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Distribution and quantification of activated platelets in platelet-rich fibrin matrices

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Abstract

Platelet-rich fibrin (PRF) has been widely applied in regenerative therapy owing to its simple preparation protocol. To date, the original protocol for preparing leukocyte-rich (L)-PRF has been modified to produce derivatives such as advanced (A)-PRF, concentrated growth factors (CGF), and horizontal (H)-PRF. However, these derivatives have not been rigorously compared to explore possible differences. We previously developed and validated a nondestructive near-infrared (NIR) imaging method to quantitatively examine the platelet distribution in PRF matrices. To further evaluate the characteristics of platelets in PRF, we herein examined the distribution of activated platelets. Four types of PRF matrices were prepared under different centrifugal conditions from blood samples obtained from the same healthy donors. After fixation and compression, the matrices were stained immunohistochemically without sectioning and visualized using an NIR imager. Qualitative morphological analysis revealed that whole platelets were distributed widely and homogeneously in H-PRF and A-PRF, but localized along the distal tube surface in L-PRF and CGF. Activated platelets were distributed as were whole platelets in A-PRF, L-PRF, and CGF, but localized mainly in the "buffy coat" region in H-PRF. Quantitative analysis revealed that there was no significant difference in the ratio of activated to whole platelets between PRF derivatives. These findings suggest that platelet activation is similarly induced in fibrin matrices regardless of centrifugal speed or rotor angulation. However, only the H-PRF group was distinguishable from the other PRF derivatives in terms of activated platelet distribution.

Keywords

Activated platelets, CD41, CD62P, distribution, platelet-rich fibrin

History

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Introduction

The development of platelet-rich plasma (PRP) was a breakthrough in the history of regenerative medicine [1,2]. However, the relatively complicated and technique-sensitive preparation protocol was the main shortcoming and has hindered its widespread application [3]. Platelet-rich fibrin (PRF), which emerged as a second generation of PRP [4], overcame this shortcoming by simplifying the protocol, without requiring anticoagulants, coagulation factors, or specific operator skills. The lack of definition or classification of PRF [5] and future vision for standardization [2,6] has unexpectedly resulted in non-evidence-based modification of the original PRF protocol and uncontrolled development of PRF derivatives. The commercially-driven, and often less scientific, competitions have distorted the accurate evaluation of PRF therapy and has delayed more widespread approval from various regulatory authorities [2].

While materialogical characterization of individual PRF derivatives may not directly lead to the ultimate goal of standardizing the PRF preparation protocol, it is important to thoroughly understand individual PRF derivatives and optimize their clinical application for more predictable outcomes. To our knowledge, only a few published studies have reported rigorous scientific comparisons among several PRF preparations without commercial bias. For the purpose of protocol optimization, Miron and his coworkers have vigorously demonstrated the differences between the derivatives mainly in terms of growth factor release in a series of studies [7–10]. Based on their findings, it was concluded that centrifugal speed and the quality of blood collection tubes are the most impactful factors affecting the quality of PRF preparation [10].

These studies have inspired our group to develop a nondestructive visualization method to compare platelet distribution among individual PRF derivatives. In previous studies [11–13], we observed similar findings in that silica-coated plastic tubes and low-speed centrifugation distributed platelets widely and homogeneously in PRF matrices [12,13]. To further expand on these findings and better understand blood cell layer separation following centrifugation, in this study, we attempted to visualize and evaluate the differences in the distribution of activated platelets using commonly utilized PRF preparation protocols.

Materials and Methods

Preparation of PRF Matrices

Blood samples were collected from twelve healthy, nonsmoking male and female volunteers aged 20 to 71 years and used for PRF

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preparation. Depending on the purpose of each experiment, the minimum essential number of donors was randomly selected for sample collection. The study design and consent forms for all procedures performed (project identification code: 2019–0423) were approved by the Ethics Committee for Human Participants of Niigata University (Niigata, Japan) in accordance with the Helsinki Declaration of 1964 (as revised in 2013).

