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Original article

Morphological and biological properties of platelet-rich fibrin membrane produced from different centrifugation protocols

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Abstract

Background: Platelet-rich fibrin (PRF) membrane is a promising tool for the treatment of corneal epithelial disease; however, a standardized production protocol remains to be established.
Objective: This study aimed to evaluate the morphological and biological properties of PRF membrane produced using different centrifugation protocols.
Methods: Four distinct low-speed centrifugation protocols (100 × g/5 min, 200 × g/5 min, 100 × g/10 min, and 200 × g/10 min) were established for PRF membrane production from blood obtained from three healthy volunteers. We assessed the fibrin and membrane dimensions, white blood cell and platelet concentrations, kinetic release of growth factors (TGF-b, PDGF-BB, VEGF, and IGF-1), and inflammatory cytokines (IL-6, IL-1, and TNF-a). Furthermore, in vitro degradation and bacterial contamination were examined.
Results: High platelet concentrations were consistently achieved in the PRF membranes produced by the four centrifugation protocols; however, there were no significant differences between these groups. Moreover, distinct release patterns were observed for each growth factor and cytokine. The PRF fibrin matrix effectively released growth factors over a sustained period of 3–10 days. Notably, no bacterial contamination was detected in any of the PRF membranes produced by the protocols.
Conclusion: Our findings definitively demonstrate that a low-speed centrifugation protocol can be employed to produce PRF membrane with high platelet concentrations that release growth factors over an extended period, thereby offering substantial therapeutic potential for ocular surface diseases.

Keywords: Ocular surface diseases, platelet fibrin membrane, platelet-rich fibrin membrane.

Over the past decade, there has been a substantial advance in biological products for ophthalmology, primarily due to their beneficial properties of lubrication, growth factor delivery, and anti-inflammatory effects. Autologous serum, which was first described in 1975, is of autologous origin and contains various growth factors, minerals, and cytokines that are essential for maintaining ocular surface health and improving homeostasis.[1] Thereafter, platelet-rich plasma was developed, offering greater benefit in that it promoted wound healing and had anti-inflammatory properties.[2] Most recently, platelet-rich fibrin (PRF) membrane, a second-generation platelet concentrate, has emerged as a promising tool in regenerative medicine.[3]

The fibrin matrix is the main advantage of PRF. It can serve as a scaffold for epithelial cell proliferation and migration, thus effectively retaining epitheliotropic growth factors within a three-dimensional fibrin network.[4] PRF membrane has been applied in various medical fields, including dentistry, orthopedics, trauma, and surgery.[3]

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In ophthalmology, previous studies have reported the efficacy of PRF membrane in supporting corneal wound healing.[5] However, a standardized production protocol for ophthalmic PRF membrane has not been established.[6] Over the past decade, various centrifugation protocols, differing in speed and duration, have been proposed.[7] Notably, high centrifugation velocity can compromise the cellular integrity of the fibrin matrix, while lower centrifugation speeds yield a PRF membrane with a higher concentration of platelets and growth factors.[8]

The objective of this study was to evaluate the morphological and biological properties, including platelet and white blood cell (WBC) content, kinetic release of growth factors and cytokines, *in vitro* degradation, and bacterial contamination, of PRF membranes produced from four different low-speed centrifugation protocols.

Materials and methods

This study was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (COI no. 0877/2023). Human blood samples and tissues were handled according to the principles of the Declaration of Helsinki. Three healthy volunteers, aged 18–80 years old with a body weight of more than 50 kg, received detailed information regarding the study's objectives, processes, benefits, and risks, and then provided written informed consent.

After enrollment, a complete blood count was performed for each volunteer. Volunteers with hemoglobin levels of less than 11 mg% or platelet concentrations of less than 150,000 cells/mm^3 were excluded.

Preparation of PRF membrane

Blood samples were collected from each volunteer via venipuncture using a sterile technique. The blood samples were immediately transferred into 8 mL glass-coated plastic tubes without anticoagulant (Xinle Medical, Hebei, China) for PRF membrane preparation. Thereafter, 6 mL of blood was aliquoted equally into each of the 12 tubes, and 1 mL of blood was transferred into a 3 mL PET tube with EDTA (Xinle Medical) for platelet and WBC count analysis using a hematologic analyzer (Sysmex xn330, Sysmex, Singapore).

Blood samples in glass-coated plastic tubes without anticoagulant were centrifuged according to the following protocols: 1) maximum relative centrifugal force (RCF) 100 × g (1,029 revolutions per minute (rpm)) for 5 min, 2) maximum RCF 200 × g (1,300 rpm) for 5 min, 3) maximum RCF 100 × g (1,029 rpm) for 10 min, and 4) maximum RCF 200 × g (1,300 rpm) for 10 min (Spectrafuge 6C, Labnet, USA). Three tubes of blood samples from each volunteer were prepared in each protocol.

