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ORIGINAL PAPER

Platelet lysate promotes re-epithelialization of persistent epithelial defects: a pilot study

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Abstract

Purpose To study the use of autologous platelet lysate prepared in a standardized method for the healing of persistent corneal epithelial defects (PED).

Study design Clinical and experimental investigation.

Methods In this prospective pilot study (ClinicalTrials.gov identifier NCT02979912), ten patients with a PED duration of a minimum 14 days were included. Autologous platelet lysate was prepared in a standardized methodology. Repeated freeze–thaw cycles were used to lyse the platelets. Patients were advised to apply the eye drops four times a day and were evaluated at baseline and on days 7, 14, 21, 28.

Results No adverse events were reported due to the use of undiluted autologous platelet lysate. A total of 70% of patients had complete re-epithelialization within 28 days. Of these, 40% healed within 14 days (effective group) and 30% within 28 days (partially effective group).

Conclusions Undiluted autologous platelet lysate, prepared according to a standardized methodology, is a safe and effective adjunct therapy for the treatment of PED.

Keywords Platelet lysate · Persistent epithelial defects · Re-epithelialization

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Introduction

Persistent corneal epithelial defects (PEDs) are associated with pain, visual impairment and potential serious complications. Tsubota defined PEDs as 'defects persisting for more than 2 weeks without improvement despite conventional treatment, such as non-preservative artificial tears or extended wear soft contact lenses' [1]. Following corneal wounding, corneal epithelial cells (CEC) migrate, proliferate and stratify closing the wounded area [2]. This re-epithelialization and wound healing process usually occurs without any complications. However, in some cases such as limbal stem cell deficiency, dry eye disease, exposure keratopathy and neurotrophic

keratopathy the defect persists and does not respond to conventional therapy [3, 4].

The complex wound healing responses are orchestrated by growth factors and cytokines, which are either secreted locally by neighboring cells or carried to the sight of injury by tears. Thus, the tear film is essential for normal CEC homeostasis and wound healing [5]. EGF, fibronectin, vitamin A, TGF-α, IGF, substance P, nerve growth factor (NGF) and neuropeptide Y are some of the essential epitheliatrophic and neurotrophic components of tears [2, 6–8]. Thus, many of the aforementioned conditions are associated with a decrease in tear production [5].

A range of treatment methods have been advanced to substitute the active components of tears and introduce a localized high concentration of epitheliotrophic wound healing growth factors [4]. Serum eye drops have been studied widely in the treatment of PED, and the rationale behind this is that serum supplies a local high concentration of factors that is preservative free, which acts as an attractive alternative for artificial tears [9–11]. Around 70% of cases with PED heal within 4 weeks following treatment with 20% diluted serum eye drops. The dilution process was thought to be necessary to lower the concentration of TGF-β to its physiological levels in normal tears [9–11]. However, later studies used 50% and 100% serum with a reported increase in efficacy and extremely minimal adverse effects [12, 13, 15].

Since α-granules in platelets contain high growth factor concentration, released upon platelet activation, autologous platelet lysate (PL) has higher epitheliotrophic factors compared to serum levels. PL enhances proliferation and migration of CECs upon culture in low concentration supplemented media [16, 17]. Additionally, it accelerates healing in corneal debridement experiments compared to controls [17]. Fea et al. [18] used 50% (v/v) diluted autologous PL to treat dry eyes of patients with Sjögren syndrome and reported significantly increased basal epithelium cells density compared to control group treated with artificial tears.

In this study, we aimed to investigate the safety and get preliminary insights into the effectiveness of undiluted autologous platelet lysate eye drops on the healing of PED.

Patients and methods

Patients and study design

This study is a single-center, non-comparative, prospective pilot study. The study was approved by the Institutional Review Board and Ethics Committee at the Cell Therapy Center. All included patients were informed about the objective and protocol of the study and gave written informed consent in accordance with the Declaration of Helsinki prior to undergoing any procedures. This trial was prospectively registered on ClinicalTrial.gov, under the identifier code: NCT02979912.