As described previously [11,12], approximately 9 mL of blood was collected from each volunteer in plain plastic vacuum blood collection tubes (Neotube #NP-PN0909; Nipro, Osaka, Japan). The sample was transferred to the same type of plain glass tube (Nichiden-Rika Glass Co Ltd., Kobe, Japan) and immediately centrifuged at ambient temperature (24–27°C) under the recommended centrifugal conditions using a horizontal centrifuge (Eppendorf 5702, Hamburg, Germany; previously referred to as "Bio-PRF" [11]) and three fixed-angle centrifuges [Medifuge (Silfradent S. r. l., Santa Sofia, Italy), Duo Quattro (Process for PRF, Nice, France), and EBA200 (Hettich, Vlotho, Germany)]. These centrifugation conditions were summarized in Table I. After centrifugation (Figure 1a), the upper PRF matrices were washed and fixed in 10% neutralized formalin (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) for 3–5 h in plastic tubes to maintain the shape of the original PRF matrix.

To evaluate the basic characteristics of individual blood samples, the number of platelets and other blood cells in the whole-blood samples and PRP preparations was determined using an automated hematology analyzer (pocH 100iV; Sysmex, Kobe, Japan).

Near-infrared (NIR) Imaging of Platelet Distribution in PRF Matrices

The fixed PRF matrices were sagittally divided into two equal pieces (Figure 1b), one of which was used for the detection of $CD41^+$ or $CD62P^+$ platelets while the other was used as an isotype control [11,12]. After blocking with 2% Block Ace (Sumitomo Dainippon Pharma, Osaka, Japan) in 0.1 T-PBS, the PRF pieces were probed with mouse monoclonal antibodies against human CD41 or CD62P (BioLegend, San Diego, CA, USA) at a 3:1000 dilution for 24 h at 4°C. The PRF isotype controls were treated with non-immunized mouse IgG (BioLegend). The PRF pieces were subsequently probed with NIR dye-conjugated goat anti-mouse IgG (1:4500) (iFluor 790; AAT Bioquest, Inc., Sunnyvale, CA, USA) for 90 min at 4°C. After washing, dehydration, and compression, $CD41^+$ or $CD62P^+$ platelets were visualized at 800 nm using a Pearl NIR imaging system (Li-Cor, Lincoln, NE, USA).

The total florescence intensity of each half of the PRF membrane was measured using software provided by Li-Cor. The specific fluorescence intensity of the CD41 or CD62P antibody-reactive proteins was expressed as the ratio of the fluorescence intensity of CD41 or CD62P to that of the corresponding isotype control. To highlight the differences in fluorescence intensity, platelet distribution is shown in pseudo-color using the WinROOF 2018 image analysis software (Mitani Corp., Fukui, Japan). Neither gamma correction nor any other retouching was performed to enhance the contrast of the images.

Table I. Centrifugation conditions for each PRF preparation.

	H-PRF	A-PRF	L-PRF	CGF
Centrifuge	Eppendorf #5702	Duo Quattro	Hettich EBA200*	Medifuge
RPM	2,200	1,300	2,700	• 2,700 • 2,400 • 2,700 • 3,000
RCF max	700 g	200 g	700 g	• 685 g • 540 g • 685 g • 845 g
Time (min)	8	14***	12	• 2 • 4 • 4 • 3
R max (mm)	130.0	109.8	85.1	84.0**

*This is the original model of the IntraSpin centrifuge.
**The radii were obtained by manual measurement, while other radii were derived from the design drawings [10] or from information from the manufacturer.
***The duration was extended to reproduce clot formation.

Statistical Analysis

The data are expressed as the mean ± standard deviation. For the fluorescence intensity data in Figure 2, the data from image analysis was evaluated using SigmaPlot (SigmaPlot 13.0; Systat Software, Inc., San Jose, CA, USA) as being parametric by both normality (Shapiro-Wilk) and equal variance testing. One-way analysis of variance was performed followed by Bonferroni's multiple-comparisons test. $P < .05$ was considered statistically significant.

Results

Figure 2 shows the pseudo-colored images of the distribution of $CD41^+$ and $CD62P^+$ platelet in H-PRF, which was prepared using a horizontal centrifuge [7] and currently designated horizontal-PRF (H-PRF) by Miron and other parties [7,14], advanced (A)-PRF, leukocyte-rich (L)-PRF, and concentrated growth factor (CGF) matrices. Although several minor individual variations were observed, the images shown in this figure are representative of other images in terms of platelet distribution. To avoid unnecessary misinterpretation of data, it should be noted that the fluorescence intensity per platelet is generally expressed by a Gaussian distribution and is not maintained at constant levels [15].