After centrifugation, the solid platelet-rich fibrin clot that formed in the middle part of the tube was carefully removed using sterile tweezers, and then sterile scissors were used to remove the remnants of red blood cells in the bottom section. To obtain the PRF membrane, the platelet-rich fibrin clot was placed between two sterile microscopic slides with a 1 mm rubber stopper at each corner. A compression force was applied over the slide to reshape the platelet-rich fibrin clot into a PRF membrane. All preparation steps were performed at 25°C.

Morphological characteristics

The maximum length and width of the PRF clots and membranes were measured immediately after centrifugation and clot compression, respectively. Photographs of the PRF clots and membranes were used to analyze the areas of clots and membranes using ImageJ software (NIH, Wisconsin, US).

The concentration of platelets and WBCs in the PRF membrane was evaluated using the indirect subtraction method with the following equation:

Concentration in membrane = Concentration in whole blood – (Concentration in remnant + Concentration in exudate)

Histological analysis was performed to evaluate the distribution of platelets, fibrin fibers, and WBCs across the long axis of each PRF membrane obtained from each protocol. In brief, the PRF membranes were fixed in 10% neutrally buffered formalin for 24 h at room temperature. These were then embedded in paraffin and cut along the center of the PRF membrane long axis. The samples were stained with hematoxylin & eosin (H&E) (CV Laboratories, Bangkok, Thailand) and evaluated under a light microscope (CX23, OLYMPUS, Tokyo, Japan).

The *in vitro* degradation of the PRF membranes obtained from each centrifugation protocol was observed. After PRF membrane preparation, the membranes were weighed to obtain the initial weight and photographed for the initial dimension assessment. Then, the membranes were incubated in a humidified incubator for up to 10 days at 37°C. The membranes were weighed and photographed every day until they were completely degraded. All photographs were processed using ImageJ software.

Biological characteristics

The release kinetics of several growth factors and cytokines related to corneal epithelial regeneration from the PRF membranes obtained from each centrifugation protocol were evaluated. The growth factors and cytokines, including insulin-like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF-BB), vascular endothelial growth factor (VEGF), interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), were determined using enzyme-linked immunosorbent assay (ELISA) evaluations (Bio-Plex Pro™ Human Cytokine Panel, Bio-Rad, USA). Briefly, the PRF membrane was incubated in 4 mL of Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Grand Island, NY, USA) for 2 h. Then, the PRF membrane was transferred to a new sterile tube and stored at 37°C, and the obtained medium was stored at −80°C until use. Finally, the medium collected at Days 1, 3, 7, and 10 after PRF membrane preparation was evaluated for the growth factors and cytokine concentrations according to the manufacturer's instructions. Three PRF membranes obtained from each protocol were used, and the experiments were performed twice, four weeks apart.

PRF membrane contamination

The PRF membranes were incubated on blood agar, chocolate agar, and brucellar agar under anaerobic or aerobic conditions, according to the agar type, using an automated culture system (VITEK MS, BIOMERIEUX, Netherlands). Organism growth in the culture system was evaluated daily until Day 14 of incubation.

Statistical analysis

A two-way analysis of variance was used to analyze the differences between the morphological and biological characteristics of the PRF and PRF membranes among the protocols and different observation times. The interclass correlation coefficient was used to evaluate the test and retest reliability between the two ELISA experiments. Differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 10.4.1 for Windows (GraphPad Software, Boston, Massachusetts, USA).

Results

The three healthy participants consisted of two females and one male with ages of 35, 49, and 62 years old and weights of 54, 76, and 60 kg, respectively. Their hemoglobin concentrations were 12.5, 14.2, and 13.00 mg%, respectively. The platelet concentrations were 414,000, 301,000, and 313,000 cells/mm^3, and WBC concentrations were 6,500, 6,000, and 4,300 cells/mm^3, respectively.

Morphological properties of PRF membranes

All of the centrifugation protocols produced PRF in the middle layers of the blood tubes. The PRF was then separated from the other layers and compressed to produce a PRM membrane **(Figure 1A-B)**. Among the four protocols, there were statistically significant differences in the mean fibrin length, width, and weight ($P < 0.01$, 0.03, and 0.02, respectively), while the PRF area revealed no difference ($P = 0.10$). The 100 × g/10 min protocol exhibited the highest mean PRF fibrin length, width, area, and weight among the four protocols **(Figure 1C).**

Among the four protocols, the PRF membrane showed significant differences in width and weight ($P = 0.03$ and 0.01, respectively), while no significant differences in the PRF membrane length and area were demonstrated ($P = 0.07$ and 0.14, respectively) (**Figure 1D**). All PRF membrane weights were approximately 80.0% - 85.0% of the original PRF weight. Among the four protocols, the 100 × g/10 min protocol revealed the highest PRF membrane mean membrane length, width, area, and weight.

