Ophtalmology

R&D studies

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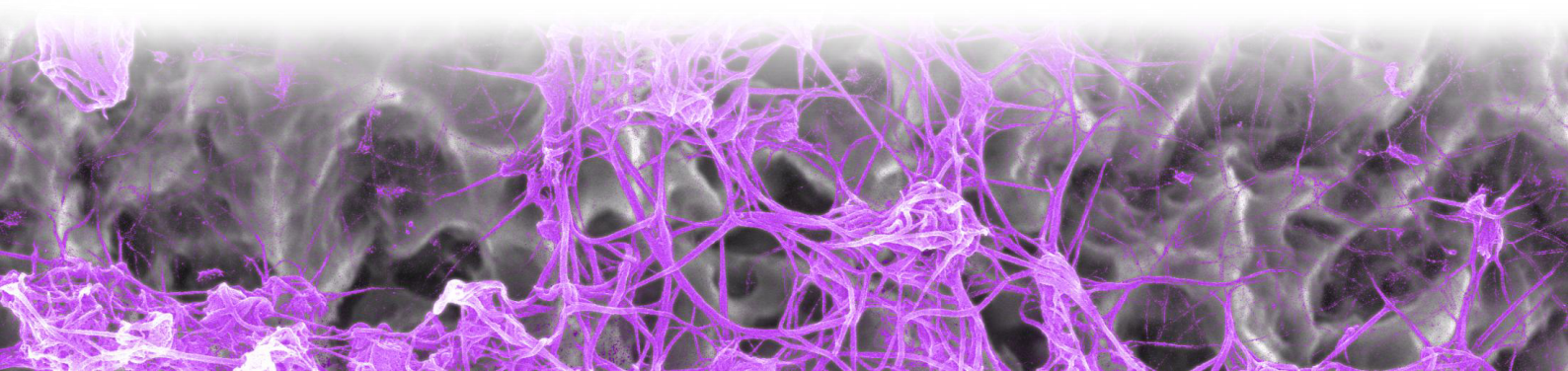

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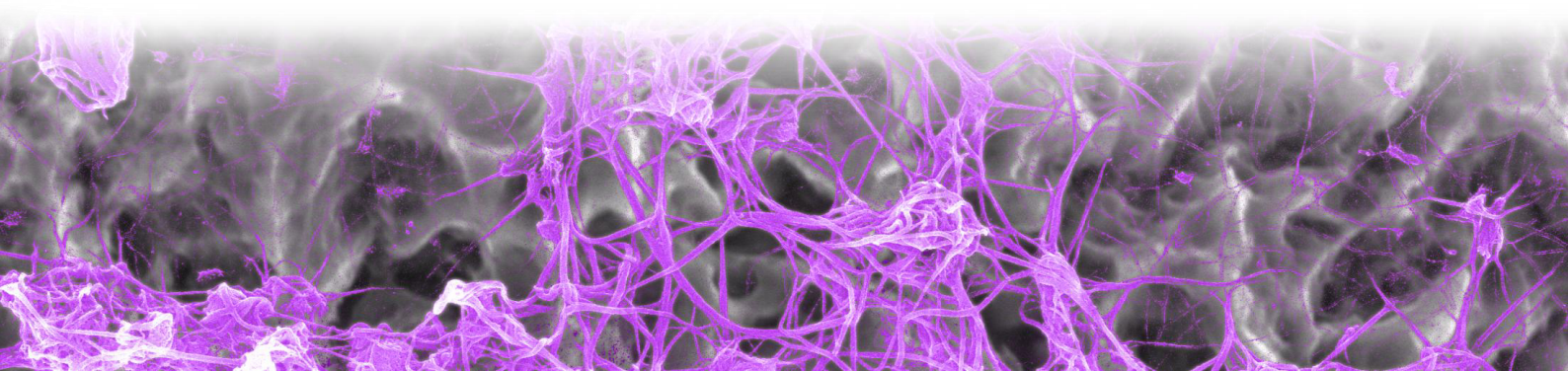

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Plasma Rich in Growth Factors as a Therapeutic Agent for Persistent Corneal Epithelial Defects

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Objective: To evaluate the efficacy of topically applied autologous plasma rich in growth factors (PRGF) as a treatment for persistent epithelial defects (PEDs) of the cornea.

Methods: A series of prospective noncomparative cases.

Participants: Twenty eyes from 18 patients with PED with various underlying etiopathologies: neurogenic, iatrogenic, associated with burning or secondary to severe dry eye. Patients were treated with a PRGF eyedrop solution. Serial photographs of the cornea were taken until epithelialization was complete. We had previously characterized the levels of a panel of growth factors (platelet-derived growth factor, epithelial growth factor, vascular endothelial growth factor, hepatocyte growth factor, fibroblast growth factor, and nerve growth factor) in the PRGF of 11 of these patients. The following variables were additionally recorded: (1) duration of PED before treatment, (2) previous treatments, (3) time for complete epithelialization, and (4) treatments required concomitantly with PRGF.

Results: Epithelial defects healed in 17 of 20 cases (85%), with a mean therapeutic time of 10.9 weeks (range 2–39 weeks). Mean progression time before treatment was 26.7 weeks (range 2–104 weeks). Growth factor concentrations were platelet-derived growth factor 12645.9 ± 1690.0 pg/mL, epithelial growth factor 468.9 ± 97.6 pg/mL, vascular endothelial growth factor 204.5 ± 119.4 pg/mL, hepatocyte growth factor 149.5 ± 173.5 pg/mL, fibroblast growth factor 82.6 ± 95.9 pg/mL, and nerve growth factor 37.7 ± 18.6 pg/mL.

Conclusion: PRGF, when applied as eyedrops, is a highly effective therapeutic agent for the treatment of a broad etiopathological spectrum of corneal PEDs.

Key Words: persistent epithelial defect, neurotrophic corneal ulcer, growth factor, plasma rich in growth factors

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Persistent epithelial defect (PED) of the cornea is defined as a lesion that measures more than 2 mm in diameter, persists for more than 2 weeks, and is resistant to conventional treatments.[1,2] The etiopathology of PED can be very variable, but the 2 principal causes are alterations in the tear surface and neurogenic dysfunctions.[1,3] Other causes include burns because of chemical agents, immunological alterations, dystrophies of the epithelium and basal membrane, metabolic alterations, iatrogenia, trauma, and infections.[1–3]

Not infrequently, PEDs do not respond to the application of conventional treatments such as artificial tears, therapeutic contact lenses, antiinflammatory agents, oral antibiotics, inhibitors of collagenolytic enzymes, or tarsorrhaphy. Resistant PEDs continue to degenerate, in many cases toward progressive stromal lysis and subsequent perforation; therefore new therapeutic alternatives are urgently being sought. Many of these consist of natural preparations, which are rich in growth factors or of these factors alone, derived by synthetic processes. Among the topically applied natural preparations, autologous serum has been used in recent years for the treatment of PED and dry eye with satisfactory results in most cases.[1,2]

More recently, a novel treatment has been proposed, consisting of plasma rich in growth factors (PRGF).[4–6] This treatment involves the application of autologous platelet protein extracts, which are rich in growth factors. However, the use of PRGF has been limited up to now to the repair of lesions of the skin, mucous membranes, and subcutaneous tissue in oral, maxillofacial, and orthopedic surgery. Thus, the objectives of the present article are to (1) describe the manner of preparation of PRGF, (2) characterize PRGF in terms of its levels of a panel of growth factors, and (3) analyze the clinical response of PED to PRGF treatment, thereby evaluating the possibility that this treatment may serve as a new efficacious and safe alternative to enhance reepithelialization.

MATERIALS AND METHODS

Patients

This study included 18 patients (20 eyes) diagnosed with PED, studied between 2004 and 2007 at the Instituto Clínico-Quirúrgico de Oftalmología (Bilbao, Spain). The mean age of patients was 61.2 years (range 33–88 years). Thirteen were men and 5 were women.

All patients gave their written consent after being informed about the treatment. Our study was approved by the Ethics Committee of the Instituto Clínico-Quirúrgico de Oftalmología, and the principles of the Helsinki Declaration

were complied with. The etiopathology of the epithelial defects was varied: neurotrophic ulcers (9; 6 nonherpetic and 3 herpetic), iatrogenic (6 cases; after ocular surgery), secondary to chemical burn (3), and secondary to severe dry eye (2).

The criterion for inclusion was PED with no improvement after conventional treatments, the latter including both medical (artificial tears, topical steroids and antibiotics, oral antibiotics or antiviral agents, therapeutic contact lenses, autologous serum, and so on) and surgical (amniotic membrane patch and eyelid surgery) treatments. Among the 20 eyes included in our study, 18 eyes (90%) had been receiving medical treatment consisting of artificial tears and an association of topical antibiotics and topical steroids. Two cases had also been treated with contact lenses and 2 with topical antiviral agents. Six patients had previously been treated with 20% autologous serum but without success. Surgical treatment had also been performed in 6 cases. Surgical approaches included lateral tarsorrhaphy, amniotic membrane patch, and lateral pexia of the inferior eyelid. Cases involving active corneal infection were excluded.

We decided that the application of PRGF did not need to preclude the use of other concomitant treatments (such as tarsorrhaphy, punctal occlusion, oral tetracyclines, antibiotics, antiinflammatory agents, and so on) when they were considered necessary. In addition, we included in the protocol the possibility of carrying out amniotic membrane transplant if at the end of a fortnight of treatment no objective improvement of the ulcer could be observed or if clinical features deteriorated at any stage.

PRGF Preparation

PRGF is a natural extract, which is obtained from the patient's own blood. The preparation process involves a 2- to 3-fold concentration of platelets found in patient blood by centrifugation, resulting in a liquid, which can be used as eyedrops for surface application. Fifty milliliters of whole blood was collected by venipuncture in 5-mL sterile tubes containing 0.5 mL of 3.8% sodium citrate. Samples were centrifuged at 460*g* for 8 minutes at room temperature. The upper fraction immediately above the erythrocyte pellet was then transferred to a sterile tube. Platelet activation and fibrin matrix formation were induced by adding calcium chloride to a final concentration of 22.8 mM, and clots were allowed to retract for 2 hours at 37°C. The released supernatant is rich in growth factors. This supernatant (PRGF) was diluted 1:1 with 0.9% sodium chloride, and 2.5 mL of this diluted PRGF were transferred into 5-mL sterilized eyedrop bottles. All procedures were carried out under highly sterile conditions, operating inside a laminar flow hood. Before initiating treatment, patients were instructed to keep the bottles at −20°C until required; the bottle in use was to be stored at 4°C and discarded after 5 days.[4]

PRGF Treatment Regime

A period of clearing before initiating PRGF treatment was not employed. PRGF was administered at a 1:1 dilution, initially at 1 drop every 2 hours (during daytime) for the first 3 days. Subsequently, the treatment regime was personalized as a function of the clinical evolution of each epithelial defect.

Ophthalmologic Evaluation

Visual parameters were not recorded for this study.

Measurement of Growth Factors in PRGF

The concentration of a panel of growth factors, which could be considered to be potentially therapeutic, was measured in the PRGF of 11 patients. Platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) were measured in undiluted PRGF using commercially available Quantikine colorimetric sandwich ELISA kits (R&D, Minneapolis, MN), according to the manufacturer's instructions. All enzyme-linked immunosorbent assays were performed in triplicate.

Evaluation Parameters

The following variables were analyzed: duration of PED progression before PRGF treatment; previous treatments (medical and/or surgical); duration of treatment until complete epithelialization; need for PRGF-associated treatments (medical and/or surgical); levels of PDGF, EGF, VEGF, HGF, FGF, and NGF in the PRGF of these patients. Means and standard deviations were calculated using Windows NT Excel software.

RESULTS

PRGF was characterized in terms of the concentration of the growth factors indicated in Table 1. The most abundant growth factor was PDGF, and the least abundant one was NGF.

The mean duration of PED progression before PRGF treatment was 26.7 weeks (range 1–104 weeks, with a median of 9 weeks). The epithelial defect was restored in 85% of cases (17 of 20 eyes), with a mean epithelialization time of 10.9 weeks (range 2–39 weeks, median of 5 weeks). Table 2 summarizes the relevant characteristics of the patients.

For the 3 cases in which the epithelial defect did not heal, follow-up time was 10, 12, and 22 weeks. In these cases, the etiopathology was neurotrophic; 1 was iatrogenic (case 9), and 2 were nonherpetic (posttrigeminal ablation, case 6 and postradiotherapy, case 18). Two cases required conjunctival flap (cases 9 and 18), and another was treated with a Boston keratoprosthesis (case 6).

PRGF was applied alone to 4 eyes (20% of cases). In the remaining cases, other medical and/or surgical treatments were employed during follow-up: topical antibiotics, topical corticoids, oral antiviral agents, oral tetracyclines, topical antiviral agents, amniotic membrane transplant, and lateral tarsorrhaphy.

The aim of the treatment with PRGF was epithelialization; once it was achieved, the treatment was discontinued except in cases 1, 2, and 3. Cases 2 and 3 correspond to PED associated with severe dry eye; when the PED was resolved, PRGF treatment was maintained in a prophylactic manner at concentration of 20%. In case 1, PED was secondary to surgical damage to both facial and trigeminal nerves; after various episodes of PED successfully treated with PRGF, we finally decided to maintain treatment continuously at a concentration of 20%.

TABLE 1. Concentrations of Growth Factors in Undiluted PRGF

Growth Factor	PDGF	EGF	VEGF	HGF	FGF	NGF
PRGF	12645.9 ± 1690.0	468.9 ± 97.6	204.5 ± 119.4	149.5 ± 173.5	82.6 ± 95.9	37.7 ± 18.6

Concentrations expressed as picograms per milliliter.

No change in corneal sensation was reported, except for patient 12, for whom anesthesia before PRGF treatment was replaced by a hypesthesia once epithelialization was complete. No differences were found regarding Schirmer test measures.

PRGF tolerance was good in 95% of cases (19 of 20). In case 18, PRGF had to be discontinued because of the patient's discomfort (redness and itching). No other complications associated with its use were detected. In particular, we had examined for the presence of overt infection and neovascularization because of the high content of VEGF in PRGF.

Slit-lamp images of representative cases are shown in Figures 1 and 2. A nonherpetic neurotrophic PED was observed in an 83-year-old man (case 15); the duration of the defect before PRGF treatment was 2 weeks. The defect measured 5.6 × 4.4 mm. Time to complete healing was 6 weeks, with PRGF being applied in the absence of other treatments (Fig. 1). An 84-year-old woman (case 17) developed a posttrigeminal ablation PED. The duration of the defect was 22 weeks. Time to complete healing was 7 weeks. PRGF was applied in conjunction with a topical antibiotic (Fig. 2).

DISCUSSION

PED is a serious eye condition because it frequently progresses to perforation of the cornea and can eventually lead to irreversible loss of vision. Despite having various causes, these defects share in common the incapacity of the cornea to close its damaged epithelium and thus constitute an important therapeutic challenge in daily clinical practice.[1,2,7,8] The forms of PED with worse prognosis have been reported to be those associated with severe dry eye and neurotrophic ulcers.[1] In the case of dry eye, the lack of tears results in growth factor deprivation; in the case of neurotrophic ulcer, factors necessary for neural metabolism, such as substance P and NGF, are absent.