In the H-PRF matrix (Figure 2a), $CD41^+$ platelets were widely distributed especially at the central region and the marginal regions adjacent to the glass surface, while $CD62^+$ activated platelets were localized mainly at the interfacial region adjacent to the red blood cell fraction. In the A-PRF matrix (Figure 2b), $CD41^+$ platelets were homogeneously distributed and $CD62P^+$ platelets were similarly distributed. In both L-PRF and CGF matrices (Figure 2c & d), $CD41^+$ platelets were localized in the distal regions of the matrix and $CD62P^+$ platelets were similarly distributed.

Figure 3 shows the quantitative comparisons of the total fluorescence intensity between $CD62P^+$ and $CD41^+$ platelets and the ratios between the intensities. The fluorescence intensity of $CD62^+$ platelets in the A-PRF matrix was higher than that in the other PRF groups. However, because $CD41^+$ platelets were similarly observed at higher levels in the A-PRF matrix, the $CD62P^+/CD41^+$ ratio calculated was similar in all PRF groups.

Discussion

In general, well-known preparation protocols of PRF from major brands require clinicians to use specified centrifuges and blood

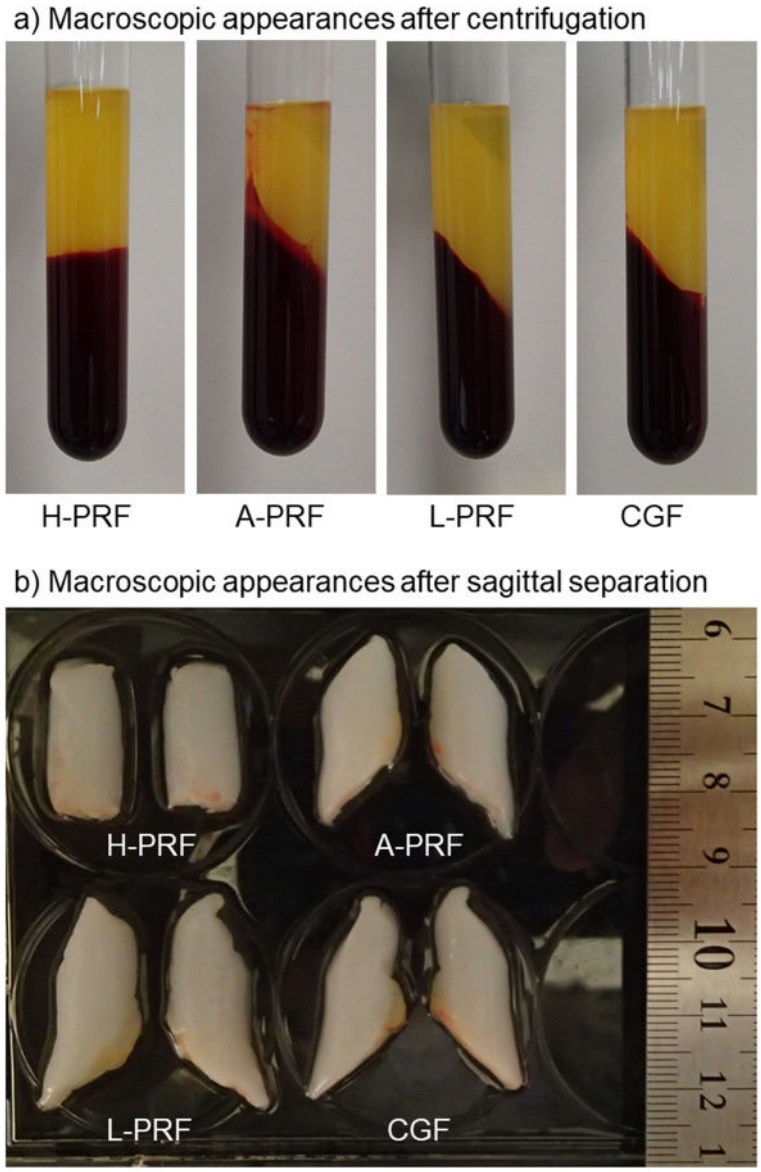

Figure 1. Macroscopic appearance of four types of platelet-rich fibrin (PRF) matrices immediately after (a) centrifugation and (b) sagittal separation.

collection tubes. "PRF-like matrices" prepared by non-specified devices are not approved as genuine "PRF matrices" even though specific centrifugal conditions are strictly followed. To rigorously compare the centrifugal conditions, the same types of glass tube were used for PRF preparation throughout this study.

