Biological effects of stored platelet-rich plasma eye-drops in corneal wound healing

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ABSTRACT

Background/aims This study aimed to assess the efficacy and sterility of stored platelet-rich plasma (PRP) eye-drops for corneal epithelial wound healing compared with those of autologous serum (AS) eye-drops.

Methods At our single institution, PRP and AS eye-drops were prepared using peripheral blood obtained from six healthy volunteers and stored at 4°C. Platelet and leucocyte counts and transforming growth factor (TGF)-β1, epidermal growth factor (EGF), and fibronectin levels were assessed during storage for up to 4 weeks. Sterility was assessed by culturing 4-week poststorage samples. PRP, AS, and phosphate-buffered saline (PBS) eye-drop efficacies were compared using corneal epithelial wound healing assays *in vitro* and *in vivo* and monitoring wound areas under a microscope every 3 hours.

Results Higher platelet and lower leucocyte counts were seen in PRP than in whole blood on the day of preparation. After storage, TGF-β1, EGF, and fibronectin levels were significantly higher in PRP than in AS eye-drops. *In vitro* and *in vivo*, PRP eye-drops used on the day of preparation significantly promoted corneal epithelial wound healing compared with PBS. Moreover, PRP eye-drops stored for 4 weeks significantly promoted corneal wound healing compared with PBS and AS eye-drops.

Conclusion PRP eye-drops stored at 4°C for 4 weeks promoted corneal epithelial wound healing with higher levels of growth factors than those observed in AS eye-drops, while maintaining sterility, suggesting that this preparation satisfies the unmet medical needs in the treatment of refractory keratoconjunctival epithelial disorders.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ There is an urgent need to develop effective therapeutic agents for refractory keratoconjunctival epithelial disorders that do not respond to existing eye-drops. Platelet-rich plasma contains growth factors, and platelet-rich plasma eye-drops may be used for managing refractory keratoconjunctival epithelial disorders; however, there is no report on the efficacy and sterility of platelet-rich plasma eye-drops after storage.

WHAT THIS STUDY ADDS

⇒ Platelet-rich plasma eye-drops stored at 4°C for 4 weeks had higher levels of growth factors than autologous serum eye-drops while maintaining a similar degree of sterility. Platelet-rich plasma eye-drops 4 weeks poststorage were superior to autologous serum eye-drops in promoting corneal wound healing in both in vivo and in vitro conditions.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study determined the efficacy and sterility of platelet-rich plasma eye-drops during storage compared with those of autologous serum eye-drops for clinical application in cases of refractory keratoconjunctival epithelial disorders.

INTRODUCTION

Refractory keratoconjunctival epithelial disorders, caused by graft-versus-host disease, corneal transplantation, Sjögren's syndrome, and severe dry eye disease,[1–5] lead to reduced visual acuity, reduced contrast sensitivity, increased susceptibility to infections, corneal perforation, and decreased quality of vision.[6–11] These disorders are treated by the frequent use of artificial tears combined with protective glasses and punctal occlusion.[12] However, many patients do not respond to these treatments, as artificial tears lack several essential therapeutic components, such as transforming growth factor (TGF)-β1, epidermal growth factor (EGF), and fibronectin, which are present in human tears.[12] Autologous serum (AS), first developed in the 1970s to treat ocular alkali burns,[13] contains therapeutic components similar to natural tears, forming the basis of AS eye-drops and showing better efficacy than conventional artificial tears.[14] AS has been used for the treatment of various keratoconjunctival epithelial disorders.[15–17]

Platelet-rich plasma (PRP) harbours high levels of growth factors.[18] PRP is used as a complement to specialised tissue-regeneration procedures, including oral and maxillofacial surgery, orthopaedics and plastic surgery.[19–22] In any eye-drop-intensive treatment regimen, the safety and efficacy of the eye-drops must be maintained during storage. As AS and PRP eye-drops do not

contain preservatives, contamination during storage is of particular concern.[16] A previous report has shown that PRP freezes at −20°C and can be stored for 3 months.[23] Plasma rich in growth factors, which is produced by the activated platelets in PRP with calcium chloride and subsequently removing the platelets, has shown storage stability and sterility by refrigeration and freezing.[24 25] However, there are no studies on the efficacy and sterility of PRP eye-drops after storage at 4°C.

We aimed to determine the effectiveness of low-temperature storage on the efficacy and sterility of PRP eye-drops compared with those of AS eye-drops as, if practical, it may expand the clinical usage of PRP to patients' households. In addition, the therapeutic advantages of PRP eye-drops compared with those of standard treatments may promote their clinical application for cases of refractory keratoconjunctival epithelial disorders.

MATERIALS AND METHODS

Blood collection

Peripheral blood was collected from six healthy volunteers (three males and three females, mean age 27.2±1.7 years) recruited between September 2019 and January 2022 at the Department of Ophthalmology, Juntendo University Hospital, Tokyo, Japan. Before drawing peripheral blood, informed consent was obtained from each donor.

AS preparation

AS was prepared according to a previous report.[26] Briefly, 20 mL of peripheral blood was collected from the median cubital vein and allowed to clot for 2 hours at 26°C in the summer and 24°C in the winter. After centrifugation for 15 min at 3000×g, the supernatant serum (5 mL) was carefully collected aseptically using a 0.22 µm filter (Merck KGaA, Darmstadt, Germany), aliquoted into three bottles (approximately 1.7 mL in each bottle), and refrigerated at 4°C.