A total of 10 patients with corneal epithelial defects persisting for more than 2 weeks despite conventional treatment were enrolled in this study as per the inclusion and exclusion criteria shown in Table 1.

Quantitative measurement of growth factor concentration

To compare PL against serum, the level of certain growth factors regarded as mitogens of epithelium was investigated. To minimize variation, PL was pooled from 22 healthy donors following complete PL preparation as described below. All participants gave a written informed consent in accordance with the declaration of Helsinki. Platelet parameters were determined using a hematology analyzer (Sysmex, Japan). Concentration of EGF, PFGF and FGF was measured in PL and serum using sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&F, USA), as per manufacturer instructions. Optical densities were measured at 450 nm using a microplate reader (Glowmax, Promega, UK).

Autologous PL preparation

Venipuncture was performed at the antecubital fossa, and 12–24 ml of whole blood was collected in 2 ml of 3.8% sodium citrate tubes. Platelet parameters were recorded. Samples were centrifuged at a low speed, and the supernatant was collected and centrifuged again at high speed to get a platelet pellet and platelet poor plasma (PPP). Following, PPP was discarded partially and the platelet pellet was resuspended in the remaining volume of PPP, to achieve a final platelet concentration of 1×10^6 platelet/μl. Platelet

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Age between 20 and 75 years	Active corneal infection
Cognitive ability to understand and sign the consent form	Lid abnormalities contributing to the PED
Corneal epithelial defect persisting for more than 2 weeks despite conventional treatment	Impending corneal perforation
Good compliance with the study regimen and availability for the duration of the entire study period	Pregnant or lactating women

activation was performed by three freeze–thaw cycles at − 80 and 37 °C, respectively. The resulting PL around 5 ml was centrifuged and filtered by passing through 0.22-μm filters. Following, PL was aspirated into four sterile eye droppers, and frozen. All procedure steps were performed under the optimal sterile conditions.

Each week, a new vial was thawed and given to the patients, and they were instructed to store eye droppers of autologous PL in their refrigerator at 4 °C. Additionally, patients were advised to apply the eye drops four times a day.

Outcome measurements

During the period of PL administration, the patients were evaluated at baseline and on days 7, 14, 21, 28. Special attention was given to record any adverse effects, including concurrent infection, itching and pain or worsening symptoms in the eye. The size of the epithelial defect was estimated by measuring the longest linear diameter and the longest one perpendicular to it, and multiplying the two dimensions. This was done following the instillation of fluorescein stain and slit lamp examination, under 10× magnification. The epithelial defects were then imaged by a digital slit lamp camera under the same magnification.

The main outcome measure was to ensure the safety and tolerability of APL eye drops and record any adverse effects. Secondary outcome measurements included complete healing of the PED, time required for corneal epithelialization and the relationship between patient characteristics and outcomes were also documented. Treatment effectiveness was determined according to the system developed by Tsubota et al. [1, 9]. The treatment was considered effective if the PED healed within 2 weeks, partially effective if the healing occurred within 1 month and failed if the PED did not heal or worsened within a month.

Statistical analysis

Data were analyzed using Prism 6 software. All data were expressed as mean ± standard deviation (SD). Differences between growth factor concentrations were evaluated by paired student's t test. In all analyses, values of $P < 0.01$ were considered statistical significant difference, while values of $P < 0.001$ were considered highly statistically significant.

Results

Growth factor concentration

The mean age of donors was 24.64 ± 4.46 (range 20–37) years, with a gender ratio of 19M: 9F. Platelet parameters were recorded (Table 2). We measured the concentration of EGF, PDGF-AA and FGF in both PL and serum (Fig. 1). The level of EGF was approximately sixfold higher in PL compared to serum with a statistically significant difference between both PL mean concentration = 1714.54 pg/ml ± 34.21 and serum mean concentration = 280.86 ± 4.88, $P < 0.00001$. PDGF-AA concentration was also higher in PL at 127% compared to serum levels, with