The platelet and WBC concentrations in the PRF from each protocol are presented in **Figure 1E**. There was no significant difference in the platelet and WBC concentrations in the PRF among the protocols ($P =$ 0.49 and 0.89, respectively). PRF derived from the 100 × g/5 min, 200 × g/5 min, 100 × g/10 min, and 200 × g/10 min protocols contained 82.0%, 80.0%, 67.0%, and 66.0% platelet concentrations of the whole blood sample.

Furthermore, histological analysis of the PRF membrane revealed the presence of three zones within the membrane. The proximal zone was mainly composed of platelets and a fibrin network, while the central zone was primarily comprised of WBCs. In addition, the distal zone was mainly composed of red blood cells. Furthermore, the fibrin network was observed in all areas where platelets were present **(Figure 2A-C).**

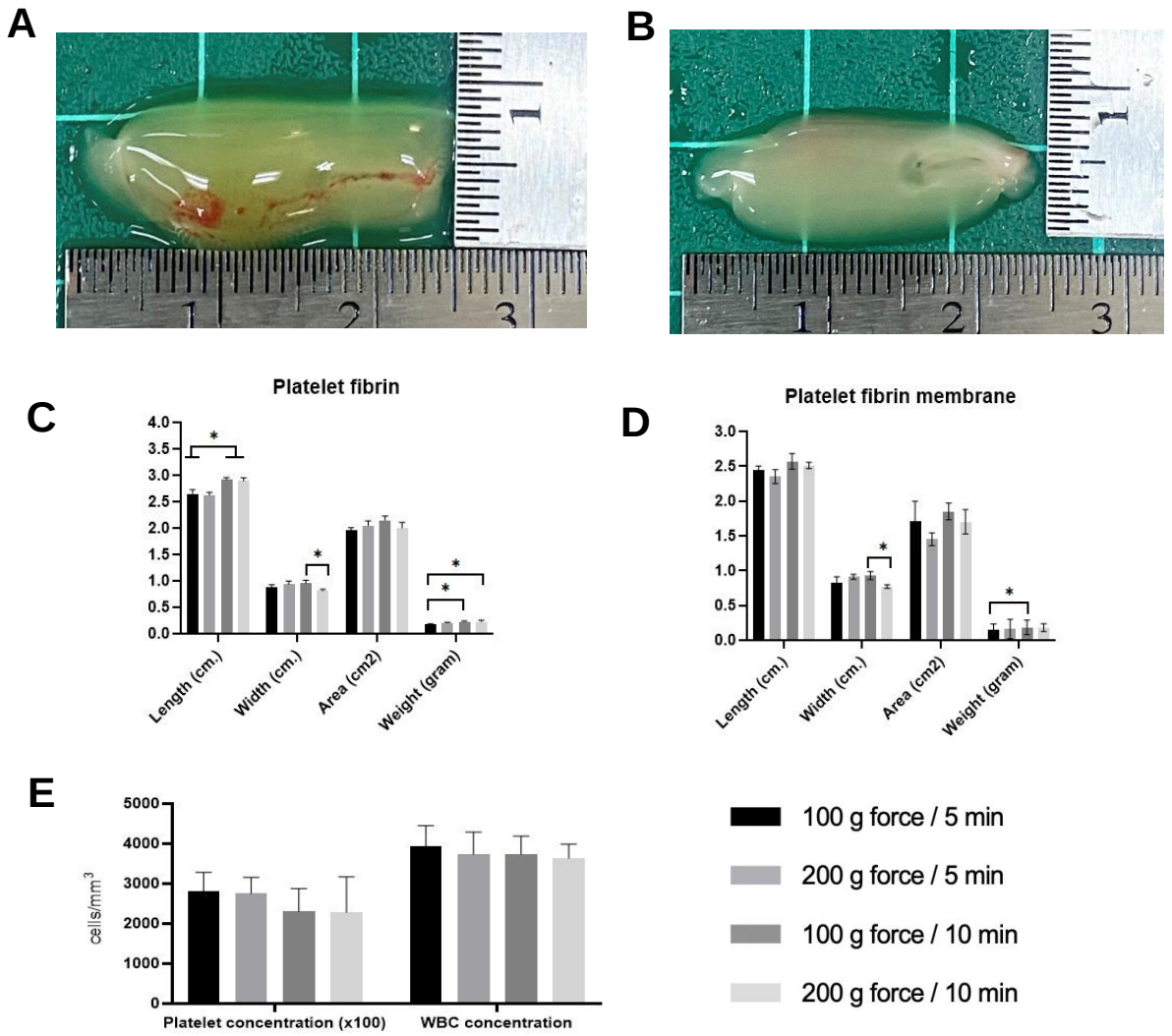

Figure 1. Morphological characteristics of PRF membrane. Example images of PRF **(A)**; and PRF membrane **(B)**; The bar graph shows the length, width, area and weight of PRF **(C)**; and PRF membrane; **(D)** of four centrifugation protocols; **(E)** The bar graph shows platelet and white blood cell concentration in PRF producing from four centrifugation protocols. (n = 3 membrane per group; *$P < 0.05$).