PRP preparation

PRP was obtained using a MyCells Autologous PRP Preparation System (Kaylight, Ramat-Hasharon, Israel). Whole blood (22 mL) was aspirated from the median cubital vein, and 5.0 mL of leucocyte-poor PRP was obtained according to the manufacturer's instructions. In brief, a 22 mL of whole blood was aspirated into the two sets of MyCells kit syringes containing 1 mL of anticoagulant dextrose solution A and separation gel, and centrifuged for 7 min at 2054×g. After aspirating the supernatant platelet-poor plasma, the residual 2.5 mL of plasma was pipetted to peel off the platelets from the surface of the separation gel. The filter column was then inserted into the separation syringe to remove the debris and filtered PRP. The PRP was aliquoted into three bottles (approximately 1.7 mL in each bottle) and refrigerated at 4°C.

Haematological analysis

Platelet and leucocyte counts were determined using an automated haematology analyzer (Ac-T diff, Beckman Coulter, Brea, California, USA). Platelet and leucocyte counts in whole blood were measured on the day of preparation (t0), whereas in the PRP preparations, they were measured on (t0), after 1 week of storage (t1), and after 4 weeks of storage (t4) at 4°C.

Microbiological analysis

Sterility was assessed by culturing the AS and PRP samples on (t0), (t1), and (t4) of storage at 4°C. Samples were cultured on 5% sheep blood agar/chocolate agar (Nissui Pharmaceuticals, Tokyo, Japan), Sabouraud agar (Eiken Chemical, Tokyo, Japan), Anaero Columbia agar (Nippon Becton Dickinson, Tokyo, Japan), and HK medium (Kyokuto Pharmaceutical, Tokyo, Japan) at 35°C.[27 28] Culture media were incubated aerobically (on sheep blood agar/chocolate agar, Sabouraud agar, and HK medium) and anaerobically (on Anaero Columbia agar) for 48 hours. Subsequently, the culture media were incubated at 20–22°C for 7 days.

TGF-β1, EGF, and fibronectin-level assessment

The stabilities of TGF-β1, EGF, and fibronectin were assessed by measuring their levels in fresh AS and PRP samples and comparing these with the levels after storage at 4°C for 1 or 4 weeks (n=6 per group). TGF-β1, EGF, and fibronectin levels were determined using commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, Minnesota, USA).[29]

In vitro corneal epithelial wound healing assay

Primary human corneal epithelial cells (HCECs) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Frozen cell vials were thawed and seeded at 5×10^3 cells/cm^2. Cells were cultured in a corneal epithelial cell basal medium, supplemented using a corneal epithelial cell growth kit (ATCC). Cultures were incubated at 37°C under 95% humidity and 5% CO_2. The culture medium was changed every 2 days.

HCECs were seeded at a density of 4×10^5 cells/mL in Essen 96-well ImageLock plates and grown to confluence in a CO_2 humidified incubator for 24 hours. A scratch was then made using the 96-pin WoundMaker (Essen BioScience, Tokyo, Japan) and wells were washed two times with phosphate-buffered saline (PBS) to remove floating cells. Immediately following wounding, media were replaced with PBS, AS (t0), AS (t4), PRP (t0), and PRP (t4). All samples were filtered using a 0.22 µm filter before being transferred to the wells and diluted 10-fold in corneal epithelial cell basal medium. Wound images were taken every 3 hours and the wound area was calculated using ImageJ V.1.53a (National Institutes of Health, Bethesda, Maryland, USA).[30 31]

In vivo corneal wound healing assay

Eight-week-old C57BL/6 (H-2b) male mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Juntendo University Graduate School of Medicine (Approval No. 310062) and conducted in accordance with the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research. Anaesthesia was administered intraperitoneally (ketamine/xylazine solution at 120 mg/kg body weight and 20 mg/kg body weight, respectively).[32 33]

A murine corneal wound model was generated by artificially creating a 2 mm (diameter) circular wound in the centre of the cornea of the right eye.[34] The effects of PRP eye-drops on corneal wound healing were compared with those of AS and PBS eye-drops in this murine corneal wound model (n=5 per group). Each eye-drop (2 µL/eye) was topically administered to the wounded cornea every 6 hours. Each corneal wound was stained with 1% fluorescein and monitored by using a slit-lamp microscope after surgery every 6 hours until the wound recovered

