

The use of a “horizontal centrifugation protocol” to prepare autologous platelet-rich fibrin membranes for corneal reconstruction surgery in dogs with complicated corneal ulcerations: A case series

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Abstract

Objective: The purpose of this case series was to describe the effect of autologous PRF membrane for corneal reconstruction surgery in dogs. PRF membranes made from two healthy dogs unrelated to the current case series were used for PRF histologic analyses.

Animals: Seven dogs with complicated corneal ulcerations.

Procedure: A complete ophthalmic examination, hematology, and fibrinogen analysis were performed pre-surgery. A PRF clot was made from autologous blood in a serum tube after centrifugation in a horizontal Bio-PRF® Centrifuge at 700 × g for 8 min. The PRF clot was processed in a PRF-Box® into a PRF membrane. The PRF membrane was sutured to the corneal ulcer bed. Each dog had a follow-up at days 5–7, 12–14, and 30–40 post-surgery. A final long-term follow-up was performed as well.

Results: A positive outcome with healing and a “good” quality PRF membrane was seen in six out of seven dogs. One dog had a fibrinogen level below normal range and the PRF membrane was of “poor” quality. This dog developed a descemetocoele 13 days post-surgery and needed rescue surgery. Mean healing time for all dogs was 9 ± 5.5 days. Minimal scarring, corneal pigmentation, and vascularization were observed at the final long-term follow-up 288 ± 44 days post-surgery.

Conclusion: PRF membrane was successful as graft material for corneal ulceration reconstruction surgery. Low fibrinogen appeared to have negative effect on the quality of the PRF membrane, showing the importance for the surgeon to evaluate the quality of the PRF membrane prior to surgery.

Louise Baadsgaard Bruun and Tommy A. Waskiw Hardon both authors have contributed equally to this study.

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KEYWORDS

clinical study, cornea, graft material, infected corneal ulceration, methodology, pedicle conjunctival graft

1 | INTRODUCTION

Corneal ulcerations are commonly seen in companion animals such as dogs and cats.^{1,2} Surgical reconstruction is recommended in progressive ulcerations and in corneal ulcerations with a stromal loss greater than 50% of the corneal depth.² The goal of surgical intervention is to promote corneal healing, provide tectonic support, maintain ocular integrity, and preserve vision with a clear optical medium.²⁻⁴ Surgical reconstruction of corneal ulcerations has been described in veterinary literature with a great variety of surgical techniques and graft materials. Each has advantages and disadvantages in terms of practicality, success rate, and preservation of corneal transparency.⁵ The platelet-rich fibrin (PRF) was originally used in human dentistry, followed by veterinary dentistry, to enhance bone regeneration at extraction sites.⁶⁻⁸ Although the (PRF) membrane technique has become part of many different surgical disciplines during the last 20 years, literature focusing on PRF membranes in human and veterinary ophthalmology is relatively limited. Table 1 gives an overview of studies using PRF membranes in veterinary and human ophthalmology for reconstruction of corneal ulceration.⁹⁻¹⁴

Platelet-rich plasma is an example of a “first-generation platelet concentrate.” These first-generation platelet concentrates are produced with protocols that have different spinning protocols and a difference in anticoagulant.⁶ The PRF membrane is referred to as a “second-generation platelet concentrate” and is a fibrin matrix made by a single spin protocol of an autologous blood sample without chemical additives.⁶ In this PRF-protocol, fibrin will be activated during centrifugation and will slowly polymerize and form a clot situated above the erythrocytes and under the supernatant of platelet-poor plasma.¹⁵ The PRF membrane was developed by Choukroun et al. in 2001 and is a “superclot” consisting mostly of platelets and leukocytes from the autologous blood sample and is, therefore, named L-PRF for leukocyte PRF.^{15,16} A biochemical analysis of the PRF membrane indicates assembly of cytokines such as platelet-derived growth factor (PDGF), transforming growth factor β -1 (TGF β -1), and insulin-like growth factor (IGF), as well as glycan chains and structural glycoproteins in a fibrin network.¹⁶

Veterinary ophthalmology studies with PRF membranes have not described their centrifugation protocol in specific detail in regard to g-force.¹²⁻¹⁴ Also, their PRF

membrane was either prepared between surgical gauze sponges^{13,14} or between two microscope glass slides.¹² The present case series will use a standardized horizontal centrifugation PRF membrane protocol described by Miron et al. in 2019.¹⁷ This protocol has not been previously used for surgical procedures in canine patients. The objective of this case series was to describe the effect of autologous PRF membrane for corneal reconstruction surgery in dogs. Additionally, PRF membranes made from two healthy dogs unrelated to the current case series were used for PRF histologic analyses.

2 | MATERIALS AND METHODS

The case series was designed as a prospective clinical study performed at two different companion animal hospitals in Denmark (Anicura Aarhus Veterinary Hospital and Haslev Veterinary Clinic) by two European College of Veterinary Ophthalmologist (ECVO) approved eye scheme examiners (LBB and TAWH). The study had a total duration of 6 months (April 1st, 2022 to September 30th, 2022). Each patient was seen at the initial presentation, the day of surgery, and at three follow-up visits on days 5–7 (first follow-up), 12–14 (second follow-up), and 30–40 (third follow-up) post-surgery. The study was approved by the Local Ethical and Administrative Committee at Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark (reference #2022–7). A written consent on study participation was obtained from the owner before treatment.

2.1 | Selection of dogs

The study population was recruited from client-owned dogs referred for complicated corneal ulceration treatment.

2.1.1 | Inclusion criteria

All dogs that were included in this study were diagnosed with complicated corneal ulceration and had surgery performed with keratectomy and PRF membrane (see below description of surgical procedure). All dogs had blood

TABLE 1 Overview of current clinical platelet-rich fibrin (PRF) membrane studies including species, diagnosis, origin of the blood used to prepare the PRF membrane, preparation protocol encountering centrifugation, process, and the fixation method of the PRF membrane.⁹⁻¹⁴

Author	Species	Diagnosis	Blood type	Centrifugation speed (rpm) and time (min)	Membrane	Fixation of membrane to donor tissue
Alio et al. 2013	11 humans	Perforated corneal ulcerations	Autologous	1600 rpm for 16 min	Membrane: Platelet-poor plasma + CaCl^2 + incubation Clot: Platelet-rich citrate-plasma + CaCl^2	Sutured to limbal conjunctiva
Can et al. 2016	3 humans	Melting stromal corneal ulcerations and Descemetocoele	Autologous	2700 rpm for 12 min (Hettich EBA-20)	Fibrin clot in PRF membrane box	Patient 1: Thick membrane on Descemetocoele and thin membrane sutured to limbal conjunctiva + contact lens. Patient 2 and 3: Membrane + contact lens (no sutures)
Da Silva et al. 2019	4 dogs	Deep corneal ulcerations	Autologous	3000 rpm for 10 min	Pressed between two pieces of surgical gauze	Membrane + contact lens (no sutures) + tarsorrhaphy or third eyelid flap
Mishra et al. 2021	10 dogs	Corneal ulcerations	Homologous	2700–3000 rpm for 10 min	Pressed between glass slides	Sutured to limbal conjunctiva
Demir et al. 2021	10 cats	Corneal sequestrum	Autologous	2700 rpm for 12 min (Hettich 200 EBA)	Pressed between two pieces of surgical gauze	Sutured to ulceration bed + third eyelid flap
Melek et al. 2022	6 Rabbits	Corneal chemical burn	Autologous	400 g for 12 min (Rotofiz 32 A Hettich)	Pressed between two pieces of surgical gauze	Sutured to limbal conjunctiva

collected for hematology and fibrinogen analysis. Dogs with uni- or bilateral corneal ulcerations and dogs with any history of previously unsuccessful medical or surgical treatment were included. Inclusion was acceptable if underlying anatomical or medical conditions predisposing for corneal ulceration was detected. All dogs were included regardless of breed, age, gender, possible infection, or if the dog had an underlying quantitative or qualitative tear film abnormality. Dogs requiring additional surgical interventions aside from corneal surgery, for example, correction of entropion, could also be included in the study.