The *in vitro* degradation of PRF membrane was assessed. All of the PRF membranes preserved their original shape, color, and odor up to day 8 after production. However, on day 9, the PRF membrane started to change in color and emitted a fetid smell. Finally, on day 10, the membrane dried and dissolved **(Figure 2D).** Changes in the PRF membrane weights and areas are presented in **Figure 2E** and **2F**. The changes in PRF membrane weight were significantly different among the four centrifugation protocols ($P = 0.03$). The PRF membrane produced using the 100 × g/5 min protocol exhibited a significant decrease in weight compared with the PRF membranes produced using the 200 × g/5 min (adjusted $P = 0.047$) and 100 × g/10 min (adjusted $P = 0.03$) protocols at day 2. In addition, the PRF membrane produced with 100 × g/5 min demonstrated a significant decrease in weight compared with the PRF membrane produced using 200 × g/10 min at day 5 after production (adjusted $P = 0.01$) **(Figure 2E).** There was no difference in the PRF membrane areas among the four centrifugation protocols during the observation periods ($P = 0.1$). Moreover, bacterial contamination was not detected in the PRF membranes produced by any of the protocols.

Biological properties of PRF membranes

The release kinetics of IGF-1, TGF-β, PDGF-BB, VEGF, IL-1, IL-6, and TNF-α were measured twice in separate experiments. The release of the growth factors and cytokines exhibited excellent test-retest reliability (all intraclass correlations > 0.8). A combination of results from both experiments was presented.