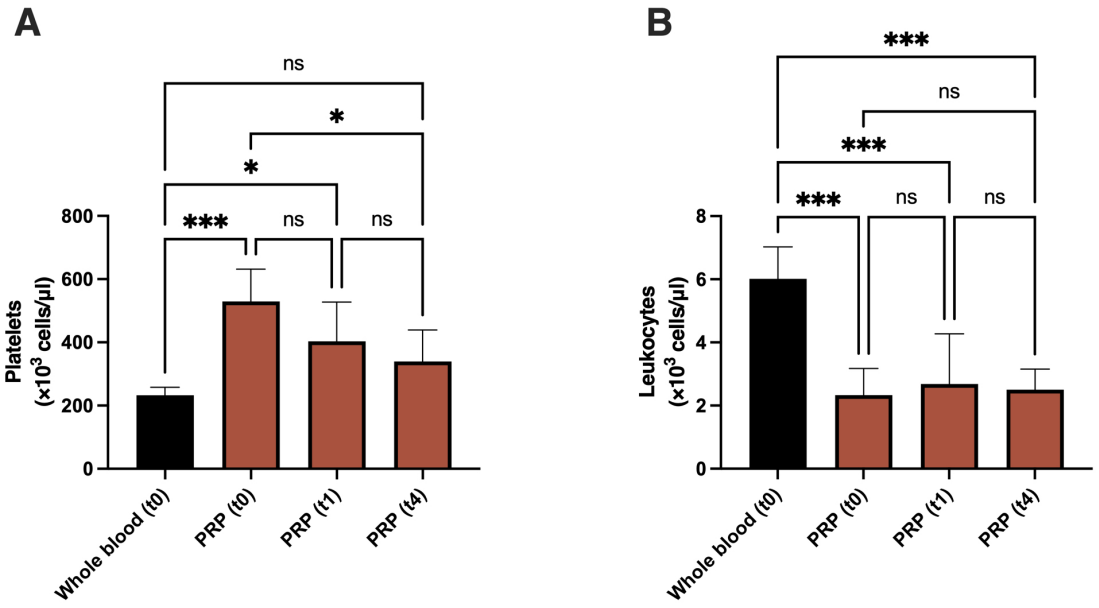

Figure 1 Platelet and leukocyte counts in whole blood, autologous serum (AS), and platelet-rich plasma (PRP) eye-drops at 4°C at various time periods. (A) Platelet levels (× 10^3 cells/μL) in whole blood, AS and PRP on the day of preparation (t0), after 1 week of storage at 4°C (t1), and after 4 weeks of storage at 4°C (t4). (B) Leukocyte levels (× 10^3 cells/μL) in whole blood, AS, and PRP (t0), PRP (t1), and PRP (t4). Results were considered statistically significant at *p<0.05 and ***p<0.001.

completely. The area of the epithelial defect was calculated using ImageJ 1.53a.[30 31]

Statistical analysis

Experiments with more than two groups were analysed using a one-way or two-way analysis of variance followed by a post hoc Bonferroni's multiple comparison test. Kaplan-Meier analysis was performed to construct corneal wound re-epithelisation curves and evaluate corneal wound re-epithelisation using a log-rank test. Data are presented as the mean±SD and differences were considered statistically significant at p<0.05. All statistical calculations were performed by using Prism V.9.1.0 (GraphPad, La Jolla, California, USA).

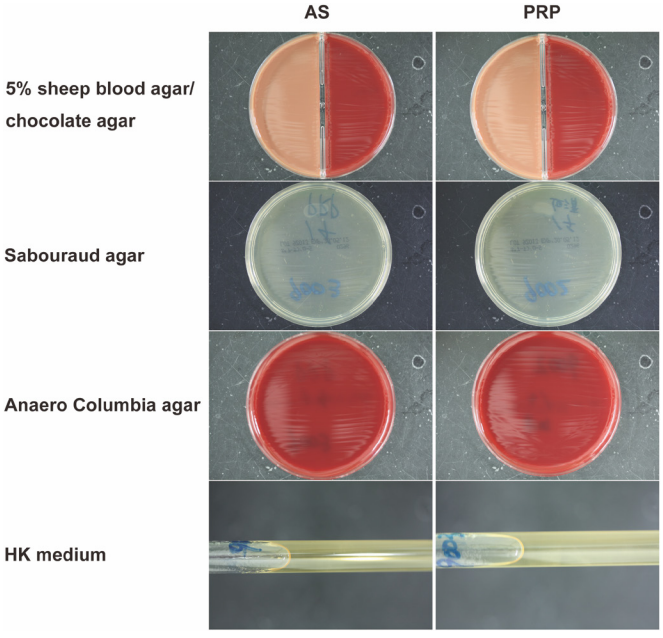

Figure 2 Bacterial culture of autologous serum (AS) and platelet-rich plasma (PRP) eye-drops during 4 weeks of storage at 4°C. Representative photograph of AS and PRP samples cultured on 5% sheep blood agar/chocolate agar, Sabouraud agar, Anaero Columbia agar, and HK medium.

RESULTS

PRP contains higher platelet and lower leucocyte counts than whole blood

Figure 1 shows the platelet and leukocyte counts in PRP (t0), PRP (t1), PRP (t4), and whole blood (t0) (n=6 per group). Platelet counts were significantly higher in PRP (t0) and PRP (t1) than in whole blood (t0) (p<0.001, p=0.030, respectively). Moreover, platelet counts in PRP decreased significantly with storage (PRP (t0) vs PRP (t4); p=0.010). Platelet counts were not significantly different between PRP (t4) and whole blood (t0) (p=0.240) (figure 1A). Leukocyte counts were significantly lower in PRP (t0), PRP (t1) and PRP(t4) than in whole blood (t0) (all p<0.001), and did not change significantly during storage (all p>0.990) (figure 1B). The mean±SD counts of the platelets and leukocytes are shown in online supplemental table S1.

Sterility of AS and PRP during the 4 weeks of storage

Figure 2 shows representative photographs of the negative culture results obtained from drops subjected to 4 weeks of storage for bacteria and fungi on the various media (n=6 per group).