2.1.2 | Exclusion criteria

Dogs presenting with a corneal descemetocoele or perforated corneal ulceration requiring surgery with additional techniques for tectonic support besides PRF membrane, for example, a conjunctival graft, were excluded.

2.2 | Preoperative evaluation

On initial presentation, all dogs underwent a complete ophthalmological examination including neuro-ophthalmic examination (menace response, dazzle reflex, direct and indirect pupillary light reflexes, palpebral reflexes), slit lamp biomicroscopy (Keeler PSL, Keeler or Kowas SL-17, Kowa Group) of the adnexal and anterior segment, as well as indirect binocular ophthalmoscopy (Heine Omega 500, Heine Optotechnik GmbH) with a 2.2 panretinal condensing lens (Volk, Eickemeyer) of the posterior segment and a fluorescein stain uptake test (Fluorescein strips, Fluorescein Sodium, Ophthalmic strips USP, Optitech). All ophthalmological examinations were performed by the same two ECVO-approved eye scheme examiners that were performing the surgery. Each dog had a sample for microbiological aerobic culture and sensitivity testing collected from the corneal ulceration and these samples were submitted to an external laboratory (IDEXX Laboratory).

Prior to the commencement of the surgical procedure, a general clinical examination and an ASA scoring (ASA Physical Status Classification System, American Society of Anesthesiologists, 2020) were performed. A pre-anesthetic blood profile with blood sample collected in IDEXX Vet-collect EDTA-tubes (IDEXX Europe B.V., EK Hoofddorp) for hematology and in Li-Heparin tubes (BD, Plymouth) for blood chemistry was performed. Samples were analyzed at the in-house laboratory with IDEXX Procyte DX and IDEXX Catalyst One (IDEXX Lab). A citrate-stabilized blood sample (BD Vacutainer Plus Plastic Citrate tube) for fibrinogen analysis was collected and shipped in a cooling

container to the IDEXX Laboratory (IDEXX Laboratory). Total blood volume collected from each dog was less than 1% of the patient's blood volume over a 24-h period.

2.3 | Preparation of the autologous PRF membrane

Blood samples were collected from the jugular vein with vacutainer in a serum tube (Bio-PRF solid PRF tube, Puredent/Puremed) without additives and placed in a desk centrifuge (Bio-PRF Centrifuge, Puredent/Puremed) within 90 s following collection. The serum tube was spun horizontally in a single cycle for 8 min at $700 \times g$ (maximum Relative Centrifugal Force [RCFmax]) at room temperature. As demonstrated in Figure 1, after the termination of spinning, two phases were visible in the tube: a solid fibrin clot as the supernatant and the coagulum of erythrocytes below. A sterile drape was used for the preparation of the membrane, and the handler was wearing sterile gloves. The PRF membrane preparation was not performed in a sterile hood. The tube content was removed and the fibrin clot was dissected from the erythrocyte coagulum with a pair of scissors and placed in a box for the preparation of the membrane (PRF-Box®, Puredent/Puremed). The clot rested on a metal plate with drainage holes and a solid metal plate was placed as a cover on the clot. The slow and gentle compression formed a membrane between the two plates as a homogenous, wet sheet of fibrin soaked with serum. This process lasted about 10 min and took place immediately prior to the surgical procedure.

2.4 | Clinical evaluation

The depth and size of the corneal ulceration were evaluated prior to surgery. The depth of the corneal ulceration was scored in four categories according to stromal loss: Less than 25%, between 25%–50%, 50%–75%, and above 75%. The size of the corneal ulcer was also scored in four categories according to surface area uptake: Less than 25%, between 25%–50%, 50%–75%, and above 75%. The location of the corneal ulceration was also evaluated to be either axial or paraxial (dorsal, ventral, medial, or lateral).

2.5 | Surgery

An anesthetic protocol was designed for each patient considering any history of previous reactions to anesthetics and analgesics, results of the clinical examination, pre-anesthetic profile, ASA scoring, breed, age, weight, and potential co-morbidities. For the surgery, the periocular