As demonstrated in previous reports [11,13] and in the present study, in the case of centrifuges equipped with fixed-angle rotors, whole $CD41^+$ platelets were localized at the distal marginal region or widely and homogeneously distributed depending on the centrifugal speed. In addition, there was no substantial difference between L-PRF and CGF, indicating that centrifuge type (e.g., rotor angulation) may not be a major factor influencing platelet distribution at higher RCF protocols.

However, the horizontal centrifuge appeared to be distinguishable from that of fixed-angle centrifuges. As for the distribution of $CD62P^+$ platelets, our findings do not necessarily indicate that most of platelets localized in the distal regions are activated. It cannot be ruled out that this distribution may solely reflect the high density of platelets

Unlike other types of PRF matrices, $CD62P^+$ activated platelets are not necessarily colocalized with whole $CD41^+$ platelets in PRF matrices prepared by the horizontal centrifuge. This is probably due to the different mechanisms of platelet activation and coagulation. As described previously [12], in the case of high-speed centrifugation using fixed-angle centrifugation, it is proposed that both platelets and coagulation factor XII are activated by gravity-dependent contact or collision with the distal glass surface to synergistically facilitate coagulation. Thus, platelets are activated mainly by the glass surface and are embedded in the fibrin network that is initially formed at the distal tube surface. By contrast, in the case of slow-speed centrifugation using a fixed-angle centrifuge, it is suggested that coagulation starts widely and precedes the gravity-dependent migration (i.e., fractionation) of platelets. Thus, platelets may be activated mainly by the newly formed fibrin network, but to a lesser degree by the glass surface [11]. On the other hand, in the case of high-speed centrifugation using the horizontal centrifuge, collision with the glass surface is avoided and platelet activation and coagulation may take place simultaneously. Platelets in the region adjacent to the interface with the red blood cell fraction may be activated at relatively higher levels by the mechanical repellence of the red blood cell fraction. These hypotheses need to be further examined and are in the process of verification.

Quantitative analysis revealed that the ratios of activated to whole platelets are not significantly different among the PRF derivatives. In addition, the images produced by the NIR imaging