The inefficacy of different treatments, such as artificial tears, topic antiinflammatories, oral tetracyclines, bandage contact lenses, or tarsorrhaphy, for the treatment of PED has driven the search in recent decades for new therapeutic approaches. Thus, the effects of individually applied synthetic growth factors, neurogenic factors, and natural extracts rich in both growth and neurogenic factors have been studied.[9] The growth factors used to favor epithelialization include EGF,[10] FGF,[9] PDGF,[11] NGF,[8] and fibronectin, a protein associated

TABLE 2. Characteristics of Patients With PED

Eye	Age	Sex	Diagnosis	Duration Before PRGF (Wk)	Time to Complete Healing (Wk)	Associated Treatments
1	33	M	Petic neurotrophic	29	23	AB, TS
2	53	F	PED associated with dry eye	78	11	AB, TS, OT
3	53	F	PED associated with dry eye	78	4	OT
4	88	M	Iatrogenic	3	10	AB, TS
5	47	M	Chemical burn	10	39	AB, TS, AMT
6	69	M	Petic neurotrophic	33	No epithelialization	AB, OT, AMT
7	60	M	Petic neurotrophic	104	2	No
8	60	M	Petic neurotrophic	104	2	No
9	60	F	Iatrogenic	11	No epithelialization	AB, TS, OAV, AMT
10	68	M	Postherpetic neurotrophic	2	5	AB, TS, OT
11	68	M	Postherpetic neurotrophic	20	2	No
12	55	M	Chemical burn	1	10	AB, TS, AMT, LT
13	45	M	Iatrogenic	3	4	No
14	40	M	Iatrogenic	1	2	No
15	83	M	Petic neurotrophic	2	6	No
16	60	M	Traumatic	3	1	AB
17	84	F	Petic neurotrophic	22	7	AB
18	61	M	Iatrogenic	2	No epithelialization	AB, TS, OT, AMT, LT
19	64	F	Postherpetic neurotrophic	8	2	TAV
20	63	F	Iatrogenic	20	10	No

AB, topical antibiotics; AMT, amniotic membrane transplantation; F, female; LT, lateral tarsorrhaphy; M, male; OAV, oral antiviral; OT, oral tetracycline; TS, topical steroids; TAV, topical antiviral.

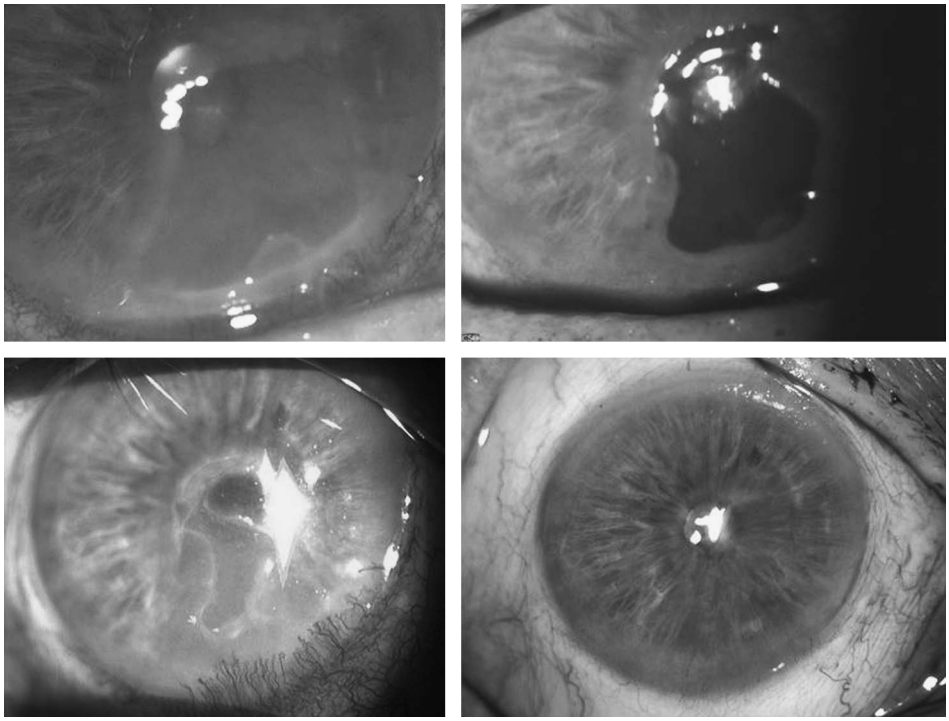

FIGURE 1. Slit-lamp images before and during treatment of the right eye of an 83-year-old man with a nonherpetic neurotrophic PED (case 15).

with the extracellular matrix.[12–14] Despite having achieved good initial results in animals, results in human patients have been somewhat contradictory and controversial to date.

One neurogenic factor that has been explored for PED therapy is substance P. This is a neurotransmitter, which is liberated by the peripheral autonomous nervous system and is present at high levels in nerve endings in the cornea. It acts in synergy with insulin-like growth factor 1 (IGF-1) and NGF.[8,15–18] Results from experiments with substance P applied with IGF-1 in humans are scarce. Recently, a study has been published reporting promising findings regarding the use of tetrapeptides derived from substance P and IGF-1.[19] NGF has been used in neurotrophic ulcers since 1998, with quite promising results; however, NGF treatment has still not yet been approved for use in humans. Nevertheless, the essential limitation of these treatments is that because of their mechanism of action, they are only applicable to PEDs of neurogenic origin.

As a result of the wide range of etiologies of PEDs, an ideal treatment would be that which provides the largest quantity and variety of growth factors. The search for natural extracts that are rich in these substances began as early as in 1940, with the application of amniotic membrane for conjunctival defects.[20] Amniotic membrane has since then been used for the treatment of PED with notable success. However, it has a number of disadvantages; on the one hand, it involves a surgical procedure, with all of the inconveniences that this implies for both the ophthalmologist and the patient, particularly when cases are chronic and involve long-term treatments. On the other hand, it is unknown if growth factors survive the process of cryoconservation implicit in this technique. Moreover, because amniotic membrane is not an autologous tissue, it also has the risk of being a source of currently undetectable pathogens.[21]

In contrast, autologous serum, obtained from the patient's own blood, has none of the aforementioned disadvantages. The use of autologous serum for the treatment of dry eye was reported in 1984. In 1999, it was proposed for the treatment of PED and dry eye, with satisfactory results.[1,2,22,23] More recently, the use of serum from fetal umbilical cord has also been reported.[3,24] The results are encouraging, but the technique presents the same disadvantages as amniotic membrane because such serum is a heterologous substance.

In more recent times, a novel treatment based on PRGF is being examined for its therapeutic potential. The technique involves the use of autologous platelet protein extracts, which are rich in growth factors.[4–6] However, it has been applied only to the repair of lesions of mucous membranes, skin, and subcutaneous ulcers in oral, maxillofacial, and orthopedic surgery. Our group reported the first case of PRGF application in ophthalmology,[25] and Alió et al[26] reported the use of a similar treatment, which was called autologous plasma rich in platelets. The principal difference between both treatments resides in platelet activation. Thus, in PRGF treatments, the physiological activation of platelets is achieved by adding calcium, whereas a controlled activation of platelets is not induced in treatments based on autologous plasma rich in platelets; instead, the platelet extract is frozen. After defreezing, platelets will lyse, releasing their growth factors. However, these are accompanied by lysosomal enzymes responsible for their degradation. We have measured the concentration of some growth factors in PRGF to attempt to correlate their presence with PED improvement. PDGF and EGF were found to be the most stable between patients; other factors were also

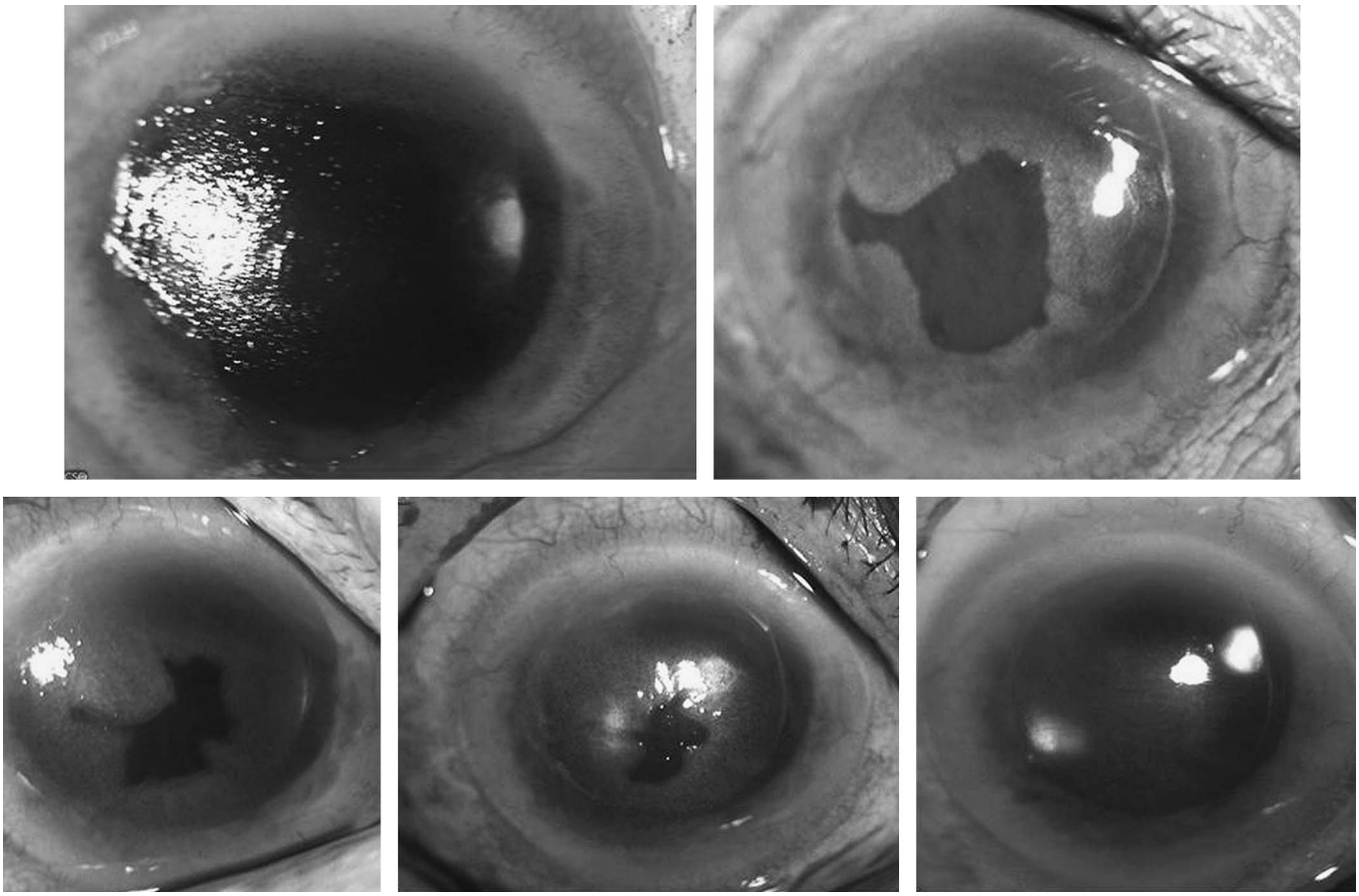

FIGURE 2. Slit-lamp images of an 84-year-old woman with a posttrigeminal ablation PED (case 17).

found to be present, but at highly variable levels (some factors were even undetectable in some patients), making their association with the therapeutic effect somewhat more difficult to correlate.

In our experience, the application of 50% PRGF is effective as a treatment for the reestablishment of the corneal epithelial surface in patients with PED of various etiologies, even in cases in which prior treatment with autologous serum was ineffective, as occurred with 6 patients in our study. PRGF can also be considered to be a cost-effective treatment because it avoids or at least diminishes the frequency of surgical interventions, which PEDs often require. Infection (mainly because of manipulation of the product by the patient) and neovascularization are 2 of the potential side effects of PRGF, but in our hands, neither of these was detected.

PRGF healing appears to be because of the presence of elements, which contribute to epithelial closure. Liu et al[27] quantified a variety of substances in different blood derivatives and concluded that the concentration of growth factors is higher when platelets are activated. Platelet activation may thus be the relevant difference between our treatment and that based on autologous serum. In our study, the concentration of growth factors in the extracts was found to be highly variable among patients, with HGF and FGF presenting most variation (Table 2). Despite the theoretical importance of this finding, its significance remains to be determined.

The diversity of the clinical types of PED involved in our study (such as age, etiology, severity, prior treatments, and associated treatments) makes it difficult to analyze in more detail the clinical efficacy of PRGF. Differences in etiology and clinical characteristics and the need for other additional therapeutic interventions in some cases, limited the homogeneity of our study group. For this reason, it is not possible in our study to establish a correlation between the etiopathology of the PEDs and their response to PRGF treatment. For the same reason, it is not possible to compare results among different treatments, as has been shown in previous studies.[28]

Nevertheless, the fact that our sample of patients was so heterogeneous and that healing took place in the majority of treated cases clearly indicates that PRGF is a promising and effective therapeutic agent for the treatment of a wide range of PEDs in ophthalmology.

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Efficacy of Plasma Rich in Growth Factors for the Treatment of Dry Eye

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Purpose: To evaluate the efficacy of plasma rich in growth factors (PRGF) for the treatment of moderate/severe dry eye.

Methods: PRGF treatment was administered to 16 patients who had moderate/severe dry eye diagnosed and who had not responded previously to other standard treatments. We quantified several growth factors present in the PRGF of each patient and obtained quantitative registers of the symptoms (modified score dry eye questionnaire), both before and after PRGF treatment. We also performed impression cytology to determine the degree of squamous metaplasia before and after PRGF treatment.

Results: PRGF treatment was associated with a statistically significant improvement in score dry eye questionnaire values ($P < 0.001$). Results from impression cytology corroborated this improvement, but the reduction in the degree of squamous metaplasia was not statistically significant. In 75% of patients treated with PRGF, no further treatments were required, whereas in the remaining 25% other ocular treatments could be reduced.

Conclusions: PRGF led to symptom improvement in patients with moderate/severe dry eye. Surprisingly, the symptoms recorded in the dry eye questionnaire do not always agree with the degree of squamous metaplasia measured by impression cytology.

Key Words: dry eye, plasma, growth factors, questionnaire, impression cytology, squamous metaplasia

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Dry eye is one of the most common ocular pathologies and frequently motivates the patient to seek clinical advice. However, despite advances in our understanding of its pathophysiology and in therapeutic options, the treatment of dry eye continues to be somewhat unsatisfactory because of essentially 3 reasons: first, the multifactorial etiology of dry eye; second, the lack of correspondence between symptoms reported by the patient and observable clinical parameters; and third, the commercial unavailability of a tear substitute that is ideal in terms of its composition and duration of effect.[1,2]

Tear film is a dynamic and complex solution with specific antimicrobial, mechanical, and optical properties. It includes a range of growth factors like epidermal growth factor, transforming growth factor beta, nerve growth factor, and others, as well as fibronectin and vitamins that promote the proliferation, migration, and differentiation of the corneal and conjunctival epithelium.[3] Ideal artificial tears should emulate the physicochemical properties of natural tears, in addition to having the highest possible retention capacity. They should also allow the addition of biological agents, which may be necessary for the metabolism of the cells of the ocular surface.