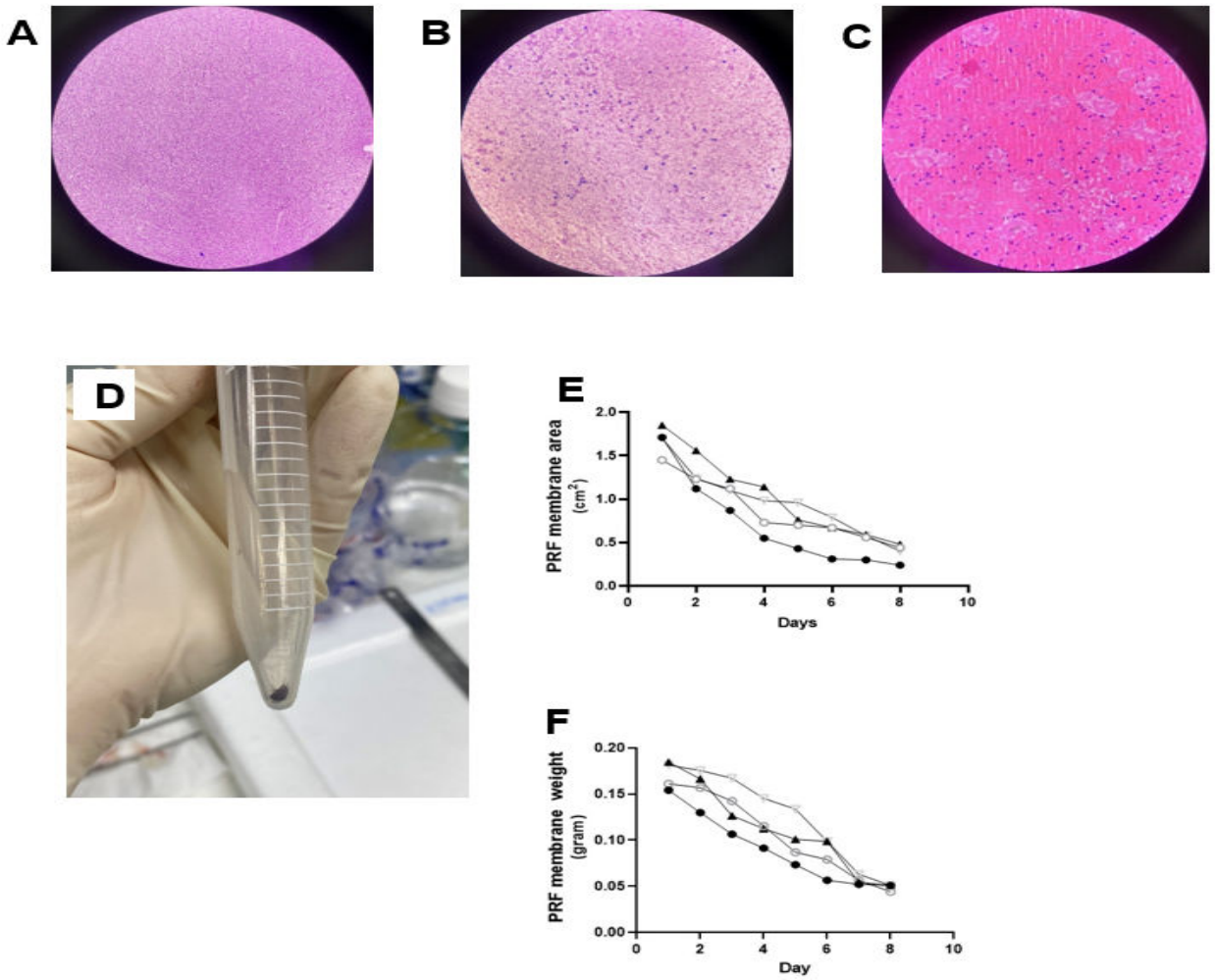

Figure 2. H&E staining of PRF membrane and *in vitro* degradation of PRF membrane. H&E staining showed differences in composition of the section derived from **(A)** proximal zone; **(B)** central zone; and **(C)** distal zone of PRF; **(D)** *In vitro* observation of PRF in humidified incubator at 37°C demonstrated PRF was dried and dissolved on day 10 after production. Graphs showed the changes in PRF membrane; **(E)** area; and **(F)** weight during in vitro degradation observation. (n = 3 membrane per group).

The release of IGF-1, TGF-β, VEGF, IL-1, IL-6, and TNF-α showed significant changes over time ($P \leq 0.01$, < 0.01, 0.03, < 0.01, < 0.01, and < 0.01, respectively), while the release of PDGF-BB did not demonstrate a significant change ($P = 0.10$).

The highest release of IGF-1, TGF-β, and TNF-α occurred on day 1. Thereafter, IGF-1 decreased rapidly, while TGF-β and TNF-α decreased slowly after PRF membrane production in all centrifugation protocols. In contrast, the release of VEGF, IL-1, and IL-6 gradually increased in all protocols. The release of growth factors and cytokines at days 1, 3, 7, and 10, and the cumulative releases of growth factors and cytokines, showed no significant differences among the four protocols **(Figures 3** and **4).**