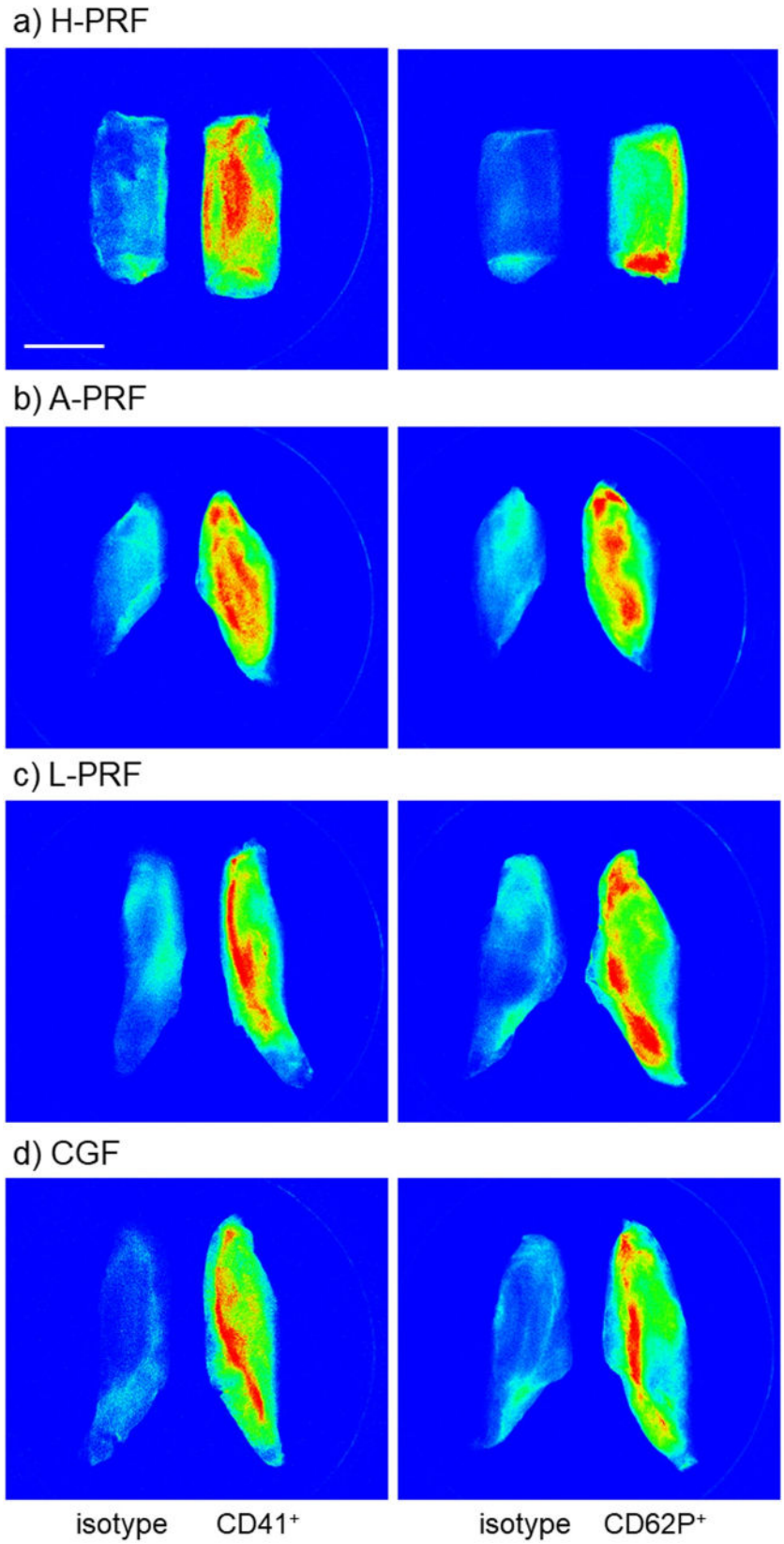

Figure 2. Pseudo-colored images of the distribution of $CD41^+$ (*left*) and $CD62P^+$ (*right*) platelets in (a) H-PRF, (b) advanced (A)-PRF, (c) leukocyte-rich (L)-PRF, and (d) concentrated growth factor (CGF) matrices. The colors represent the levels of fluorescence intensity (red: high, blue: low). Scale bar = 10 mm.

technology provide relative, but not absolute, evaluation of platelet activation levels. Thus, it is not conclusive that platelets distributed in the upper regions of H-PRF matrices are not activated. Our observations simply suggested that these platelets are "relatively less" activated than those accumulated at the interfacial region.

Finally, technical limitations are noted for better data interpretation. Undoubtedly, double-staining provides clearer evidence of activated platelets. However, in this preliminary study, we observed that all of the polyclonal antibodies that were tested showed non-specific detection at non-negligible levels. In addition, the other NIR imaging channel at a wavelength of 700 nm reduced the fluorescence intensity through the PRF matrix by approximately 50% (c.f., 20% reduction at 800 nm). Because of these technical limitations, we chose the method of single-channel visualization.