The initial reports of the efficacy of autologous serum for the treatment of dry eye promoted interest in the search for an ideal tear substitute.[4] The ensuing research not only corroborated the efficacy of autologous serum for the treatment of a variety of pathologies of the ocular surface[5–8] but also stimulated new applications for blood derivatives in ophthalmology. In recent years, further progress has been made by including factors derived from platelets in the composition of a novel blood derivative, that is, plasma rich in growth factors (PRGF).[9] This product is being successfully applied in a variety of medical areas, such as maxillofacial surgery, traumatology, and so on.[10,11] More recently, its application in ophthalmology for the treatment of persistent epithelial defects has been reported.[12,13]

This study represents a first approach to evaluate the efficacy of PRGF for the treatment of different dry eye forms in terms of the resolution of symptoms, which were evaluated subjectively by means of a questionnaire about dry eye symptoms. We also measured the efficacy in terms of improvements in specific clinical parameters. Because previous studies have indicated that impression cytology is one of the objective methods that best correlates with symptoms perceived by the patient,[14] we used this test as a principal objective marker.

PATIENTS AND METHODS

Patients

We studied 16 patients (15 women and 1 man) who presented with moderate/severe dry eye at the Instituto Clínico-Quirúrgico de Oftalmología (Bilbao, Spain). The mean age of the population was 64.63 ± 12.21 years. We evaluated both the severity of the perceived symptoms and the gravity of the clinical features. All these patients had presented a weak or null

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response to conventional treatments, which had been applied previously. Following the recommendations of the Helsinki Declaration and approval by the Instituto Clínico-Quirúrgico de Oftalmología Ethical Committee, we obtained the informed consent of these patients before commencing the study.

The pathophysiological classification of patients according to the type of dry eye is represented in Table 1. In summary, the population consisted of patients with non-Sjögren-type aqueous tear–deficient dry eye syndrome (n = 5), Sjögren syndrome (Sjögren-type aqueous tear–deficient dry eye syndrome) (n = 4), meibomian gland dysfunction (n = 4), and mixed pathology (non-Sjörgren-type aqueous tear–deficient dry eye syndrome plus meibomian gland dysfunction) (n = 3). This population could be considered to be a representative sample of the distinct pathologies, which can be found in the clinical practice.

PRGF Preparation and Treatment Regime

Whole blood was collected (30 mL) by venipuncture in 5-mL sterile tubes containing 0.5 mL of 3.8% sodium citrate. Samples were centrifuged at 460*g* for 8 minutes at room temperature. The resulting plasma was recovered, and platelets were activated with 22.8-mM calcium chloride as reported elsewhere.[9] After the formation of a clot and the liberation of the growth factors, the supernatant was recovered and diluted to 20% with 0.9% sodium chloride. This diluted PRGF (2.5 mL) were transferred into 5 mL sterilized eyedrop bottles. All procedures were performed under highly sterile conditions, operating inside a laminar flow hood.

Before initiating the treatment, the patients were instructed to keep the bottles at −20°C for a maximum of 3 months; the bottle in use was to be stored at 4°C and used for 5 to 7 days. The solution was to be applied 4 times per day.

Measurement of Growth Factors in PRGF

The concentration of a panel of growth factors that could be considered to be potentially therapeutic was measured in the PRGF of patients. Platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor, hepatocyte growth factor, and fibroblast growth factor were measured in undiluted PRGF using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN), following the manufacturer's instructions. All enzyme-linked immunosorbent assays were performed in triplicate. Means and SDs were calculated using Windows NT Excel software (Microsoft, Redmond, WA).

Clinical Follow-up of Patients

We evaluated patient discomfort using a questionnaire based on the score dry eye questionnaire (SDEQ)[15] modified according to the demographic characteristics of the region, both before PRGF treatment and 3 months later. In this questionnaire, the patient evaluated each of the following dry eye symptoms using a scale of 0 to 4: dryness, sensation of foreign body, stinging, pain, itchiness, photosensitivity, blurred vision, and redness (total 32 points). We have defined moderate/severe dry eye based on the SDEQ (score >10) and on the absence of improvement with conventional previous treatments (unpreserved artificial tears, punctal plugs, lid hygiene, systemic tetracycline, and/or unpreserved topical steroid). We also performed a biomicroscopic exploration including staining with Lissamine green (van Bijsterveld scheme[16]) and the Jones test, both at the beginning and end of the first treatment with PRGF.

Conjunctival Impression Cytology Follow-up/ Monitoring

We performed impression cytology on the first visit and after 3 months of PRGF treatment. These analyses were

TABLE 1. Etiopathological Characteristics, Previous Treatments, Effects of Treatments on Symptoms, and the Degree of Squamous Metaplasia in Each of the Patients Included in This Study

Patient Number	Age (Yr)	Sex	Diagnosis	Previous Treatment	Symptoms Test		Squamous Metaplasia	
					Pretreatment	Posttreatment	Pretreatment	Posttreatment
1	72	M	MGD	T, TC, OT, CS	22	4	0	0
2	69	M	MGD	T	26	22	2	2
3	69	M	MGD	T, TC, OT, H	18	8	2	1
4	63	M	MGD	T, TC, H, OT	16	17	1	0
5	86	M	Non-SS ADDE	T	18	4	3	2
6	58	M	Non-SS ADDE	T	31	16	3	3
7	82	M	Non-SS ADDE	T, TP	20	15	1	1
8	69	M	Non-SS ADDE	T, TC	11	12	2	2
9	63	M	Non-SS ADDE	T	13	6	1	0
10	77	M	SS ADDE	T, TC, LP, OT	18	12	1	1
11	63	M	SS ADDE	T, TC	23	16	3	3
12	68	M	SS ADDE	T	16	15	2	0
13	45	M	SS ADDE	T, TC	24	11	3	3
14	41	V	Non-SS ADDE + MGD	T	11	1	3	0
15	55	M	Non-SS ADDE + MGD	T	13	9	2	1
16	54	M	Non-SS ADDE + MGD	T, TC, H, OT	14	5	2	0

CS, conjunctival surgery; H, hygiene of the eyelids; MGD, meibomian gland dysfunction, Non-SS ADDE, non–Sjögren-type aqueous tear–deficient dry eye syndrome; OT, oral tetracyclines; SS ADDE, Sjögren type aqueous tear–deficient dry eye syndrome; T, tears; TC, topical corticosteroids; TP, tear plugs.

TABLE 2. Concentrations of Growth Factors in Undiluted PRGF

Growth Factor	PDGF	EGF	VEGF	HGF	FGF
PRGF	15,097.7 ± 3299.4	479.8 ± 113.9	91.5 ± 44.5	36.0 ± 17.3	32.6 ± 27.3

Concentrations expressed as picograms per milliliter.
EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

independent from the SDEQ results. Samples were obtained by instilling a drop of topical anesthetic (0.1% tetracaine chlorhydrate + 0.4% oxybuprocaine chlorhydrate) and a small strip of filter paper (Millipore HAWP3O4 de 5 × 5 mm, Millipore Corporation, Billerica, MA) was placed on the superior, inferior, temporal, and nasal bulbar conjunctiva, applying light pressure for 3 seconds. These strips containing samples were then fixed in absolute ethanol and stained following the protocol described.[17] Photographs of ocular surface samples were taken at ×200 magnification.

Although the process of squamous metaplasia is continuous, we divided it into different degrees. We used the criterion proposed by Oroza[18] to diagnose 4 degrees of squamous metaplasia in the samples obtained by impression cytology. This scale of 0 to 4, with degree 0 representing normal, coincides with the clinical grading proposed by Murube,[19] in which degree 1 denotes slightly altered, 2 represents moderately altered, 3 represents severely altered, and 4 denotes completely degenerated and keratinized.

Statistical Analysis

Means and SDs of all variables were calculated using Windows NT Excel software (Microsoft). Scoring on the questionnaire and the degree of pretreatment and posttreatment squamous metaplasia were compared using the paired 2-tailed Student *t* test to determinate the degree of statistical significance.

RESULTS

A summary of the clinical characteristics of the patients and the treatments that they had received before inclusion in this study is represented in Table 1. PRGF was administered as a single treatment in 12 patients (75%). In 2 patients, it was combined with palpebral hygiene and in 2 others it was combined with tear plugs.

Regarding the duration of PRGF treatment, 13 of the 16 patients were administered only 1 treatment cycle (3 months). The remaining 3 patients continued with the treatment for a mean of 10.66 ± 4.04 months (range, 7–15 months). Of the 13 patients who received only 1 cycle of PRGF treatment, 2 had to be retreated because of worsening of symptoms after having finalized the first cycle of treatment.

The concentrations of growth factors in undiluted PRGF of each patient were quantified. The corresponding mean and SD values are shown in Table 2.

PRGF treatment led to an improvement in the symptoms of dry eye in the majority of patients in this study (Table 1). Thus, these patients had reduced scores in the modified SDEQ 3 months after PRGF treatment, indicating symptom improvement after treatment. The mean questionnaire score before treatment (18.38 ± 5.68) was significantly different ($P < 0.001$) from the mean score after treatment (10.81 ± 5.84) (Fig. 1A).

Improvements, as measured in the questionnaire, could be classified as minimal or without change (<25% score reduction), moderate (score reduction between 25% and 50%), or substantial (reduction >50%). The improvement was minimal in 25% of patients, moderate in 31.25%, and substantial in 43.75% of patients.

Regarding biomicroscopic exploration tests, the mean value of the Jones test (Schirmer with anesthesia at 5 minutes) was 4.67 ± 5.14 before treatment and 6.91 ± 6.36 after PRGF treatment. The mean obtained with lissamine green dye according to the van Bijsterveld scale was 3.31 ± 2.96 before treatment and 3.07 ± 2.60 after PRGF treatment.

We found no cases of poor tolerance or undesirable effects that could be attributed to the use of PRGF. Conjunctival samples analysis by impression cytology revealed a mean squamous metaplasia grade of 1.94 ± 0.93 before treatment and 1.19 ± 1.17 after treatment, but this difference was not statistically significant (Fig. 1A). We found that, in general, reduced symptoms reported by patients were associated with a reduction in the grade of squamous metaplasia after PRGF treatment (Fig. 1B), but this association was not statistically significant either. Thus, in 8 of 16 patients

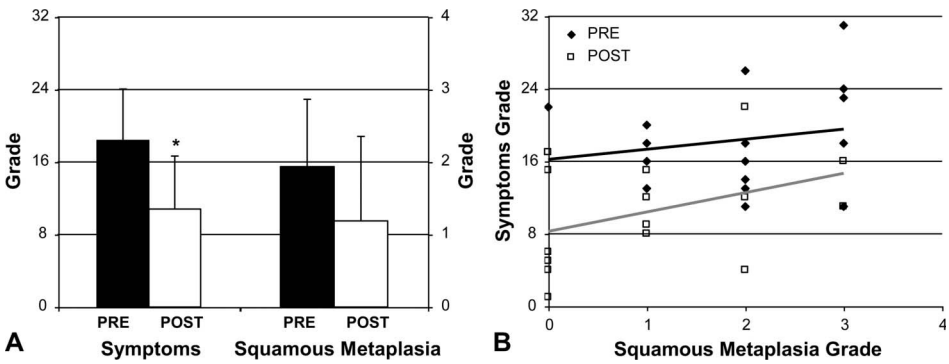

FIGURE 1. Effect of PRGF as a treatment for moderate/severe dry eye. A, Mean ± SD of the degree/grade of symptoms expressed by the patient in the modified SDEQ and of the degree of squamous metaplasia before and after treatment. B, Correlation between symptomatology and squamous metaplasia before and after PRGF treatment.

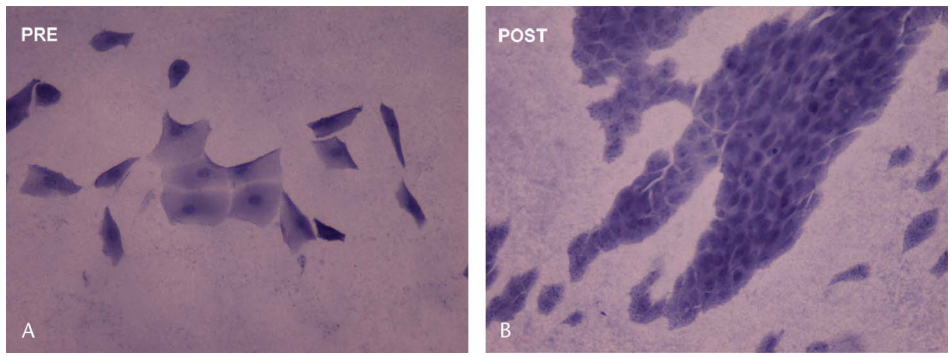

FIGURE 2. Conjunctival impression cytology of a 41-year-old man with non-Sjögren-type aqueous tear–deficient dry eye syndrome plus meibomian gland dysfunction. The improvement expressed in the evaluation of symptoms (from 11 to 1) corresponds with the reduction observed in the degree of squamous metaplasia from 3 (A) to 0 (B) after PRGF treatment.

(50%), symptom reduction was found to be directly proportional to the observed reduction in metaplasia (Table 1). However, in the other 50% of patients, a direct correlation was not found; 3 of the 16 patients had improved symptoms but in the absence of an improvement in metaplasia grade, whereas 5 of the 16 patients reported symptoms that did not correspond with the low grade of metaplasia observed in the impression cytology. In Figures 2 to 4, the 3 situations described are shown.

DISCUSSION

Dry eye is a condition involving a multifactorial alteration of the tear film, leading to an alteration of the ocular surface and the presence of symptoms that may affect visual function and the quality of life of the patient.[20] Objective tests that are currently at the disposal of the clinical practitioner frequently do not establish a correlation between damage to the ocular surface and symptoms.[21–23] Thus, both of these parameters should be kept in mind during the diagnosis and when deciding the optimal therapeutic approach.

Because different types of dry eye often can be associated with the same symptoms, it is necessary to correctly identify the principal etiopathogenic factors involved, to administer the most appropriate treatment. Thus, the use of tear plugs, topical steroids and immunosuppressors, mucolytics, secretagogs, and so on will be more or less appropriate, depending on the nature of each case, but the essence of therapy, common to all cases, will always be the substitution of the defective tear with a product that is as similar as possible to the natural tear. Herein lies the principal therapeutic limitation; currently, such an ideal substitute for the tear does not exist.

A number of artificial tear–related pharmaceutical products are available (carboxymethylcellulose, hyaluronic acid, polyvinyl alcohol, and so on). Despite the fact that currently these rarely include chemical preservatives, another class of compound known as conservation stabilizers is invariably present, making them potentially toxic for the ocular surface. In addition, tissue growth and repair factors and antimicrobiological properties are absent from these artificial tears.[24]

The principal revolution in the search for a substitute for the natural tear came when Fox et al[4] reported the application of autologous serum for the treatment of dry eye. Subsequent publications motivated more extensive clinical use of autologous serum as a therapeutic agent for cases of severe or very symptomatic dry eye, as well as for cases of persistent epithelial defects and other pathologies of the ocular surface. These studies demonstrated the superior capacity of autologous serum to maintain the integrity of the corneal and conjunctival epithelial cells, as well as to regenerate the ocular surface, because of its contribution of growth factors, whose presence in these diseases of the ocular surface are very much diminished.