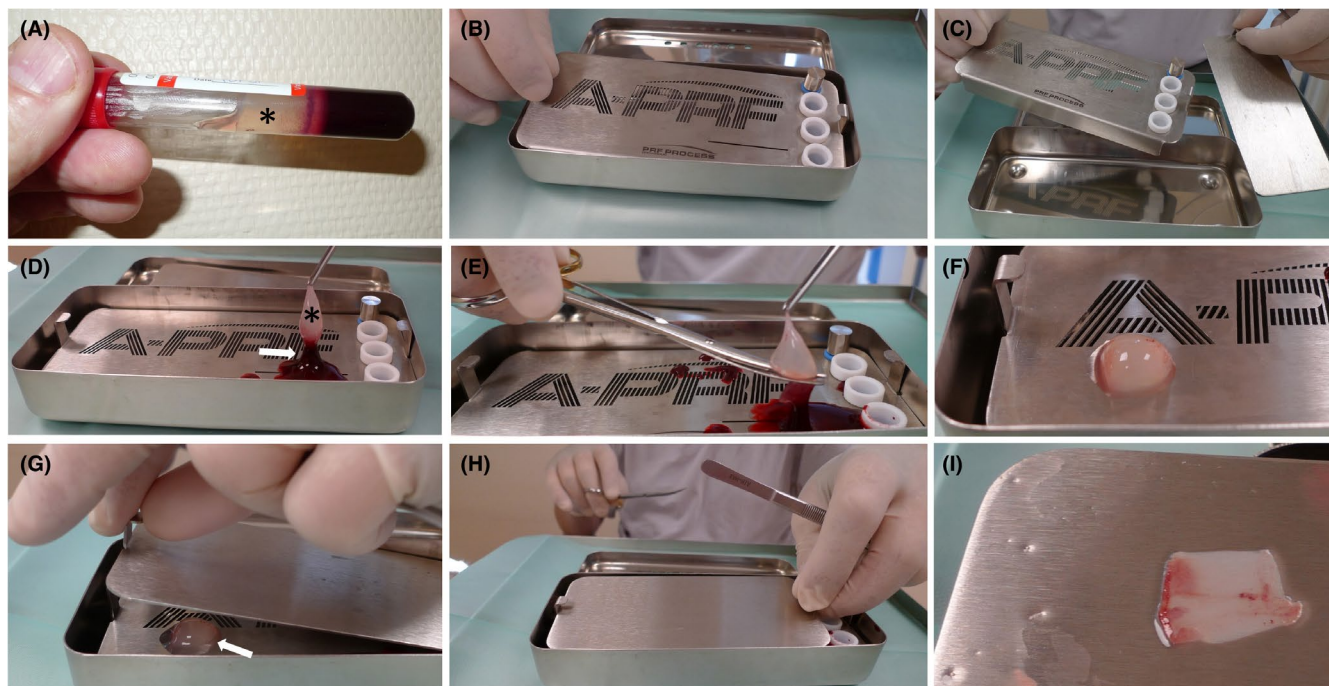


FIGURE 1 Preparation of the platelet-rich fibrin membrane. (A) Tube immediately after spinning cycle. Supernatant (asterisk) is a solid fibrin clot and a coagulum of erythrocytes is seen underneath. A small amount of serum is seen above the solid fibrin clot. (B) PRF-Box® (Puredent/Puremed). (C) The PRF-Box contains a metal plate with drainage holes and a metal cover plate. (D) Content from the tube is retrieved with a forceps grasping the fibrin clot (asterisk) and placed over the drainage plate with erythrocyte coagulum (arrow) resting on the plate. (E) With a pair of scissors, the erythrocyte coagulum is dissected from the fibrin clot. (F) Solid fibrin clot resting on drainage plate. (G) Metal cover plate is placed over the fibrin clot (arrow). (H) Fibrin clot rests under the metal cover plate for about 10 min. (I) The platelet-rich fibrin membrane soaking in serum immediately after removal of metal cover plate and ready for grafting.

area underwent standard aseptic preparation with 0.2% povidone-iodine ophthalmic solution (Skanderborg Apotek), followed by a flush with 0.9% sterile saline (Fresenius Kabi AG). The dogs were placed under the surgical microscope in dorsal recumbency with cornea in a horizontal position and the surgical site was draped. **Figure 2** demonstrates the surgical procedure: At the beginning of the surgery, a drop of topical analgesic (Oxyburprocain Minims 0.4%, Specific Pharma A/S) was instilled to the eye. A colibri-type eyelid speculum was placed, and the stromal ulceration was initially debrided with eye spears (Beaver-Visitec International Inc.). An incision was made with a disposable biopsy punch (Kai Medical, Kifu), encircling the corneal ulceration. The punch biopsy was 1–2 mm larger than the corneal ulceration. A lamellar keratectomy with a Beaver Blade 6400 (Beaver-Visitec International Inc.) or an angled crescent knife 2.2 mm (Sidepharm) with bevel up was performed and devitalized stromal tissue removed. None of the lamellar keratectomies were performed deeper than the deepest part of the corneal ulcer. From the prepared PRF membrane—still stored in the PRF-Box—a graft was harvested with the same biopsy punch as used for the corneal incision. The PRF membrane graft was sutured in the ulcer bed with monofilament resorbable 10–0 suture (Luxcryl 10–0 PGA with a spatulated needle, An-Vision).

At the end of the surgery, a drop of atropine 1% (Atropine Minims 1%, Specific Pharma A/S) was instilled in the eye for cycloplegic effect. Additional surgery for eyelid correction, ectopic cilia, distichiasis, etc., was performed after reconstruction of the corneal ulcer. Finally, a temporary tarsorrhaphy with a horizontal mattress or single interrupted suture (Ethicon polyglactin 910 coated, resorbable vicryl 5–0 FS-2, Johnson+Johnson, Diegem, Belgium) was placed for corneal and PRF membrane protection.

The quality of the PRF membrane was assessed subjectively after preparing the membrane and before suturing the PRF membrane into the ulceration bed. The quality was categorized as “good” or “poor.” A good-quality PRF membrane was sufficient in size, with a sufficient thickness, tensile strength, and non-transparent. Opposite, a poor-quality membrane was thin, more gel-like, and porous in consistency, as well as being transparent.

2.6 | Postoperative care and follow-up

Following surgery, all patients were treated with Ciprofloxacin 3 mg/mL ophthalmic solution QID (Ciloxan®, Novartis Healthcare A/S) and ophthalmic lubrication BID to TID (Aptus Sentrx eye gel, Orion Pharma Animal

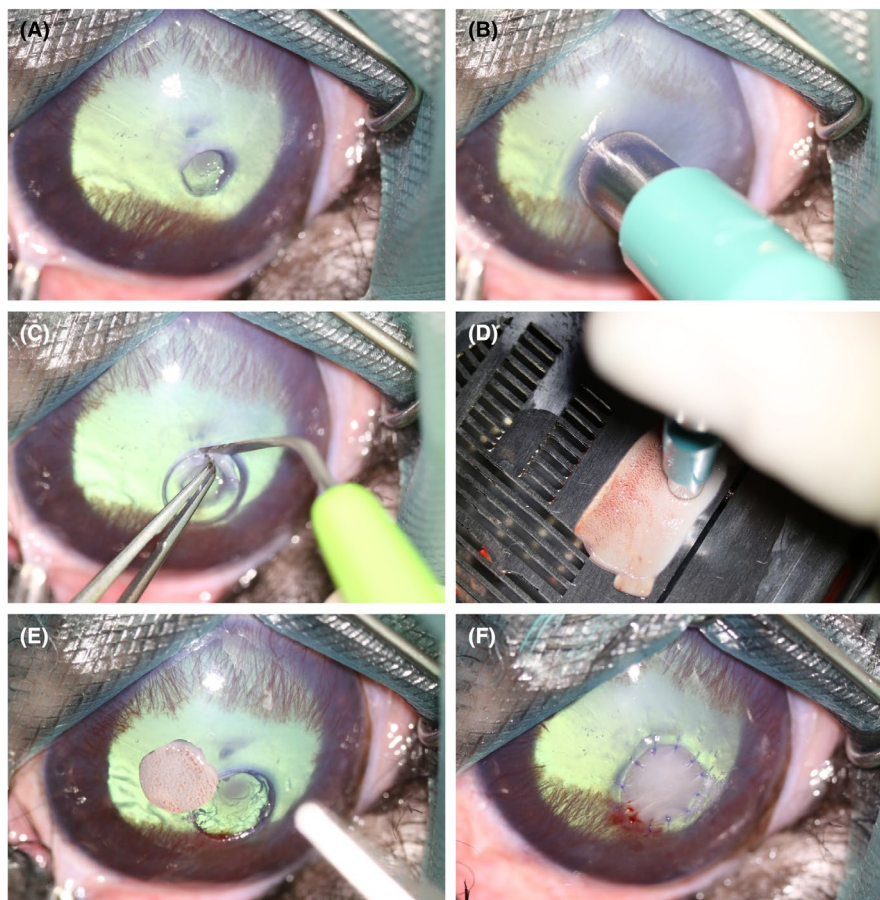


FIGURE 2 Surgical procedure as visualized under the surgical microscope. (A) After draping a colibri type of eyelid speculum was placed, and the ulcer centralized and fixed with two Halsted curved forceps situated in the perilimbal conjunctiva. (B) The stromal ulcer was debrided with eye spears (BVI) followed by incision with a disposable biopsy punch (Kai Medical) encircling the ulcer bed. (C) The devitalized corneal tissue was removed by superficial lamellar keratectomy with an angled crescent knife 2.2 mm (Sidapharm). (D) The graft was harvested from the PRF membrane resting in the PRF-Box[®] using the same biopsy punch as depicted in (B). (E) The graft was placed on the cornea. (F) The graft is in place in the ulcer bed immediately after completion of interrupted suturing with monofilament resorbable 10–0 suture (Luxcryl 10–0 PGA with spatulated needle, An-vision).

