimally observed on that of G-PRF (D). The data presented are representative of three independent experiments. Bar = 100 μm.

In the C-PRF, the fibrin meshwork was fully covered by many platelet aggregates and lymphocytes (Fig. 4A), while in the G-PRF, the fibrin meshwork was completely pressed into a film-like form, and only a few platelets could be observed (Fig. 4B). These findings were supported by the immunocytochemical detection of CD41-positive cells. In the C-PRF, on one side of the surface, numerous CD41-positive platelets were accumulated, and some platelets were found inside the membrane (Fig. 4C). However, on the other side of the surface, platelets were rarely detected. In the G-PRF, in contrast, platelet aggregates were not detected on either side of the membrane surface (Fig. 4D). Some platelets were found only inside the membrane. The density of the platelets in the G-PRF appeared higher than in the C-PRF, but this observation was likely due to the reduced thickness from the compression.

3.2. Bioactivity of PRF membranes

To determine the preservation of bioactive growth factors, both C-PRF and G-PRF were tested in a bioassay using Gin-1 cells and antibody array analysis. Gin-1 cells are widely accepted to be a normal, non-malignant cell type, and therefore, these cells require supplementation with FBS to grow. The crude exudates obtained from the G-PRF significantly suppressed the proliferation of Gin-1 cells, while those from the C-PRF significantly increased the number of Gin-1 cells (Fig. 5A). In comparing the growth factor content, the C-PRF contained a greater level of growth factors when compared to the G-PRF (Fig. 5B). Because the samples were not activated by an acidic solution, the TGF-β isoforms, which indicate the presence of platelets, were not detected or preserved in either PRF membrane preparation. However, the PDGF isoforms, another indicator of platelet levels, were found to be abundant (Fig. 5B). In this antibody array, PDGF-BB, EGF, FGF-4, IGF-II, PDGF-AB, and VEGF-D were detected at higher levels in C-PRF than in G-PRF (Fig. 5B).

The angiogenesis bioactivity of the C-PRF was then examined in the CAM assay (Fig. 6). In the non-treated control at day 3 (Fig. 6A), compared with the initial time point (day 0), new blood capillary vessels had formed and had subsequently cross-linked with one another to form networks. In the CAM treated with G-PRF (Fig. 6B), some major vessels were newly formed and induced to the G-PRF piece. In the CAM treated with C-PRF (Fig. 6C), more major and minor vessels were induced to the C-PRF piece to form a typical hub-and-spoke relationship. The number of major and minor blood vessels induced into the PRF pieces was counted and graphed (Fig. 6D). In the control, the central regions were chosen for quantitation. The C-PRF had a significantly higher angiogenic activity in this bioassay system.

The angiogenic activity of the C-PRF exudates (and crude extracts) was further examined in the scratch assay using HUVEC cultures (Fig. 7). The gaps formed in the 90% confluent cultures of HUVECs were gradually and spontaneously closed by cells that grew out from both sides of the gap in the non-treated control cultures. Addition of the exudates of C-PRF, but not of G-PRF, significantly facilitated the closing of the gaps.

4. Discussion

When preparing a standardized protocol proposal, the most important point to consider is the type of application. In the field of tissue engineering and regenerative medicine, there are three major applications of PRF preparations. These applications include 1) biodegradable barrier membranes for guided tissue regeneration, including alveolar ridge augmentation [15], 2) a source (or reservoir) of growth factors as a gel form of PRP for tissue
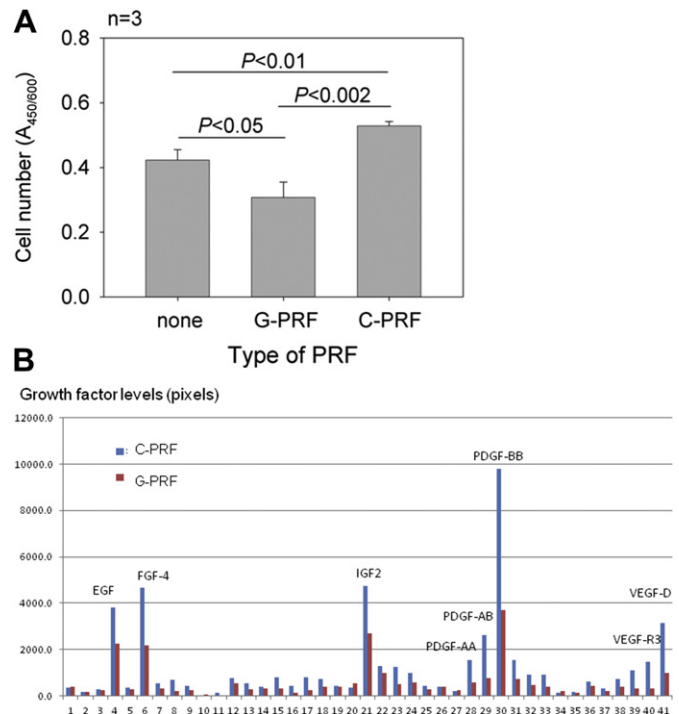