In early 2000, a deeper understanding of platelets as a principal source of the growth factors present in plasma promoted growing interest in the elaboration of autologous blood derivatives, which are rich in platelet factors.[25–27] In vitro studies demonstrated that the growth of corneal epithelial cells is stimulated more by such blood derivatives than by autologous serum.[25] Among the blood derivatives, which

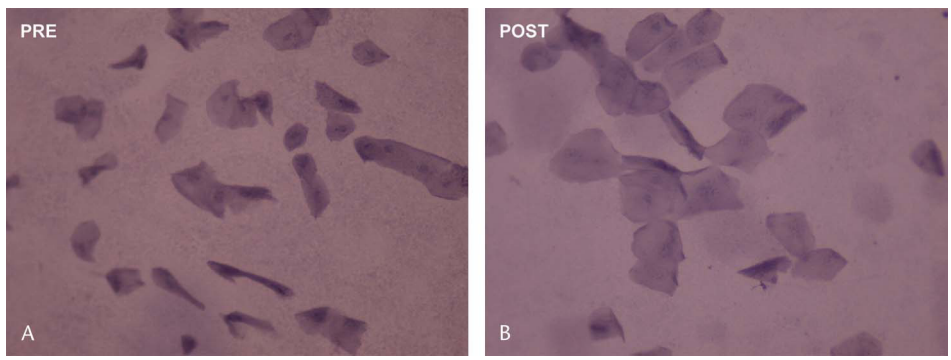

FIGURE 3. Conjunctival impression cytology of a 45-year-old woman with Sjögren type aqueous tear–deficient dry eye syndrome. The metaplasia grade before PRGF treatment was three (A). The improvement expressed in the modified SDEQ (from 24 to 11) did not correlate with the metaplasia grade observed after PRGF treatment (B), which remained fixed around 3.

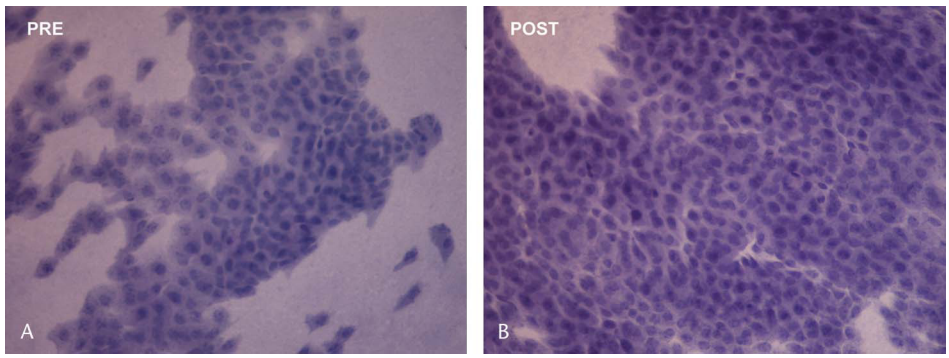

FIGURE 4. Conjunctival impression cytology of a 72-year-old woman with meibomian gland dysfunction. The intensity of symptomatology improved substantially after treatment (22 pretreatment vs. 4 post-treatment), but the metaplasia grade does not correspond with observations because metaplasia is unaltered not only after treatment (B) but also before treatment (A). Thus, the symptomatology expressed by the patient before treatment is not consistent with the images obtained by impression cytology.

include platelet factors, PRGF could be singled out because this plasma is enriched in growth factors, which are liberated from the platelets during the process of preparation of the plasma.[11] PRGF is now being successfully applied in multiple fields of regenerative medicine, with maxillofacial surgery and traumatology being the pioneer fields.[9,10] In light of all this, our group became interested in evaluating the potential application of PRGF for our field of ophthalmology. Thus, we successfully applied PRGF for the treatment of post–laser in situ keratomileusis flap necrosis in 2007.[12] In a subsequent study, we demonstrated its efficacy for the treatment of persistent epithelial defects of different origin.[13]

In the present study, we obtained preliminary results concerning the efficacy of PRGF as a treatment for dry eye. To this end, we studied a group of patients with dry eye of various etiologies in whom previous conventional treatments had not been efficacious. The group of patients, which represents different etiopathological types, comes from the general population. The prevalence of Sjögren-type dry eye syndrome in this group is higher because of the symptom severity and the absence of response of these patients to conventional treatments. Thus, the data that we present here could reasonably be extrapolated to the general population of patients with moderate to severe dry eye who do not respond to conventional treatment.

The results indicate that PRGF is an efficacious therapeutic agent for the treatment of patients who present with moderate to severe dry eye, not only because we observed symptom improvement in the majority of patients but also because in 62.5% of patients the signs of squamous metaplasia after treatment, observed by impression cytology, were absent or very light. The efficacy and good tolerance of PRGF are also corroborated by the fact that patients often request continued use of PRGF.

It is also important to underline the fact that in many cases, PRGF treatment not only leads to symptom improvement and reduced squamous metaplasia but also leads to the reduction of associated therapies, such as topical corticoids and cyclosporine. This is likely because of an indirect reduction of inflammation on reducing tear osmolarity and the dilution of proinflammatory factors, which are present in the ocular surface. It also may be because of the presence in PRGF, as in autologous serum, of potential inhibitors of inflammation, such as the interleukin-1 receptor antagonist and inhibitors of metalloproteinases[10] and other important growth factors, which are known to participate in corneal reepithelialization, such as epidermal growth factor (Table 2).

The results of this study allow us to conclude that PRGF is an interesting therapeutic alternative for the treatment of moderate to severe dry eye when compared with other commercially available agents. Because experience with the application of this blood derivative accumulates, more specific guidelines regarding the concentrations and treatment protocols will be required. In comparison to other similar plasma derivatives, our results show that PRGF is most enriched in growth factors, thus conferring additional value on this therapeutic proposal.[28] However, there are some limitations in the study. In this preliminary study, we included patients with different pathologies. In an ongoing study, we are evaluating the role of topical PRGF in the treatment of the specific types of dry eye.

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Plasma Rich in Growth Factors (PRGF-Endoret) Stimulates Proliferation and Migration of Primary Keratocytes and Conjunctival Fibroblasts and Inhibits and Reverts TGF-β1–Induced Myodifferentiation

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Purpose. Plasma rich in growth factors (PRGF-Endoret) technology is an autologous platelet-enriched plasma obtained from patient's own blood, which after activation with calcium chloride allows the release of a pool of biologically active proteins that influence and promote a range of biological processes including cell recruitment, and growth and differentiation. Because ocular surface wound healing is mediated by different growth factors, we decided to explore the potential of PRGF-Endoret technology in stimulating the biological processes related with fibroblast-induced tissue repair. Furthermore, the anti-fibrotic properties of this technology were also studied.

Methods. Blood from healthy donors was collected, centrifuged and, whole plasma column (WP) and the plasma fraction with the highest platelet concentration (F3) were drawn off, avoiding the buffy coat. Primary human cells including keratocytes and conjunctival fibroblasts were used to perform the "in vitro" investigations. The potential of PRGF-Endoret in promoting wound healing was evaluated by means of a proliferation and migration assays. Fibroblast cells were induced to myofibroblast differentiation after the treatment with 2.5 ng/mL of TGF-β1. The capability of WP and F3 to prevent and inhibit TGF-β1–induced differentiation was evaluated.

Results. Results show that this autologous approach significantly enhances proliferation and migration of both keratocytes and conjunctival fibroblasts. In addition, plasma rich in growth factors prevents and inhibits TGF-β1–induced myofibroblast differentiation. No differences were found between WP and F3 plasma fractions.

Conclusions. These results suggest that PRGF-Endoret could reduce scarring while stimulating wound healing in ocular surface. F3 or whole plasma column show similar biological effects in keratocytes and conjunctival fibroblast cells. (*Invest Ophthalmol Vis Sci.* 2011;52:6066–6073) DOI:10.1167/iovs.11-7302

Two tissues compose the ocular surface: the cornea and the conjunctiva. Both provide important functions to the eye including ocular protection, lubrication, and refractive power. After an injury, the main layers affected in both tissues are the stratified epithelium, the basement membrane, and the stroma. The stroma is one of the most important layers involved in wound healing, and it is composed mainly of fibroblasts. Keratocytes are the specialized corneal fibroblasts characterized by their low activity (quiescent) and their distribution through the stroma. Some of their main functions include maintaining the corneal transparency and producing stromal components such as collagen fibers and extracellular matrix. It has been reported that to effectively repair a damaged area after an injury, the fibroblasts adjacent to the injury need to proliferate and migrate to repopulate the area.[1,2] These processes are in part mediated by different growth factors such as epithelial growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor β1 (TGF-β1),[3–6] among others.

In some types of wounds these fibroblasts may develop actin contractile filaments, being differentiated into myofibroblasts.[7,8] During injury repair, myofibroblasts are responsible for wound contraction and extracellular matrix (ECM) deposition and organization. TGF-β has been identified as one of the main inductors of fibroblast differentiation into myofibroblasts.[9,10] However, the persistence of myofibroblastic cells after wound healing has been identified as the primary biological episode responsible for the development of scarring tissue.[11,12] The presence of fibrotic tissue at the anterior surface of the eye after an injury or a surgery may induce the opacification of the cornea (corneal haze),[13,14] or may lead to surgical failure.[15,16]

Different approaches to regenerate the ocular surface injury[17,18] and to treat the scar formation have been attempted.[19–21] One interesting alternative is to evaluate the potential of autologous plasma and platelet-derived growth factors in stimulating fibroblast-modified wound healing. The technology of plasma rich in growth factors (PRGF-Endoret, trademarks for Europe and USA, respectively) consists of the elaboration and use of a platelet-enriched plasma obtained from patient's own blood, which after activation with calcium chloride allows the in situ formation of a biodegradable fibrin scaffold and the release of a pool of biologically-active proteins that influence and promote a range of biological process in-

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cluding cell recruitment, growth, and differentiation.[22,23] Interestingly, some of the proteins secreted from α-granules of platelets, including EGF, platelet-derived growth factor (PDGF), and nerve growth factor (NGF) are necessary to promote wound repair and to maintain well-preserved ocular surface.

PRGF-Endoret technology has provided significant clinical advances in terms of wound healing and tissue regeneration in dentistry and oral implantology,[24,25] orthopedics, sport medicine,[26] and ulcer treatment,[27] among others. In all these situations, small volumes of plasma rich in growth factors and reduced number of doses are needed to achieve therapeutic efficacy. According to the technique, the fraction with the highest platelet concentration (also known as fraction 3) should be used to promote tissue regeneration as this plasma volume contains the higher amount of proteins. However, this may hamper its use as an autologous eye drop, due to the elevated number of doses necessary per day and the long period of treatment required to complete healing in several pathologies.

Assuming that function of fibroblasts is critical during ocular surface healing, the purpose of the present study is to assess the potential of PRGF-Endoret technology as an innovative approach for enhancing ocular repair and regeneration. To address these issues, the effects of fraction 3 (F3) or whole plasma (WP) column (with lower amount of proteins than F3) obtained from PRGF-Endoret technology were assessed over primary keratocytes and conjunctival fibroblast proliferation and migration. In addition, the potential of WP and F3 to inhibit and revert TGF-β1–stimulated myodifferentiation was also evaluated.

Materials and Methods

Cells

Primary human cells including keratocytes (HK) and conjunctival fibroblasts (HConF; ScienCell Research Laboratories, San Diego, CA), were cultured according to manufacturer's instructions. Briefly, cells were cultured until confluence in fibroblast medium supplemented with fibroblast growth supplement (Complete FM; ScienCell Research Laboratories) and then were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY). Cell viability was assessed by trypan blue dye exclusion. Passage 3 to 6 cells were used in all experiments.

Immunolabeling of Cells

The fibroblast-like morphology of cells and the absence of dedifferentiation were confirmed by phase-contrast microscopy and immunolabeling for collagen type I (Chemicon-Millipore, Billerica, MA), and fibronectin and vimentin (Sigma-Aldrich, St. Louis, MO). The cells were also tested against typical endothelial cells and hematopoietic progenitor cell markers: CD105 and CD34 (BD Biosciences, San Jose, CA) and against α-Smooth muscle actin (Sigma-Aldrich) to check the spontaneous differentiation to myofibroblasts in culture.

Briefly, 9500 cells per well were plated on a 24-well plate with poly-L-lysine-coated glass coverslips (BD BioCoat, BD Biosciences). Cells were fixed for 10 minutes in 4% formaldehyde for CD34, CD105, and type I collagen antigens, in methanol:acetic acid (3:1) for vimentin antigen and in methanol precooled at −20°C for fibronectin and α-smooth muscle actin (α-SMA). Cells for type I collagen staining were permeabilized with 1% Triton X-100, in phosphate-buffered saline (PBS) for 10 minutes. After that, cells were blocked with fetal bovine serum (FBS) (10% in PBS) for 30 minutes, and incubated for 1 hour with the primary antibodies in the dilutions: 1:20 for type I collagen, 1:30 for CD34 and CD105; 1:50 for vimentin, and 1:800 for fibronectin and α-SMA. Next, cells were incubated with their appropriate secondary antibodies, goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-rabbit IgG conjugated with Alexa Fluor 594 (both from Molecular Probes-Invitrogen, Grand Island, NY). Finally, cell nuclei were stained with Hoechst 33342 (Molecular Probes-Invitrogen), mounted, and visualized under a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany).

PRGF-Endoret Preparations

Blood from one healthy young male donor was collected after informed consent into 9-mL tubes with 3.8% (wt/vol) sodium citrate. The study was performed following the principles of the Declaration of Helsinki. Samples were centrifuged at 580*g* for 8 minutes at room temperature in a PRGF-Endoret system centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Half of the tubes were used to separate the whole plasma column (WP) over the buffy coat and the other half to take the immediately upper milliliter over the buffy coat called Fraction 3 (F3)—the platelet-enriched fraction (see Fig. 1). In both cases, care was taken to avoid the buffy coat containing the leukocytes. Platelet and leukocyte counts were performed with a hematology analyzer (Micros 60; Horiba ABX, Montpelier, France). Both preparations were incubated with PRGF-Endoret activator (BTI Biotechnology Institute) at 37°C in glass tubes for 1 hour. The released supernatants were collected by aspiration after centrifugation at 1000*g*

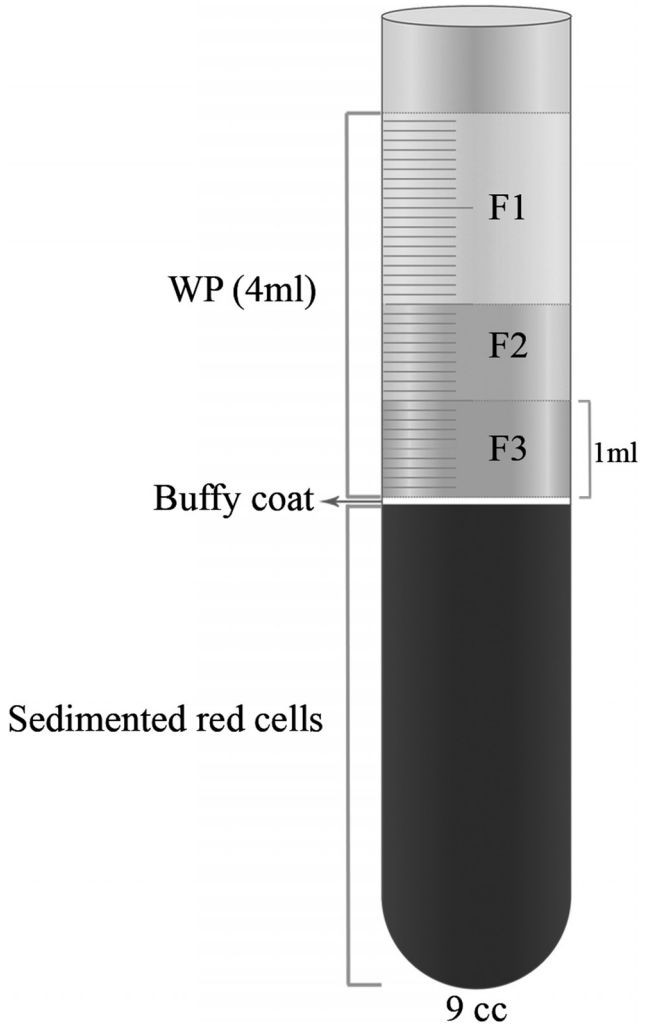

Figure 1. Scheme of the different plasma fractions obtained with the PRGF-Endoret technology. In all the different plasma preparations, care was taken to avoid the buffy coat containing leukocytes. WP, whole plasma obtained with PRGF-Endoret System. F1, fraction 1; F2, fraction 2; F3, fraction 3.

for 20 minutes at 4°C. Finally, plasma obtained from WP and F3 was aliquoted and stored at −80°C until use. Growth factors (TGF-β1, PDGF-AB, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), EGF, insulin-like growth factor I (IGF-1), and Thrombospondin 1 (TSP-1)) were measured in the supernatants using commercially available colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN; Table 1).