Health or I-Drop Vet, I-Med Animal Health). In addition to topical treatment, systemic pain and anti-inflammatory treatment were prescribed (Carprofen 4mg/kg SID PO [Carprodyl tablet, Ceva Animal Health A/S] or Meloxicam 0.1mg/kg SID PO [Meloxidyl suspension, Ceva]). All patients were provided with an e-collar postoperative (E-power, E-Vet).

At each follow-up visit, the patient received an ophthalmic examination consisting of a neuro-ophthalmic examination as described above, slit lamp biomicroscopy, fluorescein stain uptake, and tonometry (TonoVet XL, Reichert Tech. or TonoVet Plus, Icare, Tuik). The patients had at least one Schirmer tear test (STT; Ophthalmic strip, Optitech) performed at one of the follow-up visits. The ciprofloxacin was discontinued once the fluorescein stain was negative and the systemic pain medications were discontinued when the patient was estimated to be comfortable, at the corresponding

follow-up to these findings. The second follow-up visit included the removal of the tarsorrhaphy suture and sutures from any other additional surgeries. The corneal sutures were left in place for reabsorption. The only consistent medication after the second follow-up was an ophthalmic lubrication.

Clinical images were recorded at the first presentation and follow-up visits with a Leica Digilux-4 camera (Leica Cameras AG), an iPhone 11 Pro Max (Apple), or Nikon Z10 (Scandinavian Photo). Intraoperative images were recorded with a microscope-mounted Canon EOS 6D (Canon Electronics) or iPhone 11 Pro Max.

2.7 | Postoperative evaluation

The efficacy of the PRF membrane was assessed based on corneal healing time, vision, and graft opacification. The

corneal healing time measured in days was determined by corneal re-epithelialization and negative fluorescein stain uptake. Vision was assessed at the third follow-up by means of a positive menace response and was classified as “present” or “absent.” Graft opacification assessment was performed with a semi-quantitative scale at the third follow-up and a long-term follow-up visit. The graft opacification was subjectively scored into three grades: “mild” (grade 1), “moderate” (grade 2), and “severe” (grade 3). The amount of vascularization was assessed at the first presentation, at each follow-up, and the final long-term follow-up. Vascularization was subjectively scored as “absent” (grade 0), “poor peripheral” (grade 1), “moderate” (grade 2), and “severe” (grade 3). The graft opacification and vascularization grading were performed as a non-masked evaluation by the surgeon who had performed the surgery, and the score system has previously been used in two similar veterinary studies by Mezzadri et al. and Barechetti et al.^{18,19} A long-term follow-up was performed at least 6 months (>180 days) post-surgery with focus on corneal opacity and vascularization.

2.8 | Histologic analyses of PRF Membranes from healthy dogs not included in this case series

2.8.1 | PRF membrane, fibrinogen analysis, and platelet count

Two dogs (Dog A and Dog B) were used as donors for PRF membranes used in the histologic analysis of the PRF membranes, as well as for associated fibrinogen analysis. These two dogs were presented for routine health rechecks and their blood was used in this study with the owner's consent. An EDTA sample for platelet count and a citrate-stabilized blood sample for fibrinogen analysis were collected and submitted to the IDEXX laboratory (IDEXX Laboratory). Blood samples for PRF membranes were collected in Bio-PRF solid PRF tubes and PRF membranes were prepared according to the protocol described above. One sample of PRF membrane (4 mm in diameter) was prepared from Dog A; three samples of PRF membranes (4 mm in diameter) were prepared from Dog B. The four PRF membrane samples were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4, and stored in a fridge (2 to 8°C) until processing at Core Facility for Integrated Microscopy (University of Copenhagen, Denmark) for SEM. The remaining part of the PRF membrane from Dog A and Dog B were fixed in 4% buffered formaldehyde and sent for histological evaluation at the Department of Pathology's Diagnostic Center at Rigshospitalet.

2.8.2 | Histology

The formaldehyde fixed membranes were sectioned and processed according to routine paraffin technique. Sections were cut at 4 µm and mounted on glass slides. Deparaffinized hydrated sections were stained with hematoxylin and eosin (H&E) and evaluated by an ophthalmic pathologist in human medicine (SH).

2.8.3 | Scanning electron microscope (SEM)

The glutaraldehyde fixed PRF membrane samples from Dog A and Dog B were further processed with three rinses in 0.15 M sodium phosphate buffer (pH 7.4) and post-fixed in 1% OsO₄ in 0.12 M sodium cacodylate buffer (pH 7.4) for 2 hours. Following a rinse in distilled water, the specimens were dehydrated to 100% ethanol according to standard procedures and critical point dried (Balzers CPD 0.30) with CO₂. The membranes were subsequently mounted on stubs using double adhesive carbon tape (Ted Pella Inc.) as an adhesive and sputter coated with 6 nm gold (Leica, Vienna, ACE 200). The membranes were examined with a FEI Quanta 3D scanning electron microscope operated at an accelerating voltage of 2 kV.

For analysis purposes, 10 selected fibrin fiber diameter measurements in nanometers (nm) were obtained from Dog A (PRF-1 1) and each of the three PRF membranes from Dog B (PRF-2, -3, and -4). The broadest 10 fibrin fibers in each PRF membrane sample were subjectively selected for measurement. The median ± standard deviation (SD) was determined for all four groups.

2.8.4 | Cryo-scanning electron microscopy (cryo-SEM)

Freshly prepared PRF from Dog A were analyzed as a PRF membrane and a PRF clot. These two PRF tissue samples were sandwiched between two 100 µm cavity planchettes and cryopreserved in liquid N₂. The sandwiched planchettes were mounted in a planchette holder (Leica) under liquid N₂ and transferred to a cryo transfer shuttle (VCT100, Leica). The samples were cracked, sublimated for 10 min at -90°C (PRF membrane) and for 7 min at -90°C (PRF clot), and sputter coated (6 nm) with Carbon/Platinum (MED020, Leica). The two PRF membrane samples were evaluated and examined with a FEI Quanta 3D scanning electron microscope (FEI, Eindhoven) operated at an accelerating voltage of 2 kV with a cryo-stage temperature of -140°C. The cryo-SEM images were used to search for specific

cellular features in the PRF membrane and PRF clot in a descriptive manner.

3 | RESULTS

3.1 | Study population

Seven dogs were enrolled in this clinical study; two dogs had surgeries performed on the right eye (OD: oculus dexter), and five dogs on the left eye (OS: oculus sinister), with no bilateral cases. Five out of seven dogs were males (71%) and two dogs were females (29%); including male intact 57% (4/7), male neutered 14% (1/7), and female intact 29% (2/7). All breeds included in the study were brachycephalic breeds, including three shih tzu. Table 2 gives an overview of the seven dogs included in this study.