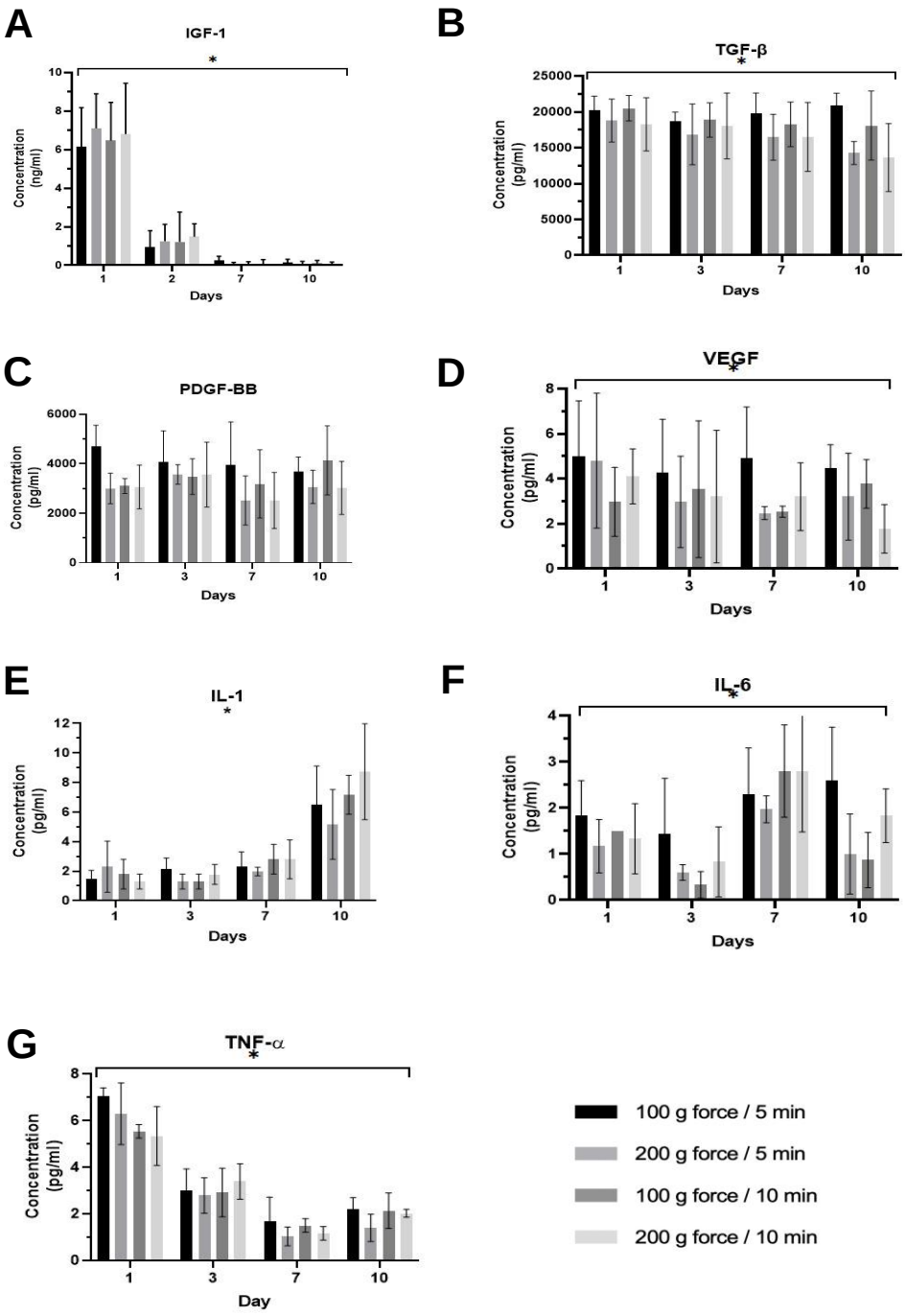

Figure 3. The releases of (A) IGF-1, (B) TGF-β, (C) PDGF-BB, (D) VEGF, (E) IL-1, (F) IL-6 and (G) TNF-α from PRF membrane producing by four centrifugation protocols at day 1, 3, 7 and 10 after production determined by ELISA assays. The releases of IGF-1, TGF-β, VEGF, IL-1, IL-6 and TNF-α showed significant changes during the observation periods. (n = 6 membrane per group; **P*< 0.05). IGF-1, insulin-like growth factor-1; IL-1, interleukin-1; IL-6, interleukin-6; PDGF-BB, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α;VEGF, vascular endothelial growth factor.