Proliferation Assay

Keratocytes and conjunctival fibroblasts were seeded at a density of 10,000 cells per cm^2 on 96-well optical bottom black plates and maintained with serum-free medium for 48 hours. Then, culture medium was replaced by serum-free medium supplemented with either the culture medium alone (FM) with 0.1% FBS as a control of nonstimulation (NS); 20% (vol/vol) WP; or 20% (vol/vol) F3. The study period was 48 hours. Density of cells in culture was estimated (CYQUANT Cell Proliferation Assay; Invitrogen, Carlsbad, CA). Briefly, medium was removed and wells were washed carefully with PBS. Then microplate was freezed at −80°C for efficient cell lysis in the assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Kunitz Units [KU]/mL) diluted in cell lysis buffer for 1 hour at room temperature. Then 2× dye/cell lysis buffer (CyQUANT GR; Invitrogen) was added to each sample well, mixed gently, and incubated for 5 minutes at room temperature protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany). A DNA standard curve ranging from 7.8 to 1000 ng/mL was included in all fluorescence quantifications. As an index of cell number, calibration curves ranging from 2500 to 90,000 cells per cm^2 were established (CyQUANT assay; Invitrogen).

Migration Assay in Response to WP and F3

To quantify the migratory potential of conjunctival fibroblasts and keratocytes, they were plated in culture inserts (Ibidi GmbH, Martinsried, Germany) placed on a 24-well plate at high density and were grown with fibroblast growth supplement (Complete FM; ScienCell Research Laboratories) until confluence. After carefully remove the inserts, two separated cell monolayers leaving a cell-free gap of approximately 500 μm thickness were created. The cells were washed with PBS and incubated with the same treatments as in the proliferation assay (0.1% FBS, 20% WP, or 20% F3) in quintuplicate for 24 hours. After this period, the different culture mediums were removed and cells were incubated with 1/500 Hoechst 33,342 in PBS for 10 minutes. To quantify the number of migratory cells, phase contrast images of the central part of the septum before treatment and phase contrast and fluorescence photographs after the treatment time were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area and the migratory cells found in this gap after the 24 hours of treatment were measured using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The results were expressed as number of cell migrated per mm^2 of area.

Myofibroblast Differentiation

Differentiation of conjunctival fibroblasts and keratocytes to myofibroblasts was induced by 2.5 ng/mL of TGF-β1 (Chemicon-Millipore) for 72 hours. Passage 4 cells were plated at a density of 5000 cells per cm^2 in 48-well tissue culture plates and maintained with serum-free culture medium for 48 hours. After this period, the medium was replaced by FM and keratocytes and conjunctival fibroblasts were stimulated with either 2.5 ng/mL TGF-β1, 2.5 ng/mL TGF-β1 plus 20% (vol/vol) WP, or 2.5 ng/mL TGF-β1 plus 20% (vol/vol) F3 supernatant for 72 hours. In the following experiments, cells were initially pretreated with 2.5 ng/mL TGF-β1 for 3 days. After this pretreatment, culture medium was replaced by FM and supplemented with either one of three treatments: 2.5 ng/mL TGF-β1, 2.5 ng/mL TGF-β1 plus 20% (vol/vol) WP supernatant, or 2.5 ng/mL TGF-β1 plus 20% (vol/vol) F3 supernatant for 3 days. In both protocols, a supplement of 0.1% FBS was added to the 2.5 ng/mL TGF-β1 treatment to maintain cell viability in the control group. Experiments were performed in quintuplicate.

After incubation time, medium was removed and cells were fixed for 10 minutes in methanol. Cells then were blocked with FBS (10% in PBS) for 30 minutes, and incubated for 1 hour with mouse anti-α-SMA antibody at 1:800, followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 488 at 1:100 for 1 hour. Finally, cell nuclei were counterstained with Hoechst 33342 mounted using an anti-fade solution (SouthernBiotech, Birmingham, AL) and visualized under a fluorescence microscope (Leica DM IRB, Leica Microsystems). Control isotype was performed by substituting the primary antibodies with 10% of FBS diluted in PBS.

For myofibroblast cell counting, two random 10× microscopic fields were photographed on each well. The digitalized images were analyzed (Image J software). Hoechst (+) cells were counted to obtain the total cell number. Hoechst (+) and α-SMA (+) cells were counted as myofibroblasts. Cells showing any kind of greenish staining were considered α-SMA–positive. Expression of α-SMA was also evaluated on cells treated with 20% (vol/vol) WP or F3 to check the myofibroblast differentiation.

Inhibition of Myofibroblastic Differentiation

Fibroblasts were seeded at a density of 5000 cell per cm^2 in a 48-well plate and were pretreated with 2.5 ng/mL of TGF-β1 plus 0.1% FBS as differentiation medium. After 72 hours, one part of the cells were fixed and stained for α-SMA and Hoechst 33342 as a positive control of differentiation. The remaining cells were then treated with FM plus 0.1% (vol/vol) FBS plus 2.5 ng/mL TGF-β1, 20% (vol/vol) plasma rich in growth factors plus 2.5 ng/mL TGF-β1, or 20% (vol/vol) F3 plus 2.5 ng/mL TGF-β1 for 3 days. All samples were performed in quintuplicate. The immunolabeling for α-SMA and Hoechst and cell counting were performed as previously described.

Statistical Analysis

Means and their respective 95% confidence intervals were calculated for each of the treatments, proceeding to review the potential differences between treatments for each experimental process (proliferation, migration, and protective and reversible effect of WP and F3).

Table 1. Platelet and Leukocyte Count and Concentrations of Several Growth Factors in the Two Different Plasma Preparations (WP and F3) of the Blood Donor

			Growth Factor Levels						
Plasma Preparation	**Leukocyte Count ($\times 10^6$/mL)**	**Platelet Count ($\times 10^6$/mL)**	**TGF-β1 (ng/mL)**	**PDGF-AB (ng/mL)**	**IGF-I (ng/mL)**	**VEGF (pg/mL)**	**HGF (pg/mL)**	**EGF (pg/mL)**	**TSP-1 (μg/mL)**
WP	<0.2	481	63	19	83	568	400	508	29
F3	<0.3	663	81	30	86	791	491	779	50

WP column enriched in platelets 2.6-fold over peripheral blood, and F3 enriched in platelets 3.6-fold over peripheral blood. Peripheral blood contained 182×10^6 platelets/mL.

Differences between treatments were considered to be significant in cases where the boundaries of the respective 95% did not overlap.

RESULTS

The human primary conjunctival fibroblasts (HConF) and keratocytes (HK) showed the typical spindle-shaped aspect in culture and did not spontaneously differentiate into myofibroblasts as confirmed by the absence of α-SMA expression (data not shown). Cells were positive for all the fibroblast markers (Collagen Type I, vimentin, and fibronectin) and negative for markers of hematopoietic and endothelial cells (data not shown).

Platelet enrichment of the PRGF-Endoret preparations were 2.6-fold for WP (481×10^6 platelets/mL) and 3.6-fold for F3 (663×10^6 platelets/mL) over the baseline concentration in whole blood. None of the preparations contained detectable levels of leukocytes. Table 1 shows platelet and leukocyte concentration for each sample (WP and F3) and the levels of some of the most important growth factors.

Cell Proliferation

Proliferation of conjunctival fibroblasts and keratocytes significantly increased after treatment with both PRGF-Endoret preparations (20% WP or 20% F3) as shown in Figure 2. In fact, conjunctival fibroblasts proliferated 3.1-fold and 3.4-fold after treatment with WP and F3 respectively. On the other hand, keratocytes showed a significant increase over basal conditions of 2.8-fold with WP and 2.7-fold with F3. No significant differences were found between the autologous treatments.

Migration Assay

WP and F3 significantly stimulated the migratory capacity of both HConF and HK. In particular, migration of HConF increased 1.8-fold and 1.7-fold over the nonstimulatory situation for WP and F3 respectively (Fig. 3A) whereas migration of keratocytes increased 2.3-fold for WP and 1.8-fold for F3 (Fig. 3B). The number of migrating cells was significantly higher with plasma preparations than with nonstimulation cells. No statistical differences were found between plasma rich in growth factors treatments. Figure 3C shows phase contrast images of HConF and HK cells after a 24-hour period of migration and highlights the potent stimulatory effect of WP and F3 over the treated cells.

Protective Effect of Plasma Preparations

The effects of plasma preparations (WP and F3) on the prevention of the TGF-β1–stimulated myofibroblastic differentiation were evaluated. Cells treated with either 20% WP or 20% F3 alone were not differentiated into myofibroblasts (data not shown). HConF and HK cells showed a spontaneous differentiation to myofibroblasts in a percentage of 16% ± 2% and 14% ± 8% respectively after 72-hour culture with 0.1% of FBS. After 3 days of stimulation with 2.5 ng/mL of TGF-β1, conjunctival fibroblasts and keratocytes showed a 61% ± 32% and a 48% ± 23% of α-SMA–positive cells respectively (Figs. 4A and 4B). The immunofluorescence for α-SMA exhibited that after culturing the cells 3 days either with 20% of WP plus 2.5 ng/mL TGF-β1 or with 20% of F3 plus 2.5 ng/mL TGF-β1, the percentage of positive HConF cells decreased drastically to 0.2% ± 0.1% and 0.1% ± 0.3%, respectively. This decrease was statistically significant for both types of fibroblasts with respect to the spontaneous transformation in culture and also with respect to the condition of TGF-β1 alone. Figure 4C shows the protective role of WP and F3 against the effect of TGF-β1 over HConF and HK cells. No significant differences were found between the responses induced by WP or F3 on fibroblast transformation to myofibroblasts.

Inhibition of Myofibroblastic Phenotype

The effects of both plasma fractions (WP and F3) on the inhibition of the TGF-β1–stimulated myofibroblast differentiation were evaluated. After pretreating the cells during 3 days with 2.5 ng/mL of TGF-β1, a 60% ± 25% of α-SMA–positive HConF cells and a 77% ± 7% of α-SMA–positive HK were found (Figs. 5A and 5B). Then cells were treated for another 3 days with either TGF-β1, a combination of TGF-β1 plus 20% WP, or a combination of TGF-β1 plus 20% F3. Interestingly, the percentage of myofibroblasts in the HConF population was reduced significantly to 7% ± 2% when WP was added and to 11% ± 3% when F3 was added (Fig. 5A). In the case of HK cells, the number of α-SMA–positive cells decreased drastically to 8% ± 2% and to 12% ± 2% when WP and F3 were added respectively (Fig. 5B). Therefore, the use of PRGF-Endoret treatments reduced significantly (with a 95% confidence interval) the number of myofibroblasts. As counterpoint, cells treated with 0.1% FBS plus 2.5 ng/mL TGF-β1 for 3 days maintained the myofibroblastic phenotype. In fact, 86% ± 8% of the HConF cells and 71% ± 7% of the HK cells were positive for α-SMA.

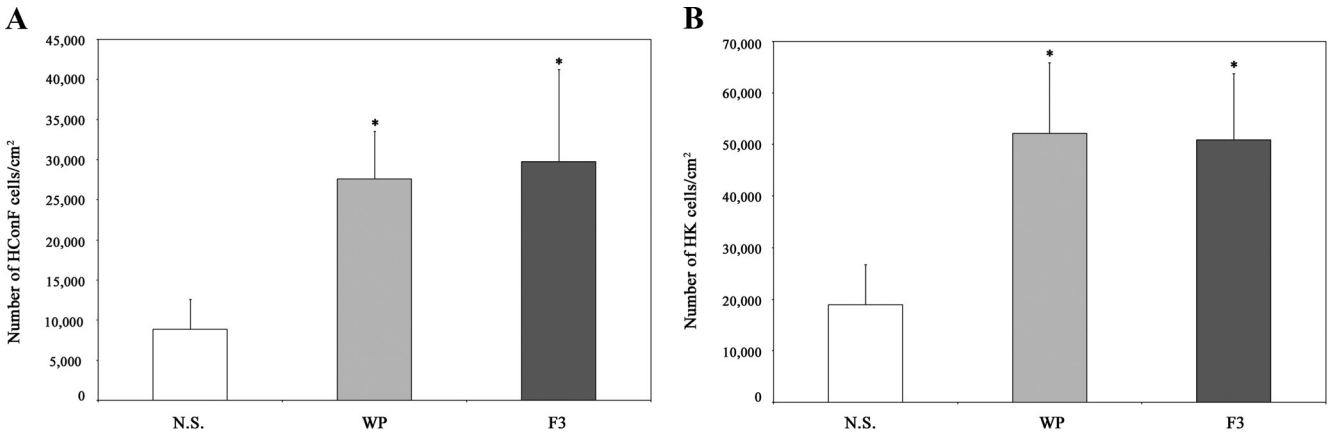

FIGURE 2. (**A**) Proliferation of HConF cells after culturing with 0.1% FBS as a control of nonstimulation (N.S.), 20% WP, or 20% F3 for 2 days. (**B**) Proliferation of keratocyte (HK) cells after culturing with 0.1% FBS (N.S.), 20% WP, or 20% F3 for 2 days. WP and F3 significantly increased proliferation of both cells compared with nonstimulatory conditions; *, 95% confidence interval. No statistical differences were found between WP and F3.