Growth factor levels are significantly higher in PRP than in AS eye-drops after storage

TGF-β1 levels were not significantly different between PRP and AS at (t0) (p=0.076); however, they were significantly higher in PRP than in AS at (t1) (p<0.001) and (t4) (p<0.001) (figure 3A). Similarly, EGF levels were not significantly different between PRP and AS at (t0) (p=0.068). However, they were significantly

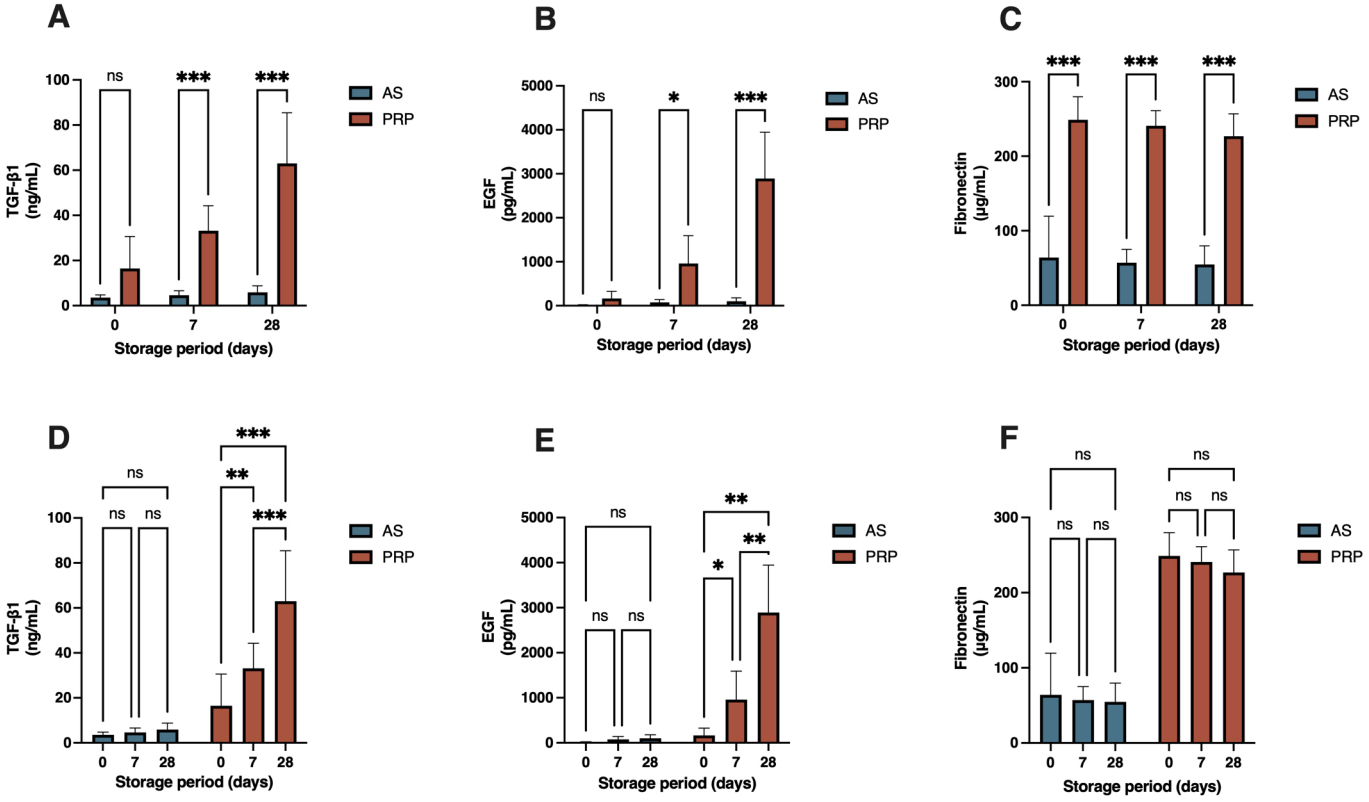

Figure 3 Levels of transforming growth factor (TGF)-β1, epidermal growth factor (EGF), and fibronectin during 4 weeks of storage at 4°C. Protein levels of (A) TGF-β1, (B) EGF, and (C) fibronectin in autologous serum (AS) and platelet-rich plasma (PRP) on the day of preparation (t0), at week 1 of storage at 4°C (t1) (A–C), and at week 4 of storage at 4°C (t4) (D–F) were assessed using an ELISA. Results were considered statistically significant at *p<0.05, **p<0.01 and ***p<0.001. ns, no significant difference.