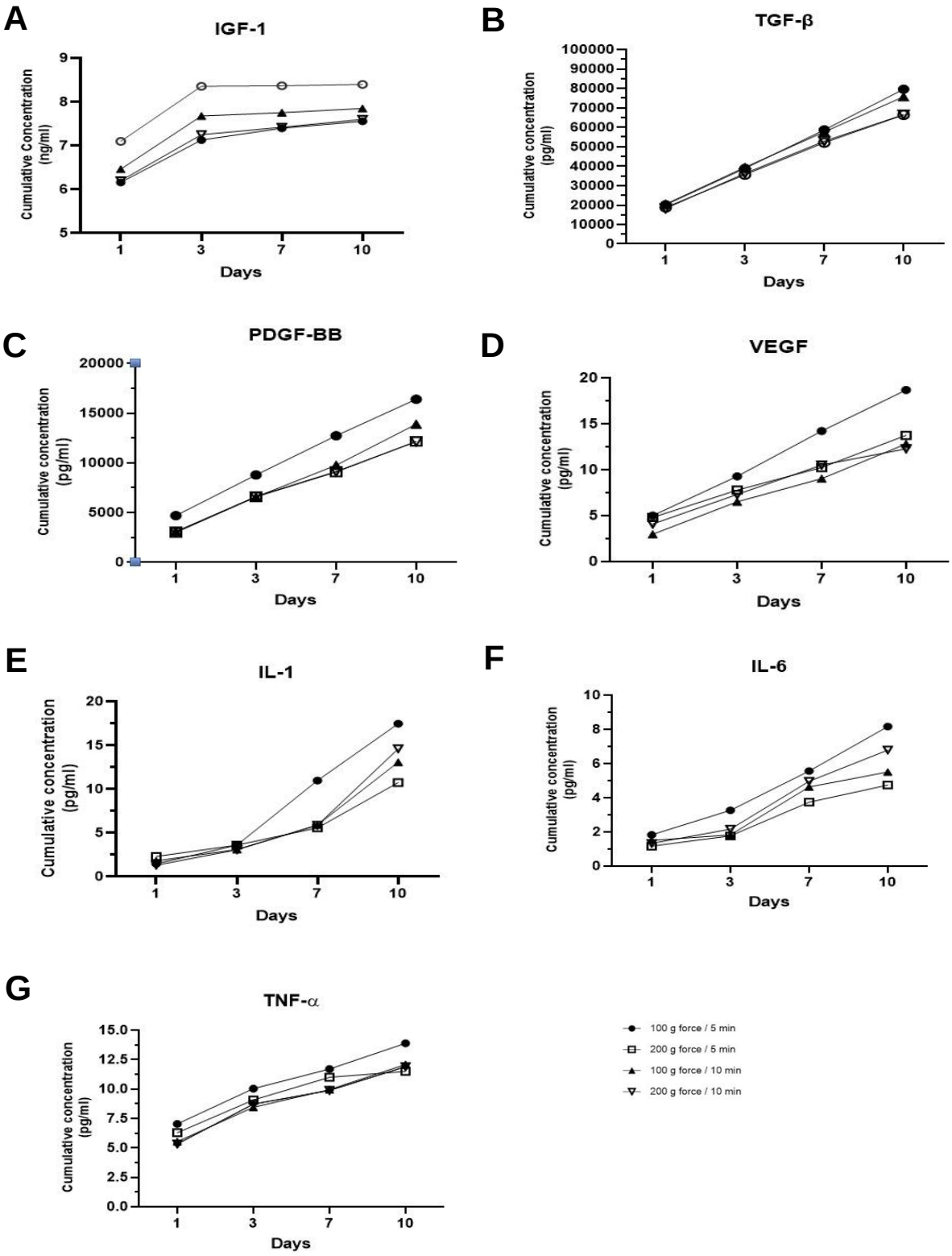

Figure 4. The cumulative releases of (A) IGF-1, (B) TGF-β, (C) PDGF-BB, (D) VEGF, (E) IL-1, IL-6 and (G) TNF-α from platelet fibrin membrane producing by four centrifugation protocols at day 1, 3, 7 and 10 after production determined by ELISA assays. There were no significant differences in the cumulative releases of IGF-1, TGF- β, PDGF-BB, VEGF, IL-1, IL-6 and TNF-α from all centrifugation protocols in all observation times (all $P > 0.05$). IGF-1, insulin-like growth factor-1; IL-1, interleukin-1; IL-6,interleukin-6; PDGF-BB, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

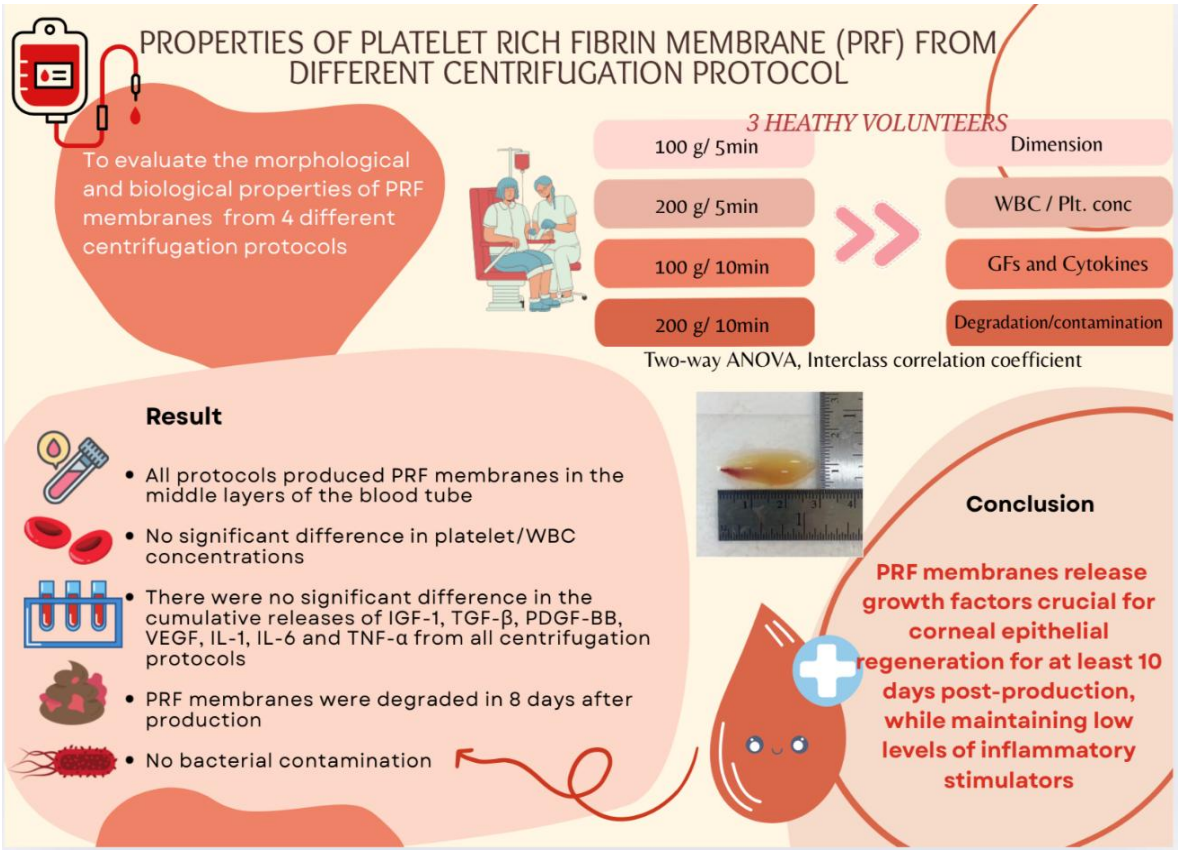

Figure 5. Graphical abstract illustrates the main concept and result of the study.