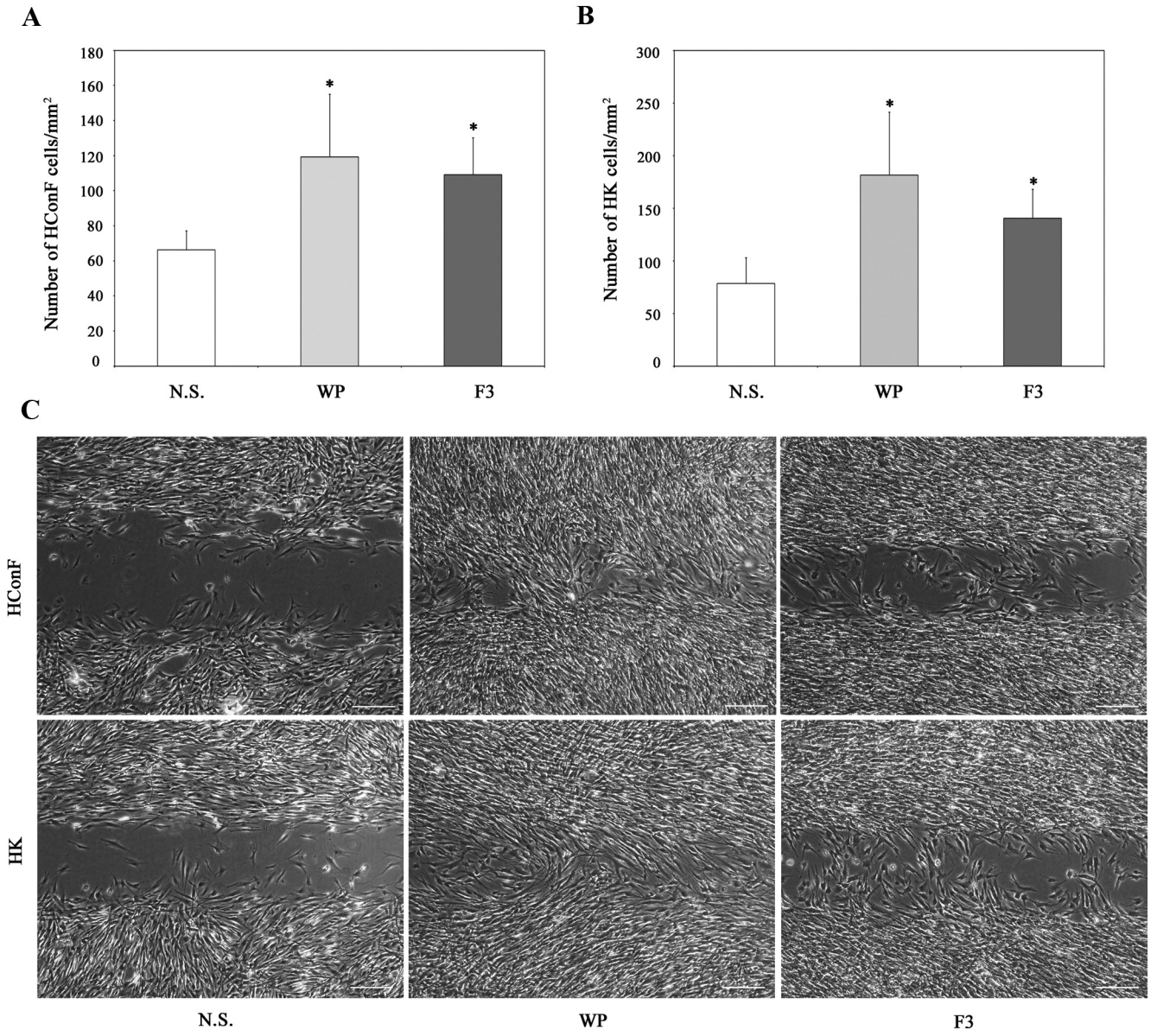

FIGURE 3. (**A**) Migration rate of HConF cells after culturing with 0.1% FBS (N.S.), 20% WP, or 20% F3 for 24 hours. (**B**) Migration rate of keratocyte (HK) cells after culturing with 0.1% FBS (N.S.), 20% WP, or 20% F3 for 24 hours. WP and F3 significantly increased migration of both cells compared with nonstimulatory conditions; *, 95% confidence interval. No statistical differences were found between WP and F3. (**C**) Phase contrast photomicrographs illustrating the migration rate of HConF and HK cells. Scale bar: 300 μm.

The immunofluorescence detection of α-SMA revealed that both WP and F3 inhibited the differentiation of the different populations of fibroblasts into myofibroblasts (Fig. 5C).

DISCUSSION

Several groups have focused their studies in understanding the different processes related to wound healing of the ocular surface. There is a general agreement that one of the main events involved in wound healing is cell proliferation as well as the migration of these cells to the damaged area.[1,28,29] There are also reports showing that different growth factors and cytokines and an intricate network of signals are implicated in the modulation of wound healing.[30]

PRGF-Endoret is an autologous platelet-rich plasma technology by which it is possible to obtain different growth factor-enriched formulations that can be used in the treatment of several disorders.[31] The effects of PRGF-Endoret on tissue regeneration have been demonstrated in dentistry, oral implantology, orthopedics, sports medicine, and treatment of skin disorders.[32]

In this study, we report for the first time the effects of PRGF-Endoret on the proliferation, migration, and differentiation of human keratocytes and conjunctival fibroblasts in vitro. Results demonstrate that the different plasma formulations evaluated (WP and F3) enhance proliferation and migration of both types of fibroblast populations and significantly protect and inhibit TGF-β1–induced myofibroblast differentiation. Interestingly, there are not significant differences between the effects induced by the platelet-enriched plasma fraction (F3) and the whole plasma fraction (WP). Although preliminary, these results may help to understand the potential of the autologous formulations in corneal wound healing.

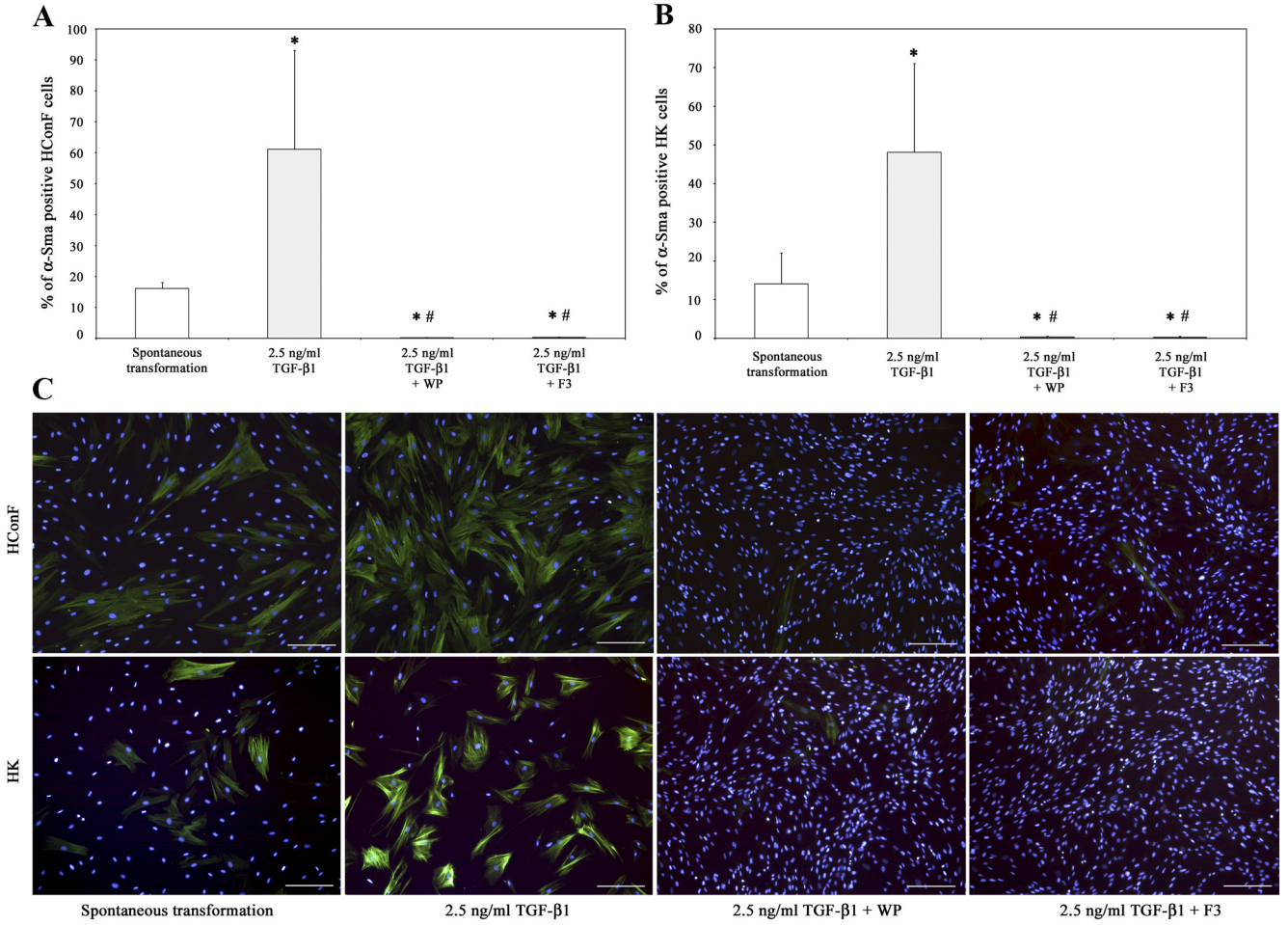

Figure 4. When cells were treated simultaneously with TGF-β1 and WP or TGF-β1 and F3, (**A**) conjunctival fibroblasts (HConF) and (**B**) keratocytes (HK), number of α-SMA–positive cells were significantly lower compared with the TGF-β1 treatment group; 95% confidence interval. There is also a significant difference between spontaneous myotransformation and the number of α-SMA–positive cells after treatment with 2.5 ng/mL TGF-β1 plus 0.1% FBS, or plus 20% WP, or plus 20% F3. (**C**) Immunofluorescence for detection of α-SMA protein in HConF and HK cultured cells. α-SMA–positive and Hoechst-positive cells are considered as myofibroblasts. Scale bar: 200 μm.

One important concern in ocular wound healing is scar formation. TGF-β has been identified as one of the most potent inductors of fibroblast differentiation into α-SMA-expressing myofibroblasts.[9,22] According to Masur et al.,[33] a spontaneous fibroblast differentiation to myofibroblasts is observed when cells are cultured at low cell density or at low concentration of FBS. In our study, a spontaneous differentiation of 16% for HConF cells and 14% for HK cells was detected. No additional differentiation was observed after adding the plasma rich in growth factors formulations (WP and F3).

Our current findings confirm that PRGF-Endoret technology protects TGF-β1–induced α-SMA– expression of keratocytes and conjunctival fibroblasts. In fact, when cells were cocultured either with 20% WP or F3 and TGF-β1 simultaneously, α-SMA– expression was under 0.3%. In addition, both WP and F3 significantly inhibited myofibroblast differentiation even when a previous 3-day culture with TGF-β1 had been carried out.

Although further research is needed to clarify the molecular events that regulate PRGF-Endoret biological activity, it seems reasonable that some of the proteins present in both WP and F3 may have played key roles in cell proliferation, migration, and differentiation. Some growth factors present in PRGF-Endoret preparations have been described as key regulators of corneal wound healing. For example, EGF, HGF, and keratocyte growth factor (KGF) stimulate epithelial cell proliferation and migration, while in the case of stroma, these processes are mediated by TGF, PDGF, and FGF.[34–38]

Some studies have discussed the mechanisms by which the myofibroblastic phenotype disappears from corneal tissue or cultured cells. Interestingly, it has been observed that FGF-1 and -2, some proteins present in plasma rich in PRGF-Endoret formulations, promote the fibroblast phenotype and reverse the myofibroblast phenotype.[11] Some other studies suggest that myofibroblast apoptosis may be one of the initial mechanisms of myofibroblast disappearance; although myofibroblasts transdifferentiation to keratocytes or corneal fibroblasts should also be considered.[39]

Another important conclusion from this study is that WP and F3 exert similar in vitro biological effects. It has to be assumed that until now, F3, that is, the plasma fraction with the highest platelet concentration, has been widely used in several medical areas[28–31] including the treatment of ocular diseases.[40–43] The data presented herein may modify the current clinical protocols as WP shows similar biological effects to F3 but represents an improvement in the yield of 400%. In fact, while only 1 mL of F3 can be obtained from 9 mL of blood, almost 4 mL of WP can be obtained from the same blood volume.

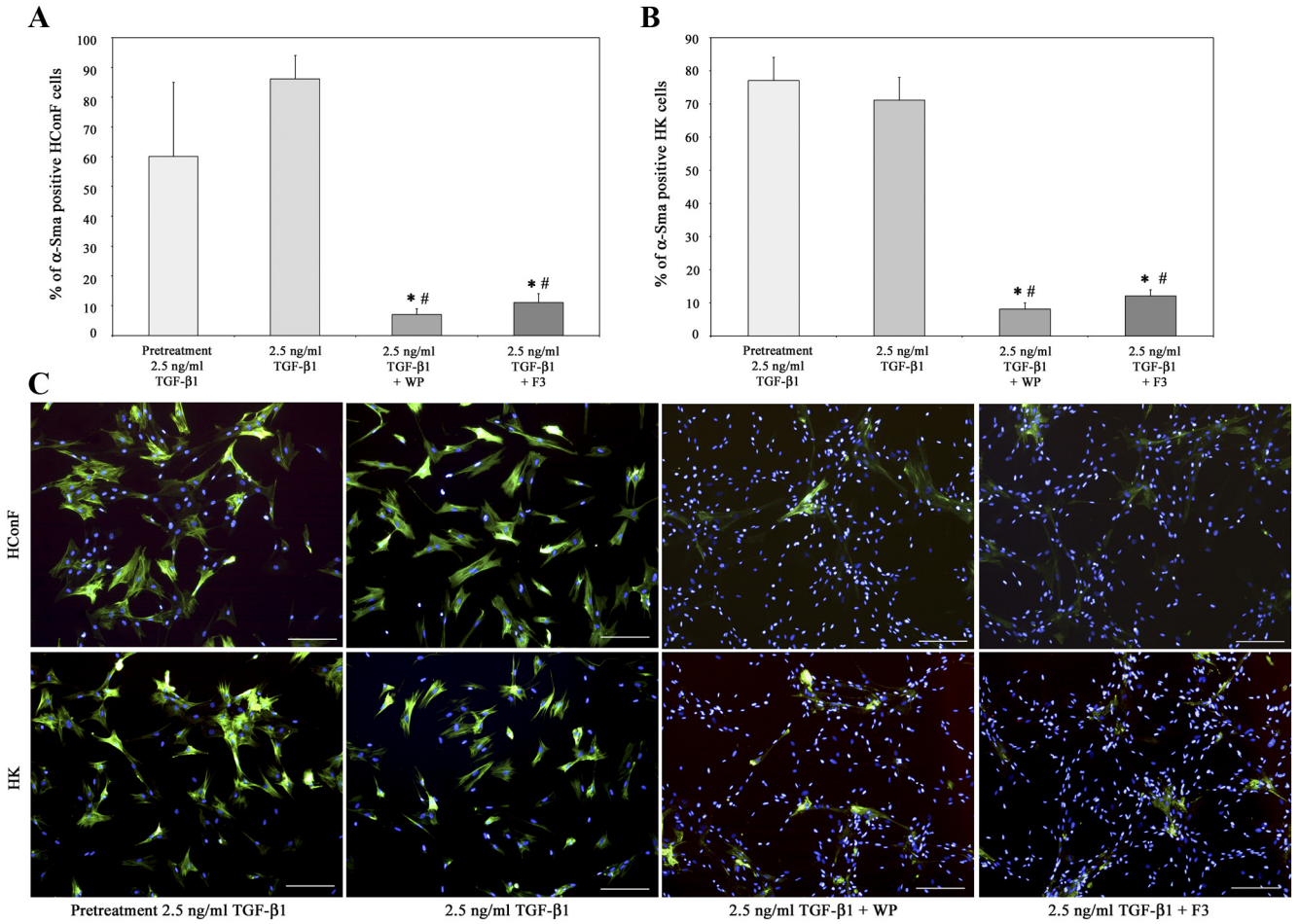

FIGURE 5. Capacity of reversion of PRGF-Endoret technology over the myofibroblastic phenotype. HConF (**A**) and HK (**B**) cells were treated for 3 days with 2.5 ng/mL TGF-β1 as a previous stimulation to get a population of myofibroblasts. Then they were cultured with 2.5 ng/mL TGF-β1 simultaneously with 20% WP or 20% F3 for another 3 days to prove the capacity of WP and F3 to dedifferentiate the cells. There is no statistical difference between the response induced by WP and F3 but there is with respect to starting point (2.5 ng/mL); *, 95% confidence interval. (**C**) Immunofluorescence of α-SMA showing positive cells before and after treatment with plasma preparations. Myofibroblasts are α-SMA–positive and Hoechst-positive cells. Scale bar: 200 μm.