3.2 | Clinical findings

At surgery, mean $\pm$ SD age was 5.4 ± 2.9 years [range 1–9 years]. The mean $\pm$ SD for STT was OD 22.7 ± 4.9 mm/min and OS 22.4 ± 7.4 mm/min [range: OD 6–30 mm/min, OS 12–32 mm/min]. Predisposing factors for the corneal ulcerations included entropion, distichiasis, ectopic cilia, corneal foreign body, tear film dysfunction, and brachycephalic ocular syndrome. All dogs had unilateral stromal corneal ulcerations with a stromal depth ranging from 25% to 75% stromal loss and an ulcerated surface area involving less than 25% of the corneal surface (Table 3). The location was either axial (57%, 4/7), paraxial ventromedial (29%, 2/7), or paraxial dorsomedial (14%, 1/7) (Figure 3).

All seven PRF membrane surgical procedures were performed successfully. The quality of the PRF membrane was classified as “good” in six out of the seven dogs and “poor” in one out of seven dogs (dog #7). Dog #7 had two PRF membranes produced with the first membrane being

too small and thin and the second membrane sufficient in size but thin, transparent, porous, and friable. Thus, a double-layer PRF membrane was used in this case only. Figure 4 demonstrates a “good” quality PRF membrane versus the “poor” quality PRF membrane from Dog #7.

The mean healing time from surgery until no fluorescein stain uptake was 9 ± 5.5 days [range 5–19 days]. The mean time from surgery until the third follow-up was 36 ± 2.6 days. Dog #7 developed a descemetocoele on day 13 and required rescue surgery with a conjunctival pedicle graft. This rescue surgery was successful and all dogs (100%, 7/7) were visual at their third follow-up. When excluding dog #7, a mean healing time from surgery until no fluorescein stain uptake was 7.8 ± 3.8 days [5–14 days] (Figure 3).

A long-term follow-up 288 ± 44 days post-surgery [range 209–328 days] was performed on six dogs (Dog #7 was excluded due to its conjunctival pedicle graft). All dogs were still visual and had no complications from surgery, except Dog #4 had developed a small epithelial inclusion cyst at one of the suture locations. Evaluation of graft opacification and corneal vascularization revealed a decrease in graft opacification and corneal vascularization in all six dogs (100%) when the third follow-up and long-term follow-up were compared to the graft immediately post-surgery and vascularization at first presentation and first follow-up. Four dogs (Dog #1,4,5,6) had developed corneal pigmentation at their third follow-up. Of these four dogs, 75% (3/4: Dog #1,4,5) had less pigmentation at their long-term follow-up. One dog (1/4, 25%: Dog #6) had developed a mild increase in corneal pigmentation (Figure 5).

3.3 | Microbiology

All dogs (100%, 7/7) had a positive microbiology culture for a single (86%, 6/7) or two (14%, 1/7) types of bacteria.

TABLE 2 Data collected from patients included in the study.

Dog #	Breed	Age (years)	Gender	Eye	STT (OD, OS)	IOP (OD, OS)	Predisposing factors
1	Pug	4	MI	OD	24, 25	13, 22	Medial lower entropion
2	Shih Tzu	3	FI	OS	23, 23	22, 19	Ectopic cilia, distichiasis
3	CKCS	1	MI	OS	23, 25	23, 14	Corneal foreign body
4	Shi Tzu	9	MI	OS	14, 6	12, 13	Qualitative and quantitative KCS
5	Shi Tzu	7	MI	OD	30, 25	22, 21	Distichiasis
6	French bulldog	8	FI	OS	25, 28	32, 22	No underlying cause
7	French bulldog	6	MN	OS	20, 25	13, 14	No underlying cause

Abbreviations: FI, Female intact; IOP, Intraocular pressure; KCS, Keratoconjunctivitis sicca; MI, Male intact; MN, Male neutered; OD, Oculus dexter; OS, Oculus sinister; STT, Schirmer Tear Test.

Table 4 shows culture results as well as topical antibiotics used at their initial presentation, sensitivity testing for these initial presentation antibiotics, and sensitivity for ciprofloxacin used post-surgery.

3.4 | Paraclinical findings

3.4.1 | Fibrinogen analysis

Four of the dogs had a fibrinogen concentration within normal reference (1.4–2.6 g/L; Reference interval:

TABLE 3 The table gives an overview of the depth (stromal loss in %) and size (surface area in %) of the different corneal ulcerations found in the seven dogs.

Corneal ulceration–depth (stromal loss in %)	# of dogs
Less than 25% stromal loss	0/7
Between 25% and 50% stromal loss	1/7
Between 50% and 75% stromal loss	2/7
Above 75% stromal loss	4/7
Corneal ulceration–size (surface area in %)	# of dogs
Less than 25% of the surface area	7/7
Between 25% and 50% of the surface area	0/7
Between 50% and 75% of the surface area	0/7
Above 75% of the surface area	0/7

1.2–2.6 g/L according to IDEXX Laboratory). Two dogs had an elevated fibrinogen concentration: Dog #2 = 3.7 g/L and Dog #4 = 3.4 g/L. One dog (Dog #7) had a fibrinogen concentration below the normal reference range = 1.1 g/L. The fibrinogen concentration in the two donor dogs (Dog A and Dog B) was within the normal range at 1.3 g/L for both dogs.

3.4.2 | Platelet count

The platelet count was within normal limits for five dogs (235–359 K/ μ L; Reference interval: 148–484 K/ μ L according to IDEXX Laboratory). One dog had an elevated platelet count (Dog #4 = 555 K/ μ L), and one dog had a decreased platelet count (Dog #3 = 134 K/ μ L). The two donor dogs (Dog A and Dog B) had a normal platelet count of 298 K/ μ L and 285 K/ μ L, respectively.

3.4.3 | Histology

The histological examination of the PRF membranes from Dog A and Dog B showed a membrane made up of a dense component and a loose component of eosinophilic material (fibrin). In both membranes, cellular elements such as erythrocytes and lymphocytes were interwoven in the loose component of the fibrin (Figure 6).