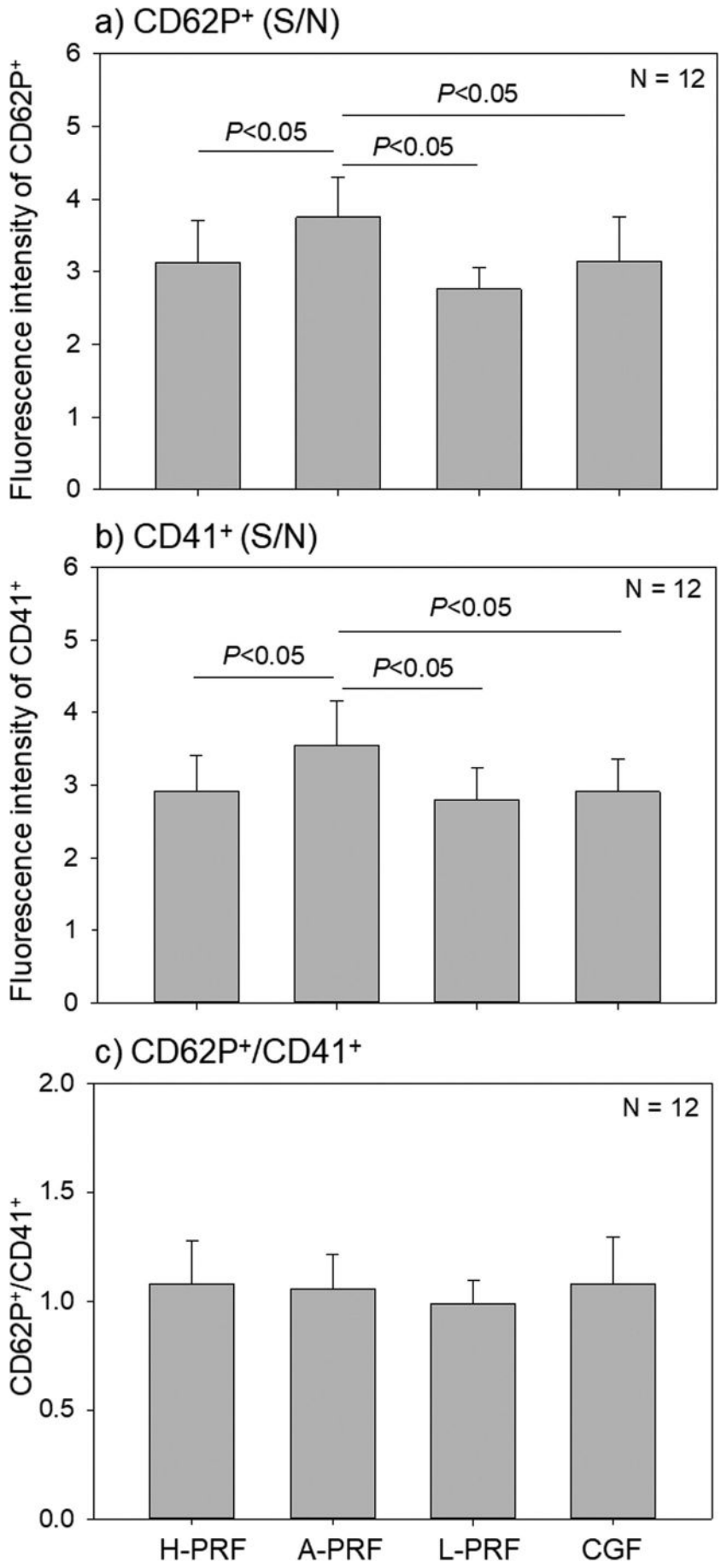

Figure 3. Quantitative comparisons of (a) total fluorescence intensity of $CD62P^+$ platelets, (b) total fluorescence intensity of $CD41^+$ platelets, and (c) the ratio of fluorescence intensities of $CD62P^+$ to $CD41^+$. "S/N" represents signal/noise ratio. N = 12.

Clinical Relevance

In PRP therapy, PRP is often activated by coagulation factors to form fibrin clots and release growth factors prior to implantation. According to this concept, the levels of activated platelets and growth factors released and retained in the PRF matrix may not be a critical issue that significantly influences the clinical outcome. However, the distribution pattern of activated platelets may be related to the controlled release of growth factors from the PRF matrix and is potentially relevant to clinical outcomes. Further study is needed to investigate the possible relevance.

Conclusions

The NIR imaging technology revealed that the distribution of activated platelets in the H-PRF, matrix prepared by the horizontal centrifuge is distinguishable from that in the other PRF groups prepared using fixed-angle centrifuges. No significant difference was observed in the relative fluorescence intensities of activated platelets (i.e., occupancy of activated platelets) among the four types of PRF derivatives.

Author Contributions

Conceptualization, A.S., H.A., and T.K.; methodology, T.K.; validation, T. T. and Y.K.; formal analysis, R.M.; investigation, A.S., H.A., H.K., T.T., T. W., K.I., Y.K., and T.K.; data curation, T.T. and K.I.; writing—original draft preparation, A.S., T.T., and T.K.; writing—review and editing, R.M. and T.K.; supervision, Y.K.; project administration, T.W.; funding acquisition, T.K. All authors have read and agreed to the published version of the manuscript.

Declaration Of Interests

The authors declare that they have no competing interests.

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