In summary, the different formulations of PRGF-Endoret enhance proliferation and migration of keratocytes and conjunctival fibroblasts while they protect and inhibit TGF-β1–induced myofibroblast differentiation. Although further studies are needed to determine the exact mechanisms underlying the effects of this autologous technology, results from this study suggest that the different PRGF-Endoret formulations (WP and F3) could improve the wound healing in ocular surface.

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In Vitro Effects of Three Blood Derivatives on Human Corneal Epithelial Cells

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Purpose. We compared the effects of three blood derivatives, autologous serum (AS), platelet-rich plasma (PRP), and serum derived from plasma rich in growth factors (PRGF), on a human corneal epithelial (HCE) cell line to evaluate their potential as an effective treatment for corneal epithelial disorders.

Methods. The concentrations of epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and fibronectin were quantified by ELISA. The proliferation and viability of HCE cells were measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Cell morphology was assessed by phase-contrast microscopy. The patterns of expression of several connexin, involucrin, and integrin α6 genes were analyzed by real-time RT-PCR.

Results. We found significantly higher levels of EGF in PRGF compared to AS and PRP. However, AS and PRGF induced robust proliferation of HCE cells. In addition, PRGF cultured cells grew as heterogeneous colonies, exhibiting differentiated and non-differentiated cell phenotypes, whereas AS- and PRP-treated cultures exhibited quite homogeneous colonies. Finally, PRGF upregulated the expression of several genes associated with communication and cell differentiation, in comparison to AS or PRP.

Conclusions. PRGF promotes biological processes required for corneal epithelialization, such as proliferation and differentiation. Since PRGF effects are similar to those associated with routinely used blood derivatives, the present findings warrant further research on PRGF as a novel alternative treatment for ocular surface diseases. (*Invest Ophthalmol Vis Sci.* 2012; 53:5571–5578) DOI:10.1167/iovs.11-7340

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The cornea is considered to be the most sensitive part of the corporeal surface and its integrity is critical for normal visual function. Many corneal surface disorders involve tear film dysfunction, the consequences of which are mainly inflammatory, but they also may include epithelial metaplasia, glandular dysfunction, and neurotrophic damage.[1,2]

Conventional treatments for these disorders include pharmaceutical tear film substitutes, therapeutic contact lenses, anti-inflammatory agents, and oral antibiotics. However, to date, an ideal substitute for natural tear film has not been developed, essentially because natural tear is a dynamic and complex solution with specific antimicrobial, mechanical, and optical properties. In addition, natural tear contains many proteins, vitamins, and growth factors, which promote proliferation, migration, and differentiation.[3] It should not be forgotten that most artificial tears contain preservatives, which potentially make them toxic for the ocular surface.[4,5]

To obtain an alternative tear substitute, several trials have been performed using recombinant nerve growth factor (NGF),[6,7] epidermal growth factor (EGF),[8] or fibronectin[9] as topical treatments. However, although some preparations have yielded successful results, the stability of these preparations was low. The possibility of stimulating cell proliferation by different factors present in plasma also has been investigated.[10] These factors, acting together, could in principle lead to a more integrated response of the affected tissue. In this sense, the introduction of blood-derived products in the field of ophthalmology has been a breakthrough. The description of autologous serum (AS)[11] was the first revolution, because its pH, osmolality, and biochemical properties were similar to those of natural tear. In addition, it contains essential nutrients, such as growth factors, vitamins, and bacteriostatic components, such as IgG and lysozyme. Since then, AS has been used frequently for topical therapy in patients with ocular disorders, as it is associated with enhanced healing.[12–17]

Subsequently, platelets that are involved in tissue repair processes were identified as a major source of the growth factors present in plasma. This fact has motivated growing interest in the development of platelet-rich plasma (PRP) preparations, with the intention of increasing the concentration of these mediators and potentiating healing processes.[18] However, there presently is no consensus about the most adequate method to obtain AS and other blood derivatives.[19] Thus, different preparations have been reported by different research groups.[13,17,20–24] Furthermore, thrombin is used routinely to stimulate the release of growth factor content from platelets. However, this practice could lead to important adverse events, such as immune reaction and the appearance of coagulopathies.[25]

More recently, a novel blood derivative has been developed that is characterized by easy and fast processing, and the ability to stimulate the release of platelet content in the absence of thrombin and leucocytes. This plasma rich in growth factors (PRGF) was introduced in maxillofacial surgery and trauma

with excellent results.[26,27] Recently, our group successfully and, to our knowledge, for the first time used serum derived from PRGF for the treatment of persistent epithelial defects[28] and dry eye syndrome.[29]

In this study, we compared the in vitro effect on human corneal epithelial (HCE) cells of three blood derivatives (AS, PRP, and PRGF), which are used in the treatment of corneal disorders. To this end, we performed several assays to characterize the growth factor content of the three different preparations, and to determine their effects on the growth and differentiation of a well characterized HCE cell line.

Materials and Methods

Preparation of Blood-Derived Products

Blood from 16 healthy volunteers was collected by venipuncture (age range 30–60 years) after patient consent had been signed, in accordance with the Declaration of Helsinki. All volunteers were healthy and not taking any medication. The blood sample from each volunteer was processed according to the following three methods to obtain the corresponding blood derivatives:

1. Autologous serum (AS): Spontaneous coagulation for 2 hours at room temperature followed by centrifugation at 1,000 g for 15 minutes. Collection of the complete supernatant fraction.[17]
2. Platelet-rich plasma (PRP): Centrifugation at 460 g for 8 minutes and collection of the complete supernatant fraction.[24]
3. Serum derived from plasma rich in growth factors (PRGF): Centrifugation at 460 g for 8 minutes, followed by collection of the complete supernatant fraction and induction of clot formation by adding calcium chloride at a final concentration of 22.8 mM (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain), in the absence of red and white blood cells. After 2 hours at 36°C, the clot was retracted and the supernatant was collected.[28,29]

For blood collection, we use tubes with sodium citrate as anticoagulant for PRP and PRGF processing or without anticoagulant for AS processing. The complement factors of all blood derivatives were inactivated at 56°C for 30 minutes. Afterwards, we pooled samples from the different volunteers to obtain representative blood preparations that provided reproducible results and minimized inter-individual variability. These pools were stored at −20°C until its use for the in vitro assays.

Quantification of Growth Factor Concentrations

The concentrations of epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and fibronectin were measured from undiluted preparations using commercially available Quantikine colorimetric sandwich ELISA kits purchased from R&D (Minneapolis, MN), except for human fibronectin, which was acquired from Chemicon International Inc. (Temecula, CA). Results were expressed as mean ± SD for each age group for the three different preparations.

Cell Culture Model

SV-40 immortalized HCE cells were provided kindly by K. Araki-Sasaki.[30] Cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's mediu (DMEM): Ham's F12 (1:1 mix; Lonza, Verviers, Belgium) with 2 mM L-glutamine (Lonza) and 1% penicillin-streptomycin (Lonza), together with 10% fetal bovine serum (FBS; Lonza). We also added to the medium 10 ng/mL EGF (Sigma, St. Louis, MO), 5 μg/mL insulin (Sigma), 0.1 μg/mL cholera toxin (Gentaur Molecular Products, Brussels, Belgium), and 0.5% dimethylsulfoxide (DMSO; Sigma). From here onwards, we refer to EGF, insulin, cholera toxin, and DMSO as "supplements." Upon confluence, cells were detached with 0.5% trypsin-0.2% EDTA (Sigma) and subcultivated 1:4–1:5 twice a week.

Cell Proliferation Assays

The effect of the three blood derivatives on HCE cell proliferation was tested at several dilutions (10, 20, and 50%) and times (24, 48, and 72 hours). Proliferation was expressed as proliferation rate ± SD of viable cells with respect to viable cells just before exposure to blood derivatives ($t = 0$ hours).

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). MTT is reduced by mitochondrial and cytosolic dehydrogenases in living cells to a purple formazan dye, which is detectable spectrophotometrically and whose absorbance is known to be directly proportional to the number of viable cells.

HCE cells were seeded at 3000 cells/well in 96-well plates and left to attach to the plastic substrate. Subsequently, to synchronize cultures, we substituted the initial culture medium containing FBS and supplements for a medium with 1% bovine serum albumin (BSA), but without supplements, and incubation proceeded overnight. Then, at this starting time ($t = 0$ hours), cells were exposed to medium containing supplements, and FBS, AS, PRP, or PRGF. After 24, 48, and 72 hours, cells were washed and incubated with 0.5 mg/mL MTT for three hours. Then the MTT solution was removed and 100 μL/well DMSO was added. Optical densities were determined at 540 nm using a microplate reader (ELx800 Microplate Reader; BioTek Instruments, Winooski, VT). All experiments were performed in quadruplicate and repeated three times.

Microscopy Assays

Phase-contrast microphotographs were taken to analyze culture morphology. Cells were incubated for 14 days with 10% FBS or with 20% of one of the three different blood preparations. Cultures were passed every three days. After washing with fresh culture medium, cell morphology was observed using a phase-contrast microscope (Nikon Eclipse TS 100; Nikon, Tokyo, Japan) and images were captured with ProgRes CapturePro 2.6 software (Jenoptik, Jena, Germany).

Real-Time RT-PCR

To compare transcription levels of a panel of genes in the differently treated HCE cells, real-time RT-PCR was performed. Thus, HCE cells were grown for 14 days with 10% FBS or 20% AS, PRP, and PRGF, in the presence or absence of supplements. Total RNA was extracted according to the manufacturer's protocol (RNeasy minikit; Qiagen Inc., Valencia, CA), and treated with DNase for 30 minutes at 37°C and 10 minutes at 65°C (Promega, Madison, WI). Total RNA was quantified, tested on an agarose gel, and stored at −80°C until use.

For each sample, cDNA was synthesized from 0.5 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR assays were performed with an iCycler PCR platform (Bio-Rad). The reaction mixture contained 1 μL cDNA from the RT reaction, together with forward and reverse specific primers (250 nM each) and iQSYBR Green Supermix (Bio-Rad) in a final reaction volume of 20 μl. Thermal cycling conditions were as follows: An initial polymerase activating step at 95°C for 3 minutes, followed by 60 cycles for 20–30 seconds each at 95°C (denaturation step), 20–30 seconds at the corresponding annealing temperature for each gene (Table 1), and 20–30 seconds at 72°C (extension step), during which data were collected. Each assay included a negative control consisting of the absence of cDNA. Expression data were generated from 4 amplification reactions with samples and controls run in triplicate, and performed on different cDNA samples reverse transcribed from RNA prepared from independent culture assays. Optical data obtained by real-time PCR were analyzed using the MyiQ Single-Color Real-Time PCR Detection System Software, Version 1.0 (Bio-Rad). The dynamic range of detection for each gene was determined by preparing 5-fold serial dilutions of

Table 1. Primers and Conditions Used for Real Time RT-PCR

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Size (bp)	Annealing Temperature °C
CX26	GCTGCAAGAACGTGTGCTAC	TGGGTTTTGATCTCCTCGAT	196 bp	65°C
CX30	TGCTTAACGTGGCAGAGTTG	GGTTGGTATTGCCTTCTGGA	244 bp	60°C
CX31.1	CCTGAGTGGGGTCAACAAGT	GGGACACAGGGAAGAACTCA	191 bp	65°C
CX43	CCTTCTTGCTGATCCAGTGGTAC	ACCAAGGACACCACCAGCAT	154 bp	60°C
Integrin α6	ATGCACGCGGATCGAGTTT	TTCCTGCTTCGTATTAACATGCT	160 bp	60°C
Involucrin	TCCTCCAGTCAATACCCATCAG	GCAGTCATGTGCTTTTCCTCTTG	126 bp	60°C
GAPDH	CCTGTTCGACAGTCAGCCG	CGACCAAATCCGTTGACTCC	102 bp	56°C
β-actin	AGATGACCCAGATCATGTTTGAG	GTCACCGGAGTCCATCACG	119 bp	60°C
RIG/S15	TTCCGCAAGTTCACCTACC	CGGGCCGGCCATGCTTTACG	361 bp	60°C

control HCE cells (undiluted, 1:5, 1:25, 1:125, and 1:625). The reliability of real-time PCR was defined by regression analysis of average Ct versus the $\log_{10}$ of the target copy number. PCR efficiency was around 93% with all primer pairs. Melt Curve analysis of each PCR assay and 1.5% agarose gel electrophoresis analysis of randomly selected samples were performed to confirm the specificity of the amplification products. The expression of three different housekeeping genes (*GAPDH*, β-actin, and *RIG/S15*) also was analyzed. They were chosen after verifying their suitability by geNorm software (version 3.5). They were used to normalize expression data obtained from the studied genes, using the Bio-Rad Gene Expression Macro Software Version 1.1 derived from the algorithms outlined by Vandesompele et al.[31] All primers were synthesized commercially (Isogen Life Sciences, Barcelona, Spain) and a BLAST search was performed to verify their specificity for their target DNA sequences. The sequences of the primer pairs, as well as the size of the corresponding amplified fragments are detailed in Table 1.

Statistical Analysis

Means and SDs of all variables were calculated using Windows NT Excel software. To determinate the degree of statistical significance, we performed a paired 2-tailed Student's *t*-test.

Results

Quantification of Growth Factors in the Blood-Preparations

Three different preparations (AS, PRP, and PRGF) were obtained from the blood of each volunteer ($n = 16$). Then, we measured by colorimetric assays the concentration of several growth factors in each blood preparation. We found that EGF levels were significantly higher in PRGF compared to the other two preparations (Table 2). Furthermore, in PRP, in which clotting had not occurred, the concentration of EGF was significantly lower than in the other two. Significant differences in FGF levels were not found among the different preparations, but the concentration of this factor was found to be age-dependent. Thus, the mean concentration of FGF in the younger volunteers (88.94 ± 9.04 pg/mL) was higher than that in the 40–49-year-old group (41.47 ± 5.89 pg/mL) and in the 50–59-year-old group (39.70 ± 3.97 pg/mL). Mean VEGF levels also were age-dependent, being highest in the group of oldest volunteers. In addition, VEGF levels were significantly lower in PRP preparation than in the other two preparations. We also observed higher levels of HGF in AS compared to PRP and PRGF, indicating that the method for obtaining AS increases the concentration of HGF. Finally, the concentrations of PDGF and fibronectin were not statistically different among the different preparations or with respect to age.

Quantification data showed important differences in the concentration of growth factors among volunteers. To avoid this variability in in vitro experiments, we used pools of the preparations for subsequent culture assays.