higher in PRP than in AS at (t1) (p=0.020) and (t4) (p<0.001) (figure 3B). Fibronectin levels were significantly higher in PRP than in AS at (t0) (p<0.001), (t1) (p<0.001) and (t4) (p<0.001) (figure 3C). TGF-β1 levels in AS did not change significantly during storage (AS (t0) vs AS (t1); p>0.999, AS (t0) vs AS (t4); p>0.999, AS (t1) vs AS (t4); p>0.999), but TGF-β1 levels in PRP increased significantly with storage (PRP (t0) vs PRP (t1); p=0.007, PRP (t0) vs PRP (t4); p<0.001, PRP (t1) vs PRP (t4); p<0.001) (figure 3D). Similarly, EGF levels in AS did not change significantly during storage (AS (t0) vs AS (t1); p=0.101, AS (t0) vs AS (t4); p=0.087, AS (t1) vs AS (t4); p=0.154), but the TGF-β1 levels in PRP increased significantly with storage (PRP (t0) vs PRP (t1); p=0.028, PRP (t0) vs PRP (t4); p=0.003, PRP (t1) vs PRP (t4); p=0.005) (figure 3E). Fibronectin levels in AS and PRP did not change significantly during storage (AS (t0) vs AS (t1); p>0.999, AS (t0) vs AS (t4); p>0.999, AS (t1) vs AS (t4); p>0.999, PRP (t0) vs PRP (t1); p>0.999, PRP (t0) vs PRP (t4); p=0.315, PRP (t1) vs PRP (t4); p=0.813) (figure 3E). The mean±SD levels of the proteins of interest are shown in online supplemental table S2.

Stored PRP eye-drops promote corneal re-epithelialisation in vitro

Figure 4A shows a representative image of the corneal epithelial wound healing assay (n=6 per group). PRP (t4) significantly promoted wound healing compared with PBS, AS (t0), AS (t4), and PRP (t0) (p<0.001, p=0.031, p=0.024, and p=0.020, respectively). PRP (t0), AS (t0), and AS (t4) significantly accelerated wound healing compared with PBS (all p<0.001). However, there was no significant difference in wound area between the groups treated with PRP (t0), AS (t0), and AS (t4) (PRP (t0) and AS (t0); p=0.490, PRP (t0) and AS (t4); p>0.990, and AS (t0) and AS (t4); p>0.990) (figure 4B).

The mean corneal wound closure time was significantly shorter in the group treated with PRP (t4) than in the groups treated with PBS, AS (t0), AS (t4), and PRP (t0) (PBS; 59.5±1.2 hours, AS (t0); 30.0±2.7 hours, AS (t4); 28.0±4.1 hours, PRP (t0); 26.5±3.5 hours, PRP (t4); 20.5±2.3 hours, p<0.001, p<0.001, p=0.002, p=0.016, respectively). Moreover, the mean wound closure time was significantly shorter in the group treated with PRP (t0) than in the PBS-treated group (PBS; 59.5±1.2 hours, PRP (t0); 26.5±3.5 hours, p<0.001) (figure 4C).

Stored PRP promotes corneal re-epithelialisation *in vivo*

Figure 5A shows a representative slit-lamp photograph of murine corneal wound healing with PRP and AS eye-drops at (t0). The corneal epithelial wound completely recovered in both groups within 42 hours after surgery. Regarding the PRP and AS eye-drops used at (t0), the total wound areas were similar between the PBS-treated, AS-treated, and PRP-treated groups at 6 hours; however, they were significantly smaller in the PRP-treated group than in the PBS-treated group 12 hours (83.3%±3.7% vs 96.6%±2.4%, p<0.001), 18 hours (52.0%±8.2% vs 64.7%±6.7%, p<0.001), and 24 hours (17.7%±9.7% vs 28.7%±9.0%, p=0.002) postsurgery (figure 5B). The wound areas were significantly smaller in the AS-treated group than in the PBS-treated group 18 hours postsurgery (54.0%±6.7% vs 64.7%±6.7%, p=0.013); however,