Fig. 5. Biological and immunological evidence for growth factors preserved in C-PRF. (A) The mitogenic action of C-PRF was examined with Gin-1 cells for 3 days in 1% FBS-containing medium. Cell numbers were evaluated by a colorimetric assay. (B) Several major growth factors were detected by the antibody array technique. Typical data are shown from a single experiment using a blood sample from a single donor. Lane 1: AR (amphiregulin), Lane 2: bFGF, Lane 3: β-NGF, Lane 4: EGF, Lane 5: EGF-R, Lane 6: FGF-4, Lane 7: FGF-6, Lane 8: FGF-7, Lane 9: GCSF, Lane 10: GDNF (glial cell-derived neurotrophic factor), Lane 11: GM-CSF, Lane 12: HB-EGF (heparin-binding EGF-like growth factor), Lane 13: HGF, Lane 14: IGFBP-1, Lane 15: IGFBP-2, Lane 16: IGFBP-3, Lane 17: IGFBP-4, Lane 18: IGFBP-6, Lane 19: IGF-I, Lane 20: IGF-I SR, Lane 21: IGF-II, Lane 22: M-CSF, Lane 23: M-CSF-R, Lane 24: NT-3 (the third neurotrophic factor), Lane 25: NT-4 (the fourth neurotrophic factor), Lane 26: PDGF-Rα, Lane 27: PDGF-Rβ, Lane 28: PDGF-AA, Lane 29: PDGF-AB, Lane 30: PDGF-BB, Lane 31: PlGF (placental-derived growth factor), Lane 32: SCF (stem cell factor), Lane 33: SCF-R, Lane 34: TGF-α, Lane 35: TGF-β1, Lane 36: TGF-β2, Lane 37: TGF-β3, Lane 38: VEGF, Lane 39: VEGF-R2, Lane 40: VEGF-R3, and Lane 41: VEGF-D. Note: The optical density data (Y-axis) were not linked to the absolute weight values of the growth factors. Therefore, the values of the same growth factors can be compared, but the amounts of the different growth factors cannot be compared between the two membranes (*i.e.*, C-PRF *vs.* G-PRF).

regeneration, such as bone induction [4], and 3) biodegradable scaffolds for tissue engineering [16].