Discussion

Our study showed that four slow centrifugation protocols consistently produced PRF membranes with a high platelet concentration and sustained integrity over 10 days at 37°C. Analysis of bioactive molecule release revealed distinct kinetics, where PDGF-BB showed sustained release over 10 days, unlike that of the other growth factors and anti-inflammatory cytokines, which peaked initially and then gradually decreased over time. Notably, the levels of IL-1, IL-6, and VEGF increased over time, while the release of TNF-α, another inflammatory cytokine, decreased over time.

We found that increasing the centrifugation time did not significantly alter the fibrin membrane size. However, all protocols with slow centrifugation speeds and varying durations consistently yielded membranes with high platelet concentrations (**Figure 5**). This optimization is crucial, as the therapeutic efficacy of the PRF membrane is directly correlated with the abundant release of growth factors from activated platelets. The reduction in platelet concentration observed with increased centrifugation speed and duration is likely due to either the displacement of blood cells into the discarded clot or direct platelet damage from excessive speed and duration.[9, 10] The resulting PRF membranes, measuring 1.5–1.9 cm², provide sufficient coverage for typical corneal epithelial defects. The ability to produce these membranes from a blood sample of only 6 mL enhances patient comfort and simplifies its preparation. For smaller defects, the selective use of the proximal, platelet-rich membrane portion allows for the targeted delivery of concentrated growth factors.

Our study revealed an initial release of growth factors and cytokines from PRF membranes produced by all the tested protocols. Notably, PDGF-BB maintained a high, sustained release for the entire 10-day period, likely because of the high platelet concentration within the fibrin matrix. While inflammatory cytokines (e.g., IL-1 and IL-6) were present, their release was initially low but gradually increased over time. This dynamic profile indicates that the PRF membrane provides a potent

regenerative stimulus, with peak benefits likely in the initial days, followed by a subsequent decline. However, the presence of inflammatory cytokines suggests that the judicious co-administration of corticosteroids could help balance the inflammation, potentially leading to enhanced healing outcomes. Furthermore, the reapplication of a PRF membrane after approximately 10 days may be beneficial if initial healing is suboptimal.

A direct quantitative comparison of growth factor releases between our study and previous standard and advanced PRF (A-PRF) investigations is challenging because of the inherent methodological variability in blood collection, preparation, and especially measurement timing and conditions (e.g., use of frozen membranes).[11, 12] Despite these variables, the low centrifugation technique demonstrated compelling advantages. The total 10-day cumulative release of TGF-β, PDGF-BB, and IGF-1 from our PRF membranes was notably higher than that reported by Kobayashi, *et al.* for standard PRF and A-PRF.[12] Our technique also yielded a greater overall content of these crucial growth factors compared to that of platelet-rich plasma.[13, 14]

Amniotic membrane (AM) grafts are a recognized treatment for corneal epithelial defects. However, our findings suggest that the PRF membrane offers distinct advantages in bioactive molecule release compared to that of AM. Specifically, our PRF membrane showed a substantially higher 10-day cumulative release of TGF-β than that of standard AM.[15] In addition, PRF membrane demonstrated a greater initial release of PDGF-BB on day 1 compared to that of processed AM.[16] Importantly, all of our PRF membrane protocols released a significantly lower amount of the pro-inflammatory cytokine IL-6 compared to that of AM.[17] These combined characteristics of elevated levels of pro-healing growth factors (TGF-β and PDGF-BB) alongside the reduced release of pro-inflammatory cytokines (IL-6) strongly indicate PRF membrane possesses a superior biological profile. Compared to that of AM, PRF membrane preparation is simpler; however, AM exhibits a slower degradation rate than that of the PRF membrane.[18]

The limitation of this study was the small sample size. In addition, this study cannot draw conclusions regarding the effect of age and sex on the morphological and biological properties of the PRF membrane.

Conclusion

In conclusion, our study demonstrates that the low centrifugation technique is highly effective for preparing PRF membrane with significant therapeutic potential for ocular surface diseases. All evaluated protocols yielded high platelet concentrations from just 6 mL of blood**,** which represents a considerable clinical advantage. Moreover, these PRF membranes released epitheliotropic growth factors that are crucial for corneal epithelial regeneration for at least 10 days post-production, while maintaining low levels of inflammatory stimulators. While our findings offer robust *in vitro* evidence, future *in vivo* studies are essential to validate these promising results.

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Conflict of interest statement

The authors have no potential conflicts of interest to disclose.

Data sharing statement

All data generated or analyzed in the present study are included in the published article. Further details are available for non-commercial purposes from the corresponding author upon reasonable request.

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