Table 2 Mean platelet parameters from 22 healthy donors

PLT (10^3/ul)	PCT (%)	MPV (fL)	PDW (fL)
252.9 ± 45.47	0.27 ± 0.05	10.78 ± 0.79	13.2 ± 1.67

PLT platelet count; *PCT* plateletcrit; *MPV* mean platelet volume; *PDW* platelet distribution width

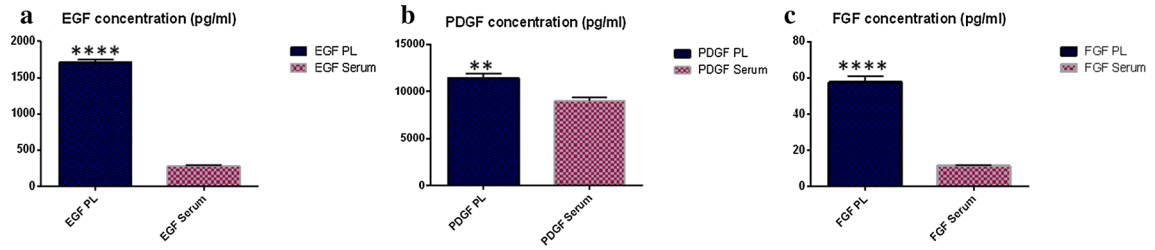

Fig. 1 Growth factor concentration in platelet lysate and serum from 22 healthy individuals. **a** EGF, **b** PDGF and **c** FGF. All concentrations are the mean ± SD. (**$P < 0.01$,****$P < 0.0001$)

statistical significance (PL mean concentration = 11,420.9 ± 450.225, serum mean concentration = 8967.91 ± 343.25, $P < 0.01$). Additionally, FGF was approximately fivefold higher in comparison with serum (PL mean concentration = 57.66 ± 3.055, serum mean concentration = 11.38 ± 0.359, $P < 0.0001$) (Table 3).

Patient outcome measurement

A total of 10 eyes from 10 patients were included in this study, and mean age of participants was 55.9 ± (range 30–70) years. Four out of the ten patients were females. All patients continued their recommended treatment regime (Table 4). Eight of the patients had PED following surgery and two following herpetic keratitis (Table 5). Patients were instructed to use APL eye drops four times daily. PED duration prior to the instigation of APL eye drops treatment was of a minimum 14 days in all treated cases. None of the patients experienced any adverse effects when using 100% APL. The mean area of PED at baseline was 16.08 ± 14.59 mm^2 (range 4–40 mm^2). Success rate following treatment was four patients with complete epithelialization (effective) (Fig. 2).

Three out of the ten patients healed within 1 month following treatment (partial effectiveness). Additionally, three patients did not achieve complete epithelialization during the course of the trial, for these patients the treatment was considered ineffective. However, 2 out of 3 of these patients had a decrease in the size of the defect and only one patient had a worsening PED. All of the patients where the treatment was considered ineffective had a PED following surgery.

Discussion

Blood-derived products have been widely studied for the treatment of ocular surface defects (OSD) including PEDs. The first study that provided the proof of principle for the use of a blood-derived product as eye drops was devised by Fox and coworkers. In their study, they used ASE for the treatment of dry eye disease in patients with Sjögren's syndrome, and they reported symptom alleviation within 3 weeks of ASE application [20]. The development of such concept is based upon the similarity between tears and serum in terms of pH and osmolarity, increased epitheliotrophic factor concentration, in addition to its viscosity and excellent lubricating properties [1, 16]. Later on, several studies reported the use of ASE for the treatment of PEDs arising from different etiologies with a success rate ranging from around 50 to 93% [1, 11–13]. Different protocols have been utilized for the preparation of ASE and a wide range of concentrations (20–100%) tested [11–13]. PRP is another blood derivative that was also investigated for the

Table 3 Mean concentration of growth factors in platelet lysate and serum

Sample	FGF concentration (pg/ml)	PDGF concentration (pg/ml)	EGF concentration (pg/ml)
Serum	11.382 ± 0.359	8967.91 ± 343.25	280.86 ± 4.88
Platelet Lysate	57.663 ± 3.055	11,420.9 ± 450.225	1714.54 ± 34.21