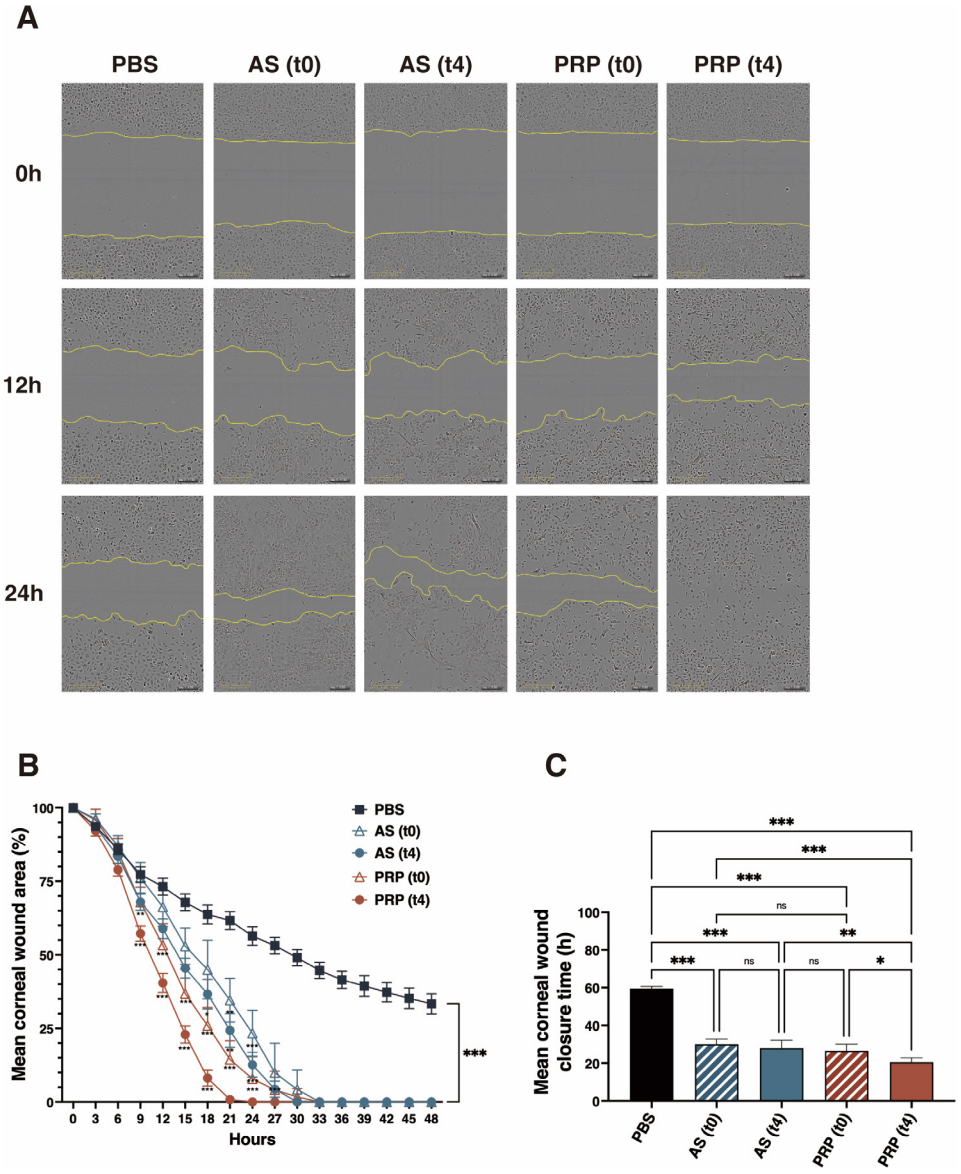

Figure 4 Corneal re-epithelialisation in a human corneal epithelial cell (HCEC) wound healing model treated with phosphate-buffered saline (PBS), autologous serum (AS), and platelet-rich plasma (PRP) eye-drops. (A) Representative image of corneal wound healing assay of HCECs at 0, 12 and 24 hours after scratching. The area between the yellow lines reflects the corneal wound area. (B) Mean corneal wound area (%) every 3 hours in each group (n=6). (C) Mean corneal wound closure time (h) in each group (n=6). Results were considered statistically significant at *p<0.05, **p<0.01, and ***p<0.001. On the day of preparation (t0); after 1 week of storage at 4°C (t1); after 4 weeks of storage at 4°C (t4). NS, no significant difference.

the wound areas were not significantly different between the AS-treated and the PRP-treated group (figure 5B). The wound healing rate was not significantly different among the three groups (figure 5C). The mean corneal wound closure time was significantly shorter in the PRP-treated group than in the PBS-treated group (35.0±5.9 hours vs 40.8±2.7 hours, p=0.025); however, the mean wound closure time was not significantly different between the PRP-treated and AS-treated groups, or the AS-treated and PBS-treated groups (figure 5D). Figure 5E shows a representative slit-lamp photograph of murine corneal wound healing using eye-drops at (t4). On using PRP and AS at (t4), wound areas were similar among the PBS-treated, AS-treated, and PRP-treated groups at 6 hours; however, they were significantly smaller in the PRP-treated group than in the PBS-treated group 12 hours (64.4%±6.1% vs 84.5%±4.4%, p<0.002), 18 hours (42.1%±5.0% vs 61.0%±9.6%, p<0.002), and 24 hours (15.4%±5.8% vs 44.4%±16.8%, p<0.05) postsurgery (figure 5F). Moreover, the wound areas in the AS group were significantly smaller than in the PBS-treated group 12 hours postsurgery (74.8%±4.0%, 84.5%±4.4%, p=0.013). They were also significantly smaller in the PRP-treated group than in the AS-treated group 12 hours (64.4%±6.1% vs 74.8%±4.0%, p=0.013) and 18 hours (42.1%±5.0% vs 56.8%±5.2%, p=0.006) postsurgery (figure 5F); however, the wound areas were not significantly different between the AS-treatd and the PRP-treated group (figure 5F). The wound healing rate did not significantly differ among the three groups (figure 5G). Furthermore, the mean corneal wound closure time was significantly shorter in the PRP-treated group than in the PBS-treated group (33.6±5.4 hours vs 40.8±2.7 hours, p=0.040); however, the mean wound closure time was not significantly different between the PRP-treated and AS-treated groups or AS-treated and PBS-treated groups (figure 5H).