We have been investigating the development of the most effective therapeutic method for periodontal regeneration, and therefore, we were most interested in application 2 noted above. This could also be applied in the case of gingival recession. To activate and facilitate the regenerative activity of the host tissue, it is necessary to both provide major growth factors and allow space for tissue regeneration. However, when the PRF is used as a barrier membrane (application 1), the mechanical toughness is of paramount importance. Although PRF prepared without exogenous thrombin stimulation according to Choukroun is not as stiff as the chemically prepared preparation, it is well cross-linked [6,7] and exhibits adequate stiffness to serve as a barrier membrane (Kawase et al. unpublished observation). In this case, the PRF would not provide as many growth factors as expected. On the other hand, when the PRF is used as a scaffolding material (application 3), both appropriate three-dimensional (3D) construction and growth factors are required. This application is similar to application 2, and the only major difference is the site where it is used, *in vitro* or *in vivo*.
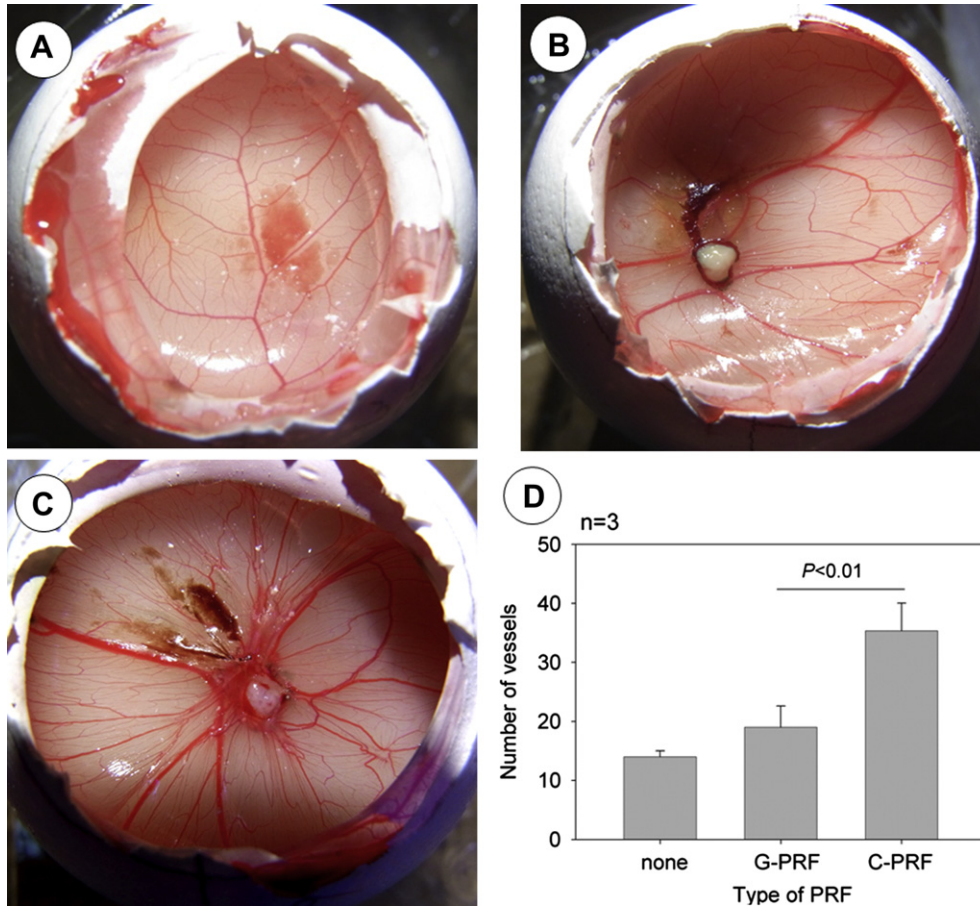
Fig. 6. Effects of C-PRF on angiogenesis in the CAMs. In controls (A), no PRF preparation was placed on the CAMs. Pieces (approx. 5×5 mm) of G-PRF (B) and C-PRF (C) were placed on CAMs and incubated at 37 °C under humidified conditions for 3 days. At the end of the incubation, the CAMs were photographed, and the number of vessels was counted. (D) The number of vessels formed during the period of incubation ($n = 3$).
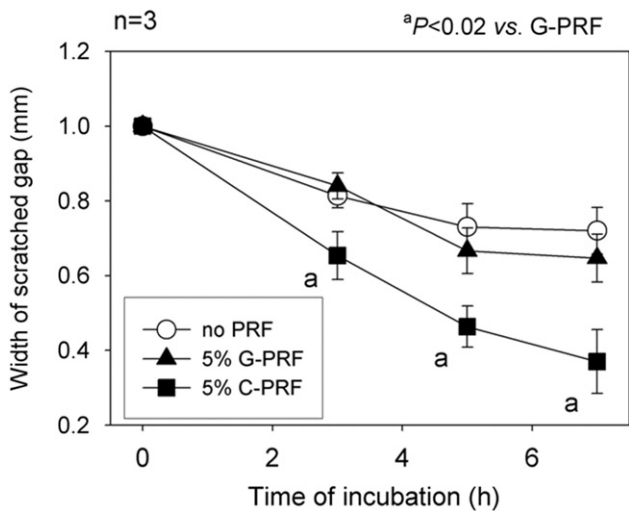

Fig. 7. Angiogenic effects of the exudates of C-PRF on the closure of scratches made in human umbilical vein endothelial cell (HUVEC) cultures. Scratches (1-mm wide) were made on the confluent monolayer cultures of the HUVECs, and the PRF exudates (crude homogenates) were added at 5% to the cultures (B, D). In the control, no PRF was added. The width of the scratch was measured over time under an inverted microscope ($n = 3$).

However, in any application, it is important to characterize the PRF prepared without exogenous thrombin stimulation according to Choukroun. The primary finding of this study was that platelets were not equally distributed inside and on the surface of the PRF clot, although a PRF clot was widely believed to be a gel uniformly concentrated with platelets. Therefore, in a clinical situation, when growth factors provided by platelets are expected and desired, the platelet-rich region adjacent to the red thrombus should be used.