Table 4 Demographics of patients treated with APL

Gender	Female/male	4/6
Age group	20–65	6
	> 65	4
Cause of PED	Post-penetrating keratoplasty	8
	Herpetic keratitis	2
Average baseline PED area/mm^2		16.08

Table 5 Clinical features and outcomes of APL-treated patients

Patient ID	Diagnosis	Drugs used during APL administration	Baseline area of PED/mm^2	Endpoint area of PED/mm^2	Days needed for healing
1	Post-PKP	Lubricant, steroid, antiglucoma, antibiotic eye drops	25	30.25	
2	Post-PKP	Lubricant, steroid, antiglucoma, antibiotic eye drops	40	19.5	
3	Post-PKP	Lubricant, steroid, antiglucoma, antibiotic eye drops	4	1	
4	Post-PKP	Lubricant, steroid, antibiotic eye drops	30.25	Healed	14
5	Herpetic keratitis	Antiglucoma, lubricants eye drops	9	Healed	21
6	Post-PKP	Lubricant, steroid, antiglucoma, antibiotic eye drops	4	Healed	7
7	Herpetic keratitis	Antiviral and lubricant eye drops	10.08	Healed	28
8	Post-PKP	Steroid, antibiotic eye drops	10.5	Healed	14
9	Post-PKP	Artificial tears	18	Healed	28
10	Post-PKP	Antibiotics	10	Healed	14
Average			16.08 ± 12.03		16.25 ± 8.31

treatment of PED following bacterial and fungal keratitis [14]. This study reported a significantly shorter healing time in patients treated with PRP (20% diluted) compared to ASE (20% diluted). Vajpayee and colleagues compared the effects of umbilical cord (UCS) serum and ASE on the healing of PED arising from varying causes [21]. In their study, they reported 1.5-fold increase in the success rate of UCS compared to ASE.

Although the mechanism of action of all of these hemoderivatives is unknown, all of such studies are based on the hypothesis that these products supply a localized high concentration of epitheliotrophic factors that promote the re-establishment of corneal epithelial barrier [22]. Based on the aforementioned information, in this study we hypothesized that autologous platelet lysate which contains a higher concentration of growth factors compared to other blood derivatives might increase the success rate of PED healing. Several studies along with ours have reported a significantly higher concentration of many growth factors compared with serum [16, 23]. Since this is the first study to assess the feasibility of using APL as eye drops for PED treatment, we utilized a non-comparative, small pilot study. Using undiluted autologous platelet lysate with a standardized platelet count for the treatment of PEDs from patients with post-PK and herpetic keratitis, our results demonstrate an overall 70% success rate, with an average of 12.6 days to achieve complete re-epithelialization.

When we analyze other trials studying the effect of hemoderived eye drops, we notice many variabilities. Coming out with a single definitive conclusion from these studies, assessing the same product is extremely