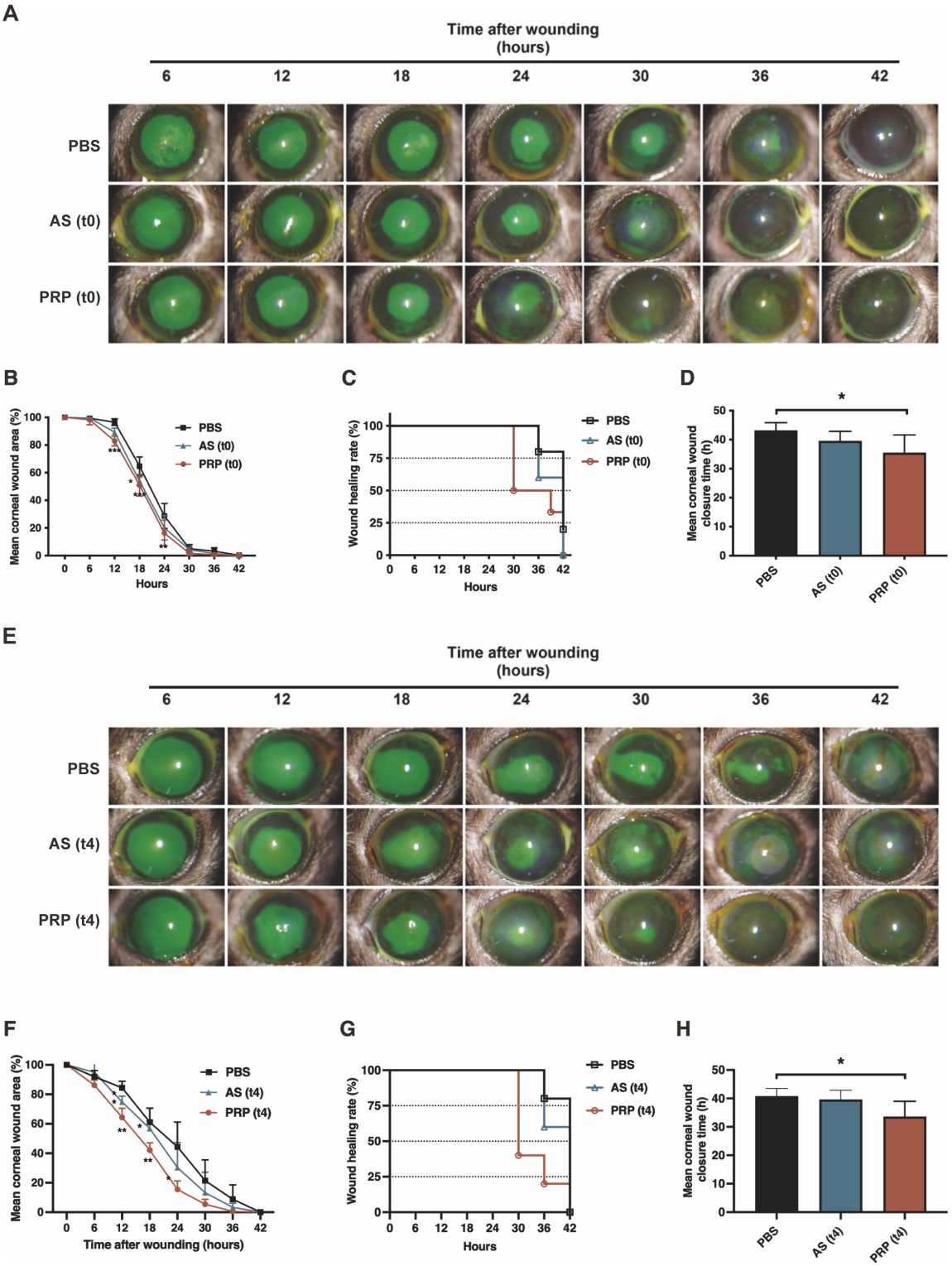

Figure 5 Corneal re-epithelialisation in a murine corneal wound healing model treated with phosphate-buffered saline (PBS), autologous serum (AS), and platelet-rich plasma (PRP) eye-drops. A–D shows the results for eye-drops used on the day of preparation (t0). (A). Representative slit-lamp photographs of the murine corneal wound healing model treated with PBS, AS, and PRP eye-drops on the (t0) every 6 hours until 42 hours post surgery. The green-stained area reflects the corneal wound area. (B) Mean corneal wound area (%) every 6 hours in each group (n=5). (C) Mean corneal wound healing rate (%) every 6 hours in each group (n=5). (D) Mean wound closure time (h) in each group (n=5). E–H shows the results for the eye-drops at 4 weeks of storage at 4°C (t4). (E) Representative slit-lamp photographs of the murine corneal wound healing model treated with PBS, AS, and PRP eye-drops on the (t4) every 6 hours until 42 hours postsurgery. The green-stained area reflects the corneal wound area. (F) Mean corneal wound area (%) every 6 hours in each group (n=5). The asterisk represents the analysis results with the PBS-treated group. (G) Mean corneal wound healing rate (%) every 6 hour in each group (n=5). (H) Mean wound closure time (h) in each group (n=5). Results were considered statistically significant at *p<0.05, **p<0.01, and ***p<0.001. On the day of preparation (t0); after 1 week of storage at 4°C (t1), after 4 weeks of storage at 4°C (t4).

DISCUSSION

There is an urgent need to develop effective therapeutic agents for keratoconjunctival epithelial disorders that do not respond to existing eye-drops. Here, the levels of different growth factors and active ingredients were examined during the storage of PRP eye-drops. Furthermore, the stability and sterility of PRP were investigated for future clinical use. There was an increased pharmacological efficacy and a complementary degree of sterility in PRP eye-drops when stored at 4°C for 4 weeks compared with those in AS eye-drops, warranting further study of PRP eye-drops.