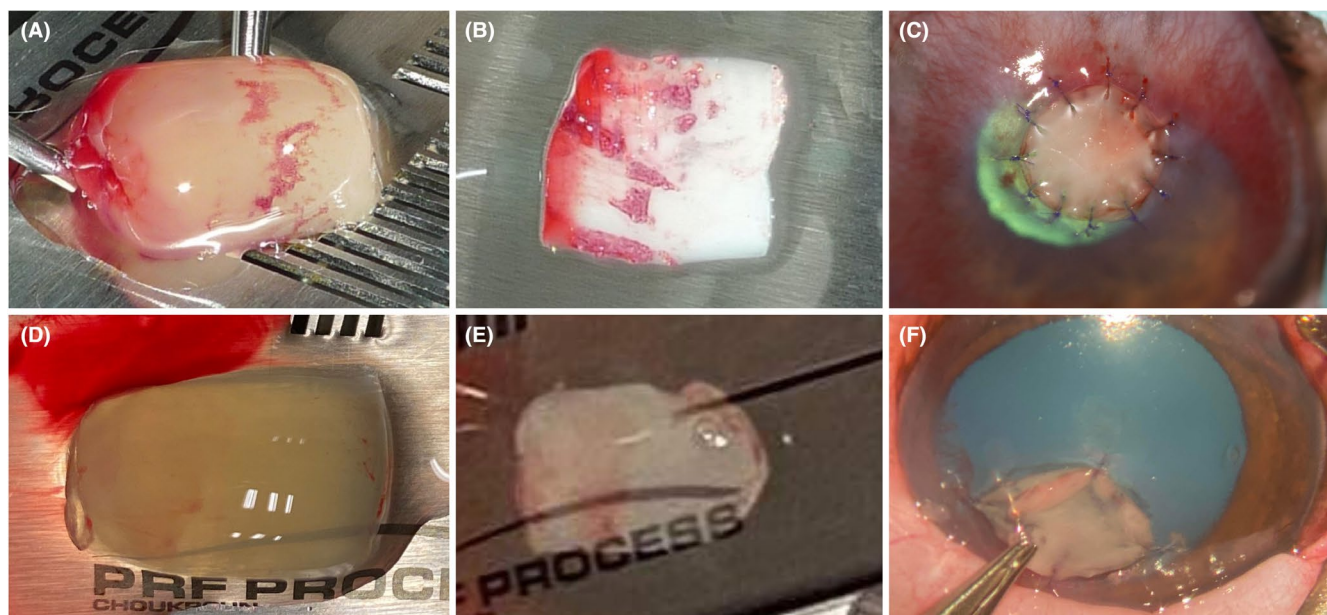


FIGURE 3 Presentation of platelet-rich fibrin (PRF) clots, membranes, and grafts with “good” and “poor” quality, respectively. (A) A good-quality PRF clot. Note the clot is non-transparent. (B) A good PRF membrane. Note the membrane is non-transparent and has a whitish appearance. (C) A good-quality PRF membrane graft sutured to the corneal ulcer bed. (D) A poor-quality PRF clot; note the clot is transparent. (E) A poor PRF membrane; note the membrane is transparent and thin. (F) A double-layer poor-quality PRF membrane graft sutured to the corneal ulcer bed. Note the broken upper layer of PRF membrane graft at the endpoint of the needle holder and thin appearance of both layers.

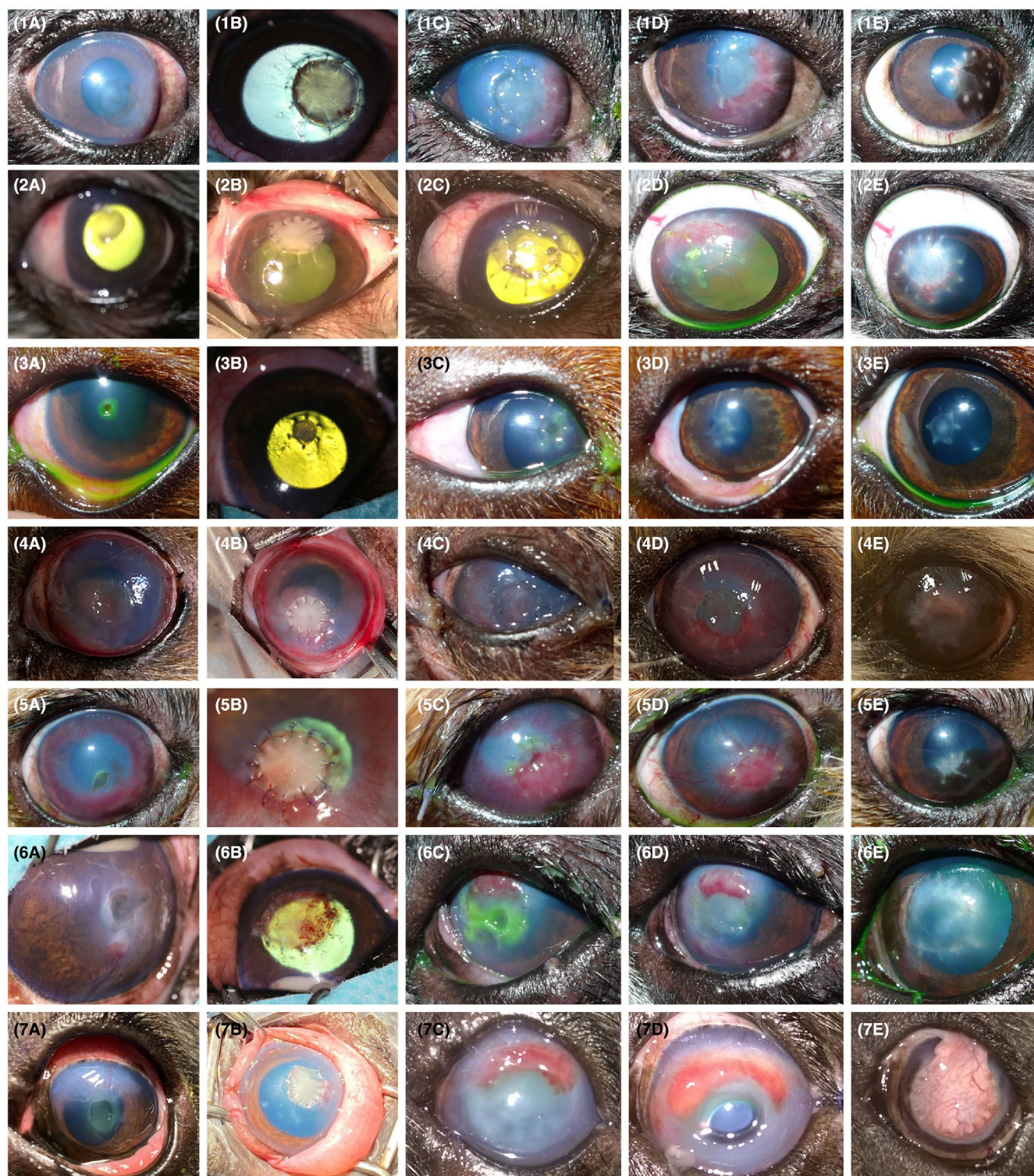


FIGURE 4 Clinical images of preoperative stromal ulcers and postoperative outcome for the seven dogs who underwent corneal reconstruction with platelet-rich fibrin membrane grafts. The letters correspond to different timepoints: (A) day 0, preoperative clinical presentation; (B) day 0, surgical reconstructed stromal ulcer with a platelet-rich fibrin membrane graft; (C) day 5–7 follow-up post-surgery (first follow-up); (D) day 12–14 follow-up post-surgery (second follow-up); (E) day 30–40 follow-up post-surgery (third follow-up).