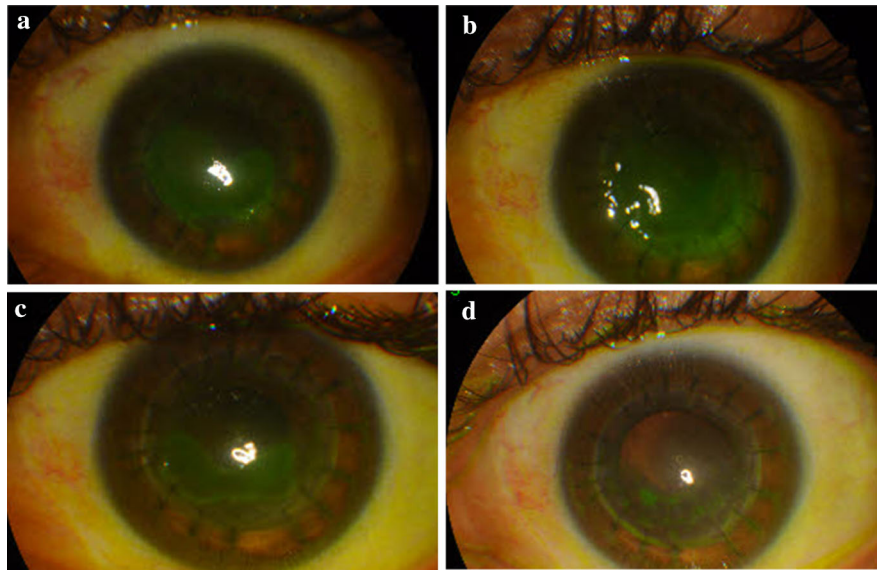

Fig. 2 Representative slit lamp images of a patient with PED following PKP. **a** baseline and at day 7, 14, 28 following APL administration (**b**–**d**), respectively

challenging. Such studies like ours utilize a non-comparative case series, each using their own protocol for product preparation, patients with different PED etiologies, defect size and duration, different application frequencies and concentrations [1, 11–13]. In terms of PED etiology and product concentration, the study conducted by Lekhanont et al. [13] is the closest to ours. Using undiluted ASE eye drops, they treated patients with postoperative PED mostly from PK, and they concluded their study with around 93% success rate. It is important to note that although the number of patients in this study was large, a negative control group was not included. Some of the reasons that might have contributed to this exceptionally high success rate include the choice of patients. Patients with postoperative PEDs tend to heal faster compared with defects associated with other ocular complication. The duration of PED is also critical, as the defect tends to respond better the shorter they persisted. Including patients with PEDs lasting for 1 week could have also contributed to this success rate. When we compare our results with Lekhanonts' study, we notice that APL has lower efficacy compared to ASE. Jeng et al. [12] utilized 50% diluted ASE eye drops for the treatment of patients for post-PK defects, with a reported 68% success rate, thus our results are closely similar. Additionally, umbilical cord blood serum (UCS) and ASE were compared in terms of efficacy for the treatment of PEDs arising from different etiologies. In post-PK, the success rate was 55.5 and 28.6% for UCS and ASE, respectively [21]. According to this, APL has higher efficacy. However, compared with other case series with PEDs with variable indications and a success rate ranging from 48 to 73%, our results come remarkably close [10, 11, 19, 24, 25].

Thus, increasing the concentration of growth factors as a whole might not be as effective as hypothesized. Since all of these products have inductive as well as inhibitory epithelial growth factors. The main reason for diluting these products is to reduce the amount of epithelial inhibitory growth factors particularly TGF-β [1]. Depleting such growth factors from these products prior to use could lead to enhanced therapeutic effect.

Some of the limitations of this study include most of our patients had PED following penetrating keratoplasty. Although this minimizes patient heterogeneity, it has been reported that patients with postoperative PED heal within an acceptable time frame, compared with patients with non-postoperative PED. Such patients usually have other ocular surface-associated problems, thus they tend to last longer. This fact might cause the skew of our data toward a higher successful rate compared to what it could be if the group of patients had a more diverse etiologies. Nevertheless, APL proves to be an effective conjunctive therapy for postoperative PED. We did not include a control group as patients already had an epithelial defect that did not respond to conventional

therapy for a minimum period of 14 days, and this study was conducted as a proof of principle. The follow-up period of this study is short. In the success group, we did not follow up to check for relapsing PED, and in the failure group we did not assess if the PED healed or worsened beyond the duration of the trial. Additionally, some studies utilize a period to wash out other used drugs, and here we assessed the use of APL in conjunction with conventional therapy to reduce the time required for epithelialization, not as a substitute.

In conclusion, based on this preliminary analysis, 100% autologous PL eye drops are effective and safe and could be used as an adjunct therapy for the treatment post-PK and herpetic keratitis PED. Additionally, we recommend any further studies using APL to standardize platelet count as in our modified product, to minimize variability and ease study comparison. However, further work must analyze all of the aforementioned points of variability in a large comparative study, in order to be able to draw clear conclusions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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