PRP has been used clinically in specialised tissue-regeneration procedures, including oral surgery and orthopaedics,[19–22 35] as well as in several ophthalmology clinical trials.[36–41] The traditional non-ophthalmic route of PRP administration is the direct injection into target tissues immediately following preparation; thus, the issue of storage is irrelevant. Conversely, in the long-term usage of PRP eye-drops, the sterility of eye-drops during storage becomes crucial for effective treatment, and because AS and PRP eye-drops do not contain any chemical preservatives, bacterial contamination during storage is of concern. Both PRP and AS eye-drops maintained complementary degrees of sterility after storage for 4 weeks at 4°C, suggesting that with respect to sterility, PRP eye-drops can be used continuously for 4 weeks if stored at 4°C. However, there have been positive culture results in some refrigerated AS eye-drops.[42 43] Although our study indicates that strictly controlled storage conditions are sufficient to maintain PRP sterility, patients using PRP eye-drops should be closely monitored for clinical evidence of ocular surface infections.

Here, a significant increase in the levels of growth factors TGF-β1 and EGF were observed in PRP eye-drops after 4 weeks of storage at 4°C. A previous study also reported that PRP is rich in growth factors, including TGF-β1, EGF, and fibronectin, which play an essential role in tissue repair.[38] PRP harbours higher levels of platelets than AS.[18] Platelet activation triggers the release of TGF-β1 and EGF.[44] We found that the platelets in PRP were activated during the storage period, leading to an increase in TGF-β1 and EGF. Therefore, the increase in TGF-β1 and EGF levels during 4 weeks of storage may offer a major advantage for choosing PRP eye-drops over traditional AS eye-drops. There was no significant change in fibronectin expression in both AS and PRP before and after 4 weeks of storage. Fibronectin expression was significantly higher in PRP than in AS at all instances. These results suggest that PRP eye-drops are effective in managing wound healing during the corneal epithelial disorders, benefiting from the higher platelet contents and their disintegration, leading to growth factor release during the 4-week storage process.

This study showed that PRP (t4) were superior in promoting corneal wound healing in both *in vivo* and *in vitro* conditions compared with AS (t0), AS (t4), and PRP (t0). The significant increase in levels of growth factors in PRP eye-drops during storage may explain the comparative benefits of PRP over AS. EGF and TGF-β1, both of which showed increased levels during storage, are important regulators that stimulate the growth, proliferation, migration, differentiation, and adhesion of corneal epithelial cells involved in wound healing.[45] These previous findings, in conjunction with our study results, suggest that 4 weeks storage of PRP at 4°C enhances its wound healing effects.

This study has several limitations. First, during sterility testing, the lids on the eye-drop vials were kept closed during the storage period. However, in clinical practice, the risk of contamination may be greater when patients handle eye-drops themselves.[42] Second, because we only used leukocyte-poor PRP, our results may not accurately reflect the efficacy and stability of PRP eye-drops prepared using other methods.[19] Third, although in previous reports AS eye-drops were used at various dilution ratios (20%–100%),[26 46–50] here, we used 100% AS eye-drops, which is one of the effective dilution ratios.[26]

PRP eye-drops stored at 4°C for 4 weeks had higher levels of growth factors than AS while maintaining a similar degree of sterility, suggesting that PRP satisfies unmet medical needs in the treatment of refractory keratoconjunctival epithelial disorders.

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Contributors YO and TI had full access to all the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: YO, TI, JZ, YS, YK, MN, and HN. Acquisition, analysis or interpretation of data: YO, KeiF, KenF, JZ, AY, HS, AM-I, AE, KN, YA, KH, TH, and MK. Drafting of manuscript: YO, TI, and JS. Critical revision of the manuscript for important intellectual content: TI, KeiF, KenF, HS, YS, YK, MN, HN, and AM. Obtained funding: YO, TI, KeiF, and HS. Statistical analysis: YO, TI, AMI, and KN. Administrative, technical or material support: TI, MN, and AM. Supervision: TI and AM. Guarantor of work: TI.

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Biological effects of stored platelet-rich plasma eye drops in corneal wound healing

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Supplemental Material

Supplemental Table S1. Platelet and leukocyte levels in whole blood and PRP at different storage periods

Storage period (n=6)	Platelets ($\times 10^3$ cells/μL)		Leukocytes ($\times 10^3$ cells/μL)	
	Whole blood	PRP	Whole blood	PRP
Day of preparation	232.5±25.7	529.7±102.1	6.0±1.0	2.3±0.8
1 week	na	403.3±124.0	na	2.6±1.6
4 weeks	na	339.3±99.9	na	2.3±0.4

PRP: platelet-rich plasma, na: not applicable.

Supplemental Table S2. Levels of TGF-β1, EGF, and fibronectin in AS and PRP at different storage periods

Storage period (n=6)	TGF-β1 (ng/mL)		EGF (pg/mL)		Fibronectin (μg/mL)	
	AS	PRP	AS	PRP	AS	PRP
Day of preparation	3.6±1.2	16.5±14.2	12.6±10.2	165.0±162.1	64.1±55.3	248.9±30.9
1 week	4.6±2.0	33.2±11.1	77.5±61.8	958.4±634.3	57.0±18.0	240.9±20.3
4 weeks	5.9±2.9	63.0±22.4	108.0±72.4	2891.8±1052.6	54.7±25.1	226.9±30.0

TGF: transforming growth factor, EGF: epidermal growth factor, AS: autologous serum, PRP: platelet-rich plasma.