3.4.4 | SEM

All of the PRF membranes evaluated showed areas with thick sheets of fibrin covering porous areas of long, thick,

and elongated fibrin fibers, following the same orientation. Large pores were seen between bundles of fibrin fibers. In areas without the thick sheet of fibrin, the bundles of fibers were directly visible. In certain areas, the fibrin fibers were

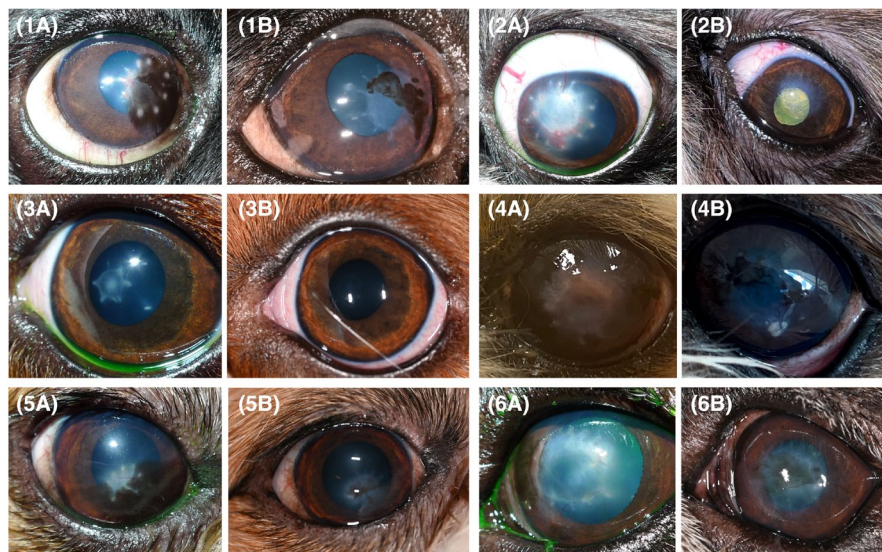


FIGURE 5 Clinical photographs of third follow-up (all [A] images) versus long-term follow-up visits (all [B] images) from Dog #1–6: 1A: Dog #1 39 days post-surgery, 1B: Dog #1328 days post-surgery, 2A: Dog #2 35 days post-surgery, 2B: Dog #2271 days post-surgery, 3A: Dog #3 35 days post-surgery, 3B: Dog #3302 days post-surgery, 4A: Dog #4 39 days post-surgery, 4B: Dog #4209 days post-surgery—notice the epithelial inclusion cyst that had developed at one of the suture locations at 6 o'clock, 5A: Dog #5 37 days post-surgery, 5B: Dog #5290 days post-surgery, 6A: Dog #6 35 days post-surgery, 6B: Dog #6325 days post-surgery.

TABLE 4 Result of microbiological, aerobic culturing, and sensitivity testing of the preoperative corneal sample.

Dog #	Bacteria	Antibiotic used before initial presentation	Antibiogram for the antibiotic used at initial presentation	Antibiogram for Ciprofloxacin used post-surgery
1	<i>Pseudomonas aeruginosa</i> (H)	None	NA	R MIC ≥ 4 mg/L
2	<i>Pasteurella species</i> (L)	None	NA	NA
3	<i>Staphylococcus pseudointermedius</i> (L)	Chloramphenicol	R ≥ 64 mg/L	S MIC ≥ 0.5 mg/L
4	<i>Pseudomonas aeruginosa</i> (L)	Chloramphenicol	R ≥ 64 mg/L	I MIC = 1 mg/L
5	<i>Escherichia coli, haemolytic</i> (H)	Fucidic Acid	NA	S MIC ≤ 0.12 mg/L
6	<i>Streptococcus canis</i> (L)	Chloramphenicol	NA	NA
7	<i>Streptococcus canis</i> (H) and <i>Serratia marcescens</i> (H)	Fucidic Acid	NA (<i>S. canis</i>), R (<i>S. marcescens</i>)	I MIC = 0.5 mg/L (<i>S. canis</i>), S MIC ≤ 0.12 mg/L (<i>S. marcescens</i>)

Abbreviations: H, High count; I, Intermediate; MIC, Minimum inhibitory concentration; L, Low count; NA, Not applicable; S, Sensitive; R, Resistant.

more compact with a small diameter of interfibrillar spaces. A high number of cross-linking fibers were present in the membranes of both dogs (Figure 6). Erythrocytes and leukocytes adhered to the surface in some areas and platelets were seen throughout most of the samples.

The mean fibrin fiber diameter was measured from both dogs (Figure 6). Dog A (PRF-1) had a fibrin fiber diameter of 159 ± 21 nm. The fibrin fiber diameter from each of the three PRF membranes from Dog B were PRF-2: 199 ± 37 nm, PRF-3: 212 ± 57 nm, and PRF-4: 164 ± 25 nm, respectively. The total fiber diameter for the four SEM-evaluated PRF membranes was 184 ± 94 nm.

3.4.5 | Cryo-SEM

A cryo-SEM processed PRF clot from Dog A showed sheets of fibrin. Thin walls of fibrin could be appreciated between crystallized fluid. Three-dimensional cross-linking fibers were observed between the fibrin walls (Figure 6).

4 | DISCUSSION

This study provides a thorough protocol for the preparation of a PRF membrane in a veterinary clinical setting.

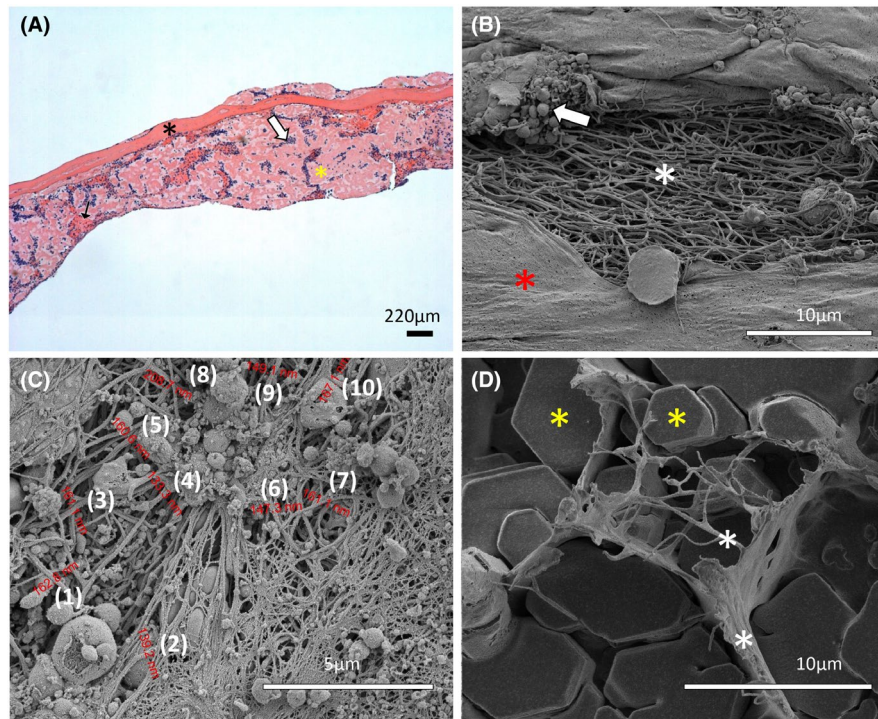


FIGURE 6 Paraclinical images of platelet-rich fibrin (PRF) membranes: (A) Histological evaluation of the PRF membranes shows a solid fibrin layer (black asterisk). Section of loosely fibrin fiber network with platelets (yellow asterisk). Leukocytes, predominantly lymphocytes (white arrow) and area with erythrocytes interwoven in the less condensed part of the fibrin (black arrow) (hematoxylin and eosin stain). (B) SEM image displays a thick sheet of fibrin (red asterisk) with openings into a porous area of long and thin elongated fibrin fibers (white asterisk). (C) The SEM images from Dog A and Dog B were used to measure the fibrin fiber diameter. The numbers (1) to (10) show the 10 areas on a membrane that were measured, and the red numbers were the actual diameter numbers (nm). (D) A Cryo-SEM image shows the three-dimensional cross-linking fibrin fibers (white arrow) and crystalized fluid (yellow asterisk).