Based on the concept that the serum reserved in the PRF clot might contain higher levels of growth factors released by platelets that are more or less activated during centrifugation [8,9], we attempted not to squeeze out all the plasma (exudates) from the PRF clots by complete compression. This technique was supported by the bioassay using Gin-1 cells and partially supported by the data from the assay using the antibody array technique. In the latter immunological semi-quantitative experiment, the tendency for higher levels of growth factors in the C-PRF than in the G-PRF can be attributed to the growth factors derived from platelets, such as PDGF-AA, PDGF-AB, and PDGF-BB. On the other hand, most growth factors tested were detected at higher levels than predicted and were maintained in the G-PRF at levels comparative to those of the C-PRF. This result may be because the fibrin and the fibrin meshwork can directly adsorb growth factors or they could entrap serum albumin or heparin and thereby indirectly retain growth factors [17].

It is well known that growth factor levels in PRP vary with individual donors [18]. However, it is possible to make a standardized higher quality PRP preparation by adjusting or preserving the
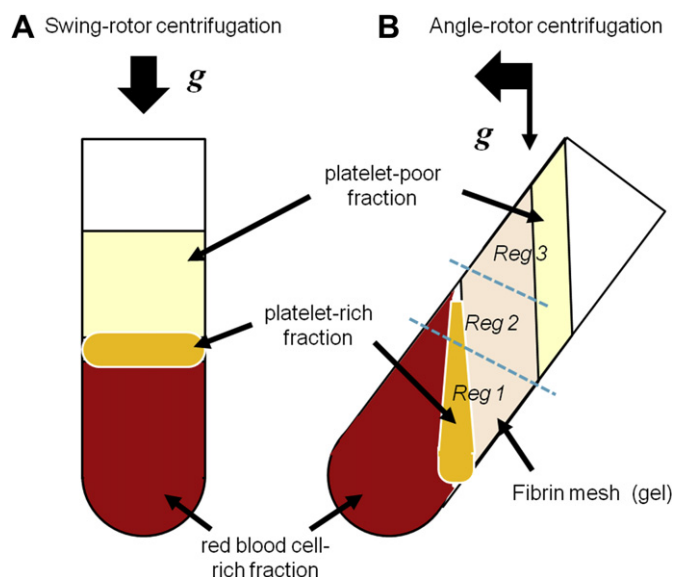
Fig. 8. Illustration of the platelet-rich portions of PRP and PRF. (A) PRP preparation with a centrifuge equipped with a swing rotor. The buffy coat, which is enriched with platelets, was isolated between the red blood cell fraction and the platelet-poor plasma fraction. "g" represents the direction of gravity. (B) PRF preparations separated by centrifugation with an angle rotor. The platelet-rich portion was placed between the red thrombus. Individual regions shown in Fig. 3 correspond to the portions shown in (B).

number of platelets. In addition, employing the same protocol would allow for better quality control of the PRP preparations. It is almost impossible to count and adjust the number of platelets in PRF preparations prior to clinical use. Therefore, the most clinically efficient way to control the quality of the results is to utilize the platelet-rich region of the PRF preparation. The three-dimensional architecture and cell composition of Choukroun's PRF preparation was recently reported by Dohan Ehrenfest et al. [19]. Based on our own findings, we have independently illustrated the difference in the direction of the gravity force during centrifugation between the conventional method of PRP preparation and the thrombin-free preparation method of PRF preparation (Fig. 8). According to this illustration, it is likely that platelets would accumulate most in Region 1 of the PRF preparations, and this was demonstrated and supported by our SEM observations. Therefore, for a higher quality and more ideal PRF preparation, we recommend that PRF clots be compressed with our novel compression device and that the region close to the red thrombus should be grafted to the target site.

In conclusion, it is difficult to accurately control the quality of human-derived materials, such as PRF preparations, but it is very important to perform the highest level of quality control on the PRF preparations prior to their application in a clinical setting. For the standardization of PRF preparations as a grafting material for tissue regeneration, we propose the use of the region of the PRF membrane with the greatest platelet enrichment and furthermore, not to squeeze out all of the plasma contained in the original PRF clots. In this regard, our novel compression device would be effective and useful for preparation of biologically active PRF; the clinical usefulness should be evaluated in clinical studies.

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