The PRF membrane was used successfully in six out of seven dogs diagnosed with complicated corneal ulcerations. The study also showed the importance of evaluating the patient's fibrinogen concentration in the peripheral blood before the preparation of a PRF membrane. The only surgery that failed in this study was with a PRF membrane from a dog that had a fibrinogen concentration below the reference level.

The PRF membrane resulted in a corneal ulcer mean healing time of 8 or 9 days (with or without Dog #7, respectively). The healing time in this study is shorter when compared to results obtained with commercially available biomaterials. Studies in dogs using bovine and human amnion membrane grafts have demonstrated a mean epithelial healing time of 26 days, acellular porcine corneal stroma 10 days, porcine urinary bladder acellular matrix 18 days, and porcine small intestinal submucosa graft 15 days.^{20–23} The comparison is biased by different severity grades of the corneal ulceration ranging from stromal to perforated ulcerations. Vanore et al. used a third eyelid flap to protect the porcine small intestinal submucosa graft in six out of seven patients and Bussieres et al. used a conjunctival graft to cover their porcine small intestinal

submucosa graft.^{21,22} The exact healing time is, therefore, difficult to compare from study to study. Other studies with PRF membranes have revealed similar corneal epithelial healing times in dogs as in our study; these studies had healing times ranging from eight to 14 days.^{10,11}

The dogs that developed corneal pigmentation post-surgery were breeds known to be at risk of increased pigmentation, one was a pug with pigmentary keratopathy and the remaining two were Shih Tzus.² The corneal ulceration depth was greater than 50% in six out of the seven dogs in our study. A study regarding corneo-conjunctival transposition observed a significant correlation between the severity of the corneal ulceration and the post-surgery opacity.²⁴ The long-term follow-up visit from all six dogs (Dog #7 was excluded) showed a decrease in fibrosis (Figure 5). This scar remodeling with increased corneal transparency is known to occur for several months following surgery for stromal corneal ulcerations.³ Interestingly, the long-term follow-up also revealed a decrease in pigmentation in three dogs and a mild increase in one dog. This clinical result several months post-surgery was obtained without any immunosuppressive treatment in any of these dogs and could indicate that the PRF membrane

could have a remodeling and anti-inflammatory effect several months post-surgery. Future studies should evaluate whether the PRF membranes have a positive chronic effect on corneal pigmentation such as seen with pigmentary keratitis and pannus.

Microbiological, aerobic culturing, and sensitivity testing were performed to observe possible adverse effects on corneal healing and any possible antibacterial effect of the PRF membrane. *Pseudomonas aeruginosa* in one dog (Dog #1) was resistant to ciprofloxacin according to the culturing and antibiogram. Despite this, no increased bacterial keratitis was observed, and the corneal healing time was 5 days. Removal of the infected cornea with a lamellar keratectomy likely contributed to the speedy healing time despite the resistance to the postoperative antibiotics, but the PRF membrane also has an antibacterial effect that likely contributed to success in Dog #1. In a recent retrospective study of melting corneal ulcerations in dogs, 57% of enucleated eyes showed pure culture of *Pseudomonas aeruginosa* isolates and was considered the main reason for enucleation despite medical and/or surgical treatment.²⁵ Studies in dogs, rabbits, and humans have shown the PRF membrane to have a positive healing effect when used for infected ulcers, even when no topical antibiotics were used.^{11,14,26} Also, a human study found that the antibacterial effect in the horizontal spun PRF clot is superior to L-PRF.²⁶

Dog #7 was the only dog with an abnormally low systemic fibrinogen level. The quality of Dog #7's PRF membrane was also graded as "poor." It, therefore, appears that low fibrinogen levels may influence the quality of the PRF membrane. Whereas a PRF membrane of "good" quality (i.e. sufficient size, thickness, tensile strength, and non-transparent as in Figure 3) can lead to a successful surgical outcome as demonstrated in the other six cases in this series. Unfortunately, private practices do not typically have the ability to perform a fibrinogen analysis in the clinic; therefore, it is important that the surgeon evaluates the quality of the PRF membrane prior to committing to using it as a graft. Alternatively, Dog #7's PRF membrane could have dehisced due to an introduction of a second infection, as a new culture was unfortunately not performed before the second surgery. This is a limitation that prevents the study from making more direct correlations. The platelet count in the present study did not appear to have an impact on the observed vascularization and healing time. Dog #3 was a Cavalier King Charles Spaniel and could have thrombocytopenia as a breed-related disease. A microscopic blood smear analysis for macrothrombocytes was not performed but could have evaluated for giant platelet disorder in this dog.²⁷ Finally, the variation of growth factors may also contribute to the variation of

PRF membrane quality observed in the present study. Epidermal growth factor (EGF) contributes to the tensile strength and healing time, which was severely altered in dog #7.¹⁵ More studies are needed to investigate the cellular aspect of the quality of the PRF membrane.

The mean fiber diameter for all four PRF membranes from Dog A and Dog B was 184 ± 94 nm, which is very similar to a human study by Can et al.²⁸ Their fiber diameter was measured to 210 ± 80 nm using the leucocyte-PRF (L-PRF) protocol.^{28,29} Consequently, the protocol described in our study will prepare PFR membranes of the same acceptable quality as being described by Can et al.²⁸ The "horizontal spinning protocol" used in our study has been shown by Miron to create a more even distribution of platelets and leukocytes in the supernatant compared to the fixed angle centrifugation.⁶ The cryo-SEM evaluation in our study supports the three-dimensional composition of sheets of fibrin interconnected with a fibrin mesh and underlines the superior horizontal spinning. Histological examinations of the PRF membranes showed no significant differences between the two dogs (Dog A and Dog B).

The present case series was limited to a smaller number of enrolled patients, no control group, and being descriptive data. These limitations cause variabilities and a prospective study with a calculated sample size for the "test" and "control" group is recommended in the future. It was not possible to comment on the specific thickness of the PRF membrane in this study and a future study could look at the thickness of the PRF membrane in correlation to its dehydration time in the metal box (PRF-Box®).

In conclusion, the PRF membrane preparation with the "horizontal centrifugation protocol" resulted in an effective graft material for corneal ulceration reconstruction surgery with a surgical success of six out of seven dogs. The study shows the importance of performing fibrinogen analysis prior to surgery or for the surgeon to evaluate the quality of the PRF membrane prior to surgery as low fibrinogen appeared to result in inferior quality of the PRF membrane.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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