Cell Proliferation

We measured the effect on HCE cell proliferation of exposure for 24, 48, and 72 hours to the different blood derivatives. Two of the three preparations (AS and PRGF) produced a clear dose-

Table 2. Concentration of Growth Factors in the Three Different Blood Derivatives

Blood Derivatives	Age (y)	EGF (pg/mL)	FGF (pg/mL)	VEGF (pg/mL)	HGF (pg/mL)	PDGF (ng/mL)	Fibronectin (μg/mL)
AS	30–39	409.52 ± 69.04	102.50 ± 69.17	127.69 ± 100.84	282.47 ± 136.57	17.06 ± 4.24	30.83 ± 6.86
	40–49	460.39 ± 114.83	36.35 ± 20.42	143.68 ± 36.92	333.13 ± 62.59	16.71 ± 2.99	30.18 ± 9.86
	50–59	398.07 ± 76.16	36.73 ± 37.21	203.35 ± 97.65	222.90 ± 22.94	18.11 ± 4.15	32.34 ± 4.45
	Mean ± SD	**417.94 ± 82.62**	**58.55 ± 54.64**	**160.06 ± 89.90**	**275.83 ± 101.65‡**	**17.36 ± 3.73**	**31.11 ± 6.76**
PRP	30–39	287.79 ± 70.88	81.87 ± 52.93	57.97 ± 43.59	93.13 ± 92.45	17.48 ± 5.44	28.65 ± 8.37
	40–49	277.79 ± 85.61	50.30 ± 35.86	47.59 ± 39.76	48.47 ± 17.71	15.80 ± 4.52	31.36 ± 2.56
	50–59	273.49 ± 93.73	36.71 ± 32.83	70.75 ± 48.20	98.90 ± 11.49	20.68 ± 8.36	31.99 ± 1.90
	Mean ± SD	**279.83 ± 78.18**	**57.04 ± 44.44**	**60.31 ± 42.50†**	**84.60 ± 63.87**	**18.26 ± 6.44**	**30.55 ± 5.10**
PRGF	30–39	480.80 ± 94.88	82.45 ± 51.61	105.06 ± 80.33	117.32 ± 118.64	16.07 ± 5.01	32.05 ± 4.60
	40–49	522.64 ± 93.75	37.76 ± 19.33	81.49 ± 42.68	37.13 ± 42.88	14.49 ± 5.00	31.47 ± 3.02
	50–59	475.09 ± 109.06	45.66 ± 29.16	169.62 ± 84.88	79.07 ± 44.11	15.80 ± 4.83	31.98 ± 3.99
	Mean ± SD	**489.12 ± 95.48***	**58.27 ± 41.25**	**124.60 ± 79.64**	**83.74 ± 79.42**	**15.58 ± 4.65**	**31.83 ± 3.56**

* Statistically significant differences between PRGF and PRP, and/or AS ($P \leq 0.05$).
† Statistically significant differences between PRP and PRGF, and/or AS ($P \leq 0.05$).
‡ Statistically significant differences between AS and PRP, and/or PRGF ($P \leq 0.05$).

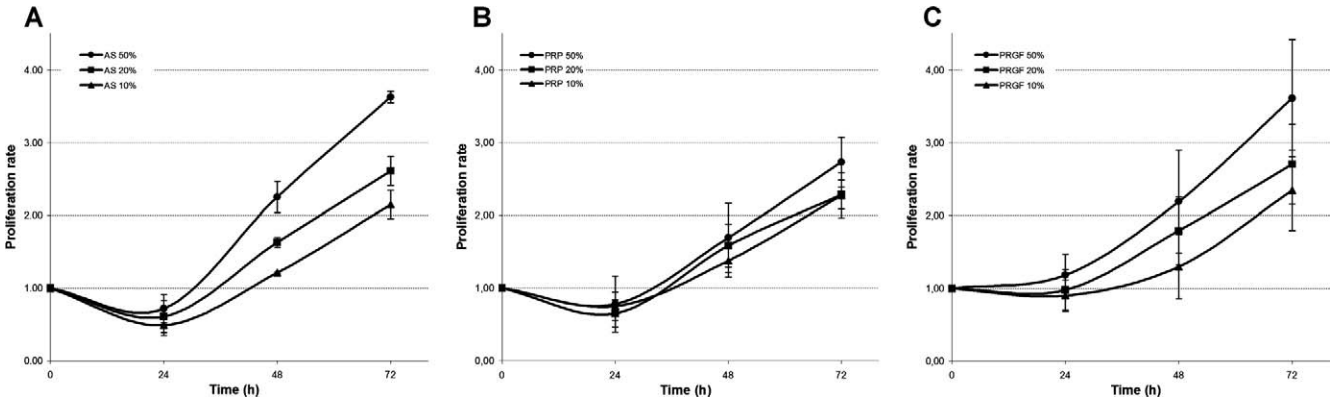

Figure 1. Patterns of proliferation (measured by the MTT assay) of HCE cells exposed for 24, 48, and 72 hours to increasing concentrations of the three blood derivatives. (**A**) AS. (**B**) PRP. (**C**) PRGF. Results are expressed as proliferation rate ± SD of viable cells with respect to viable cells at $t = 0$ hours. In each case, $n = 3$.

response growth pattern in HCE cells (Figs. 1A, 1C). However, PRP induced a weaker response, which did not appear to be dose-dependent (Fig. 1B). We observed a tendency for cells incubated with AS and PRP to exhibit reduced viability following 24 hours of incubation with different concentrations of the three preparations (Figs. 1A, 1B).

When comparing cell viability in response to the three different preparations with that in response to FBS (Fig. 2), we found that PRGF exhibited the highest cell viability at 24 hours (Fig. 2A). However, the three blood derivatives showed similar cell viability at doses of 10 and 20% at 48 and 72 hours of treatment (Figs. 2B, 2C). In contrast, doses of 50% AS and PRGF after 48 and 72 hours showed similar cell viability, which was higher than that of PRP. In cultures with 50% PRGF, cell viability was not significantly different from that observed when cells were cultured with FBS (Figs. 2A–2C).

Cell Morphology

Phase-contrast micrographs of HCE cells cultured with the three blood derivatives revealed substantial differences associated with the different treatments (Fig. 3). Thus, cells cultured with AS or PRP grew as quite homogeneous colonies of medium- and large-sized cells. In addition, PRP cultures showed a lower cell density, probably due to the weaker growth pattern of cells under this condition. In contrast, PRGF and FBS cultures grew in a similar manner, and both exhibited cell heterogeneity. Thus, two clearly distinguishable types of cell populations were observed, those with small cells that were grouped together, and those with larger and more flattened cells.

The absence of supplements from cultures was associated with a more differentiated phenotype in all cases. However, this was more evident in PRGF and FBS cultures, in which colonies of small-sized cells were observed rarely. Instead, medium- and large-sized cells coexisted in expanded colonies (Fig. 3).

Differentiation Analysis by Gene Expression Patterns

The passage of specific growth modulation signals through gap junctions may regulate the proliferation and differentiation of human epithelial cells. For this reason, we performed quantitative RT-PCR to study the expression level of several connexins, such as connexin 26 (*CX26*), connexin 30 (*CX30*), connexin 31.1 (*CX31.1*), and connexin 43 (*CX43*). These gap junction proteins are transmembrane proteins, which are differentially distributed along the corneal epithelium. In addition, we analyzed the expression of integrin α6, which is expressed mainly in the basal epithelial cell layer as a component of hemidesmosomes, and involucrin, which is expressed specifically in superficial epithelial cells in the human cornea. To this end, HCE cells were cultured for 14 days with 10% FBS, 20% AS, 20% PRP, or 20% PRGF, in the presence and absence of supplements.

Analysis of quantitative RT-PCR results revealed very distinct gene expression patterns in HCE cells cultured with PRGF in

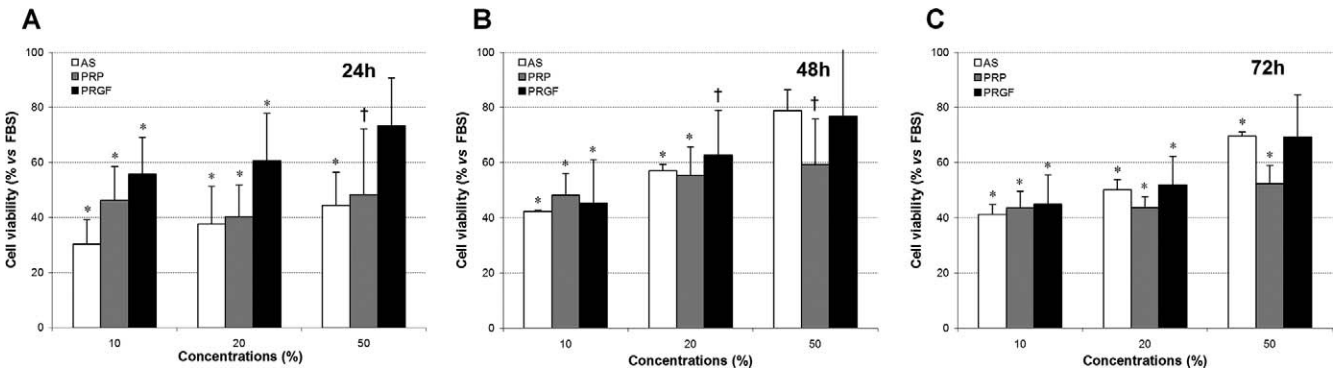

Figure 2. Viability (MTT assay) of HCE cells exposed to different concentrations of blood derivatives. Viability was measured 24, 48, and 72 hours after treatment. Results are expressed as percentage mean ± SD with respect to viability in FBS-treated cultures ($n = 3$ for each case). Statistically significant differences with respect to the FBS cultures $^*P \leq 0.01$. $\dagger P \leq 0.05$.

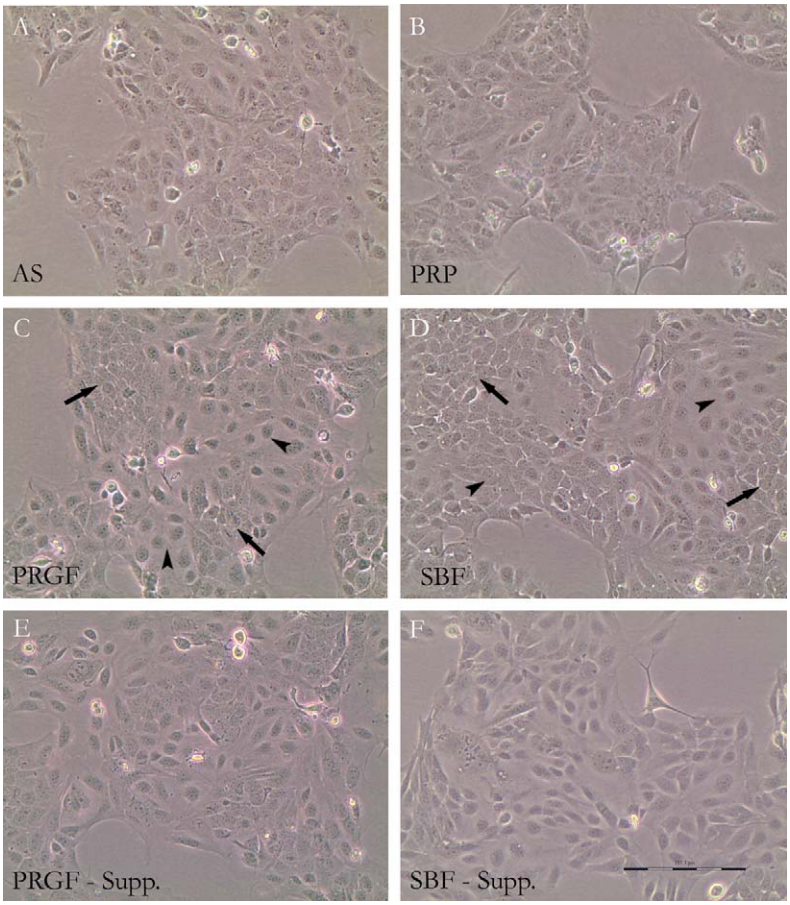

Figure 3. Phase-contrast microphotographs of HCE cell monolayers after 14 days of exposure to (**A**) AS, (**B**) PRP, (**C**) PRGF, (**D**) FBS, (**E**) PRGF without supplements (– Supp.), and (**F**) SBF – Supp. AS, PRP, and PRGF, each at a dilution of 20%, and FBS at 10%. Morphologic differences among cultures are apparent. Compacted colonies of small and roundish cells (*arrows*), and bigger and more flattened cells (*arrowheads*) are indicated. In the absence of supplements, lower density colonies were seen, and big and flattened cells were predominant. Fields illustrated in these images are representative of the whole culture. *Scale bar*: 200 μm.

comparison to cells cultured with AS or PRP. PRGF treatment was found to induce higher expression of *CX26*, *CX43*, and involucrin, with differences being statistically significant in the case of the latter two genes (Fig. 4). In contrast, expression of *CX31.1* was found to be the most variable among preparations, being significantly down-regulated in PRGF-exposed cultures in comparison to PRP and AS cultures. In addition, integrin α6 was more highly expressed in PRGF-cultured cells with respect to those cultured with AS or PRP, this difference being statistically significant for the latter case.

Finally, we also examined the effect of the absence or presence of supplements on the expression of these genes in HCE cells by real-time RT-PCR, since it is known that these factors are necessary to maintain the stable morphology of cultures. We observed that in the absence of supplements, the expression of involucrin and particularly *CX31.1* increased in all treatments (data not shown) in comparison to the corresponding treatment in the presence of supplements.

Discussion

We investigated the effect of three blood derivatives on HCE cell line. Two of the preparations are being used currently as therapeutic agents for several ocular surface diseases,[17,24] while the third preparation (PRGF) recently has been introduced into the field of ophthalmology.[28,29]

It is known that platelets are a source of a variety of growth factors with important functions in wound healing.[26,32–34] The PRP preparation that we studied is a platelet concentrate in which platelets are neither activated nor removed. However,

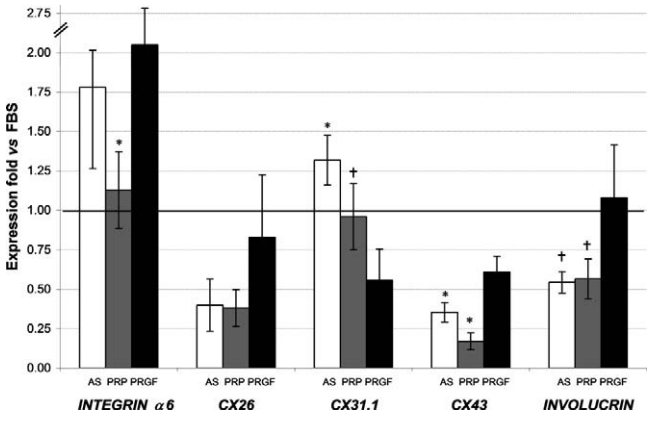

Figure 4. Real-time RT-PCR of integrin α6, several connexin and involucrin gene expression in HCE cells after 14 days of exposure to the three blood derivatives in media including supplements. Results are expressed as fold gene expression ± SD versus FBS cultures ($n = 4$). Statistically significant differences with respect to PRGF cultures. $^{*}P \leq 0.01$. $\dagger P \leq 0.05$.

platelet concentrates cannot be stored for more than a few days unless frozen. When PRP preparations are frozen, platelets break and their membranes produce a cloudy aspect in PRP after thawing. This debris is thought to induce apoptotic cell death.[35] As an alternative, our group has been using another blood derivative (serum derived from PRGF) in which the growth factors are released from platelets into the supernatant by their activation with calcium chloride, which is preferred over thrombin, because it enables a more sustained and physiologic release of platelet constituents, without immunologic reactions and the appearance of coagulopathies.[25] In addition, the release of growth factors occurs in the absence of leukocytes, thereby avoiding the pro-inflammatory effects of proteases and acid hydrolases contained in white blood cells.[36,37] Finally, we also used another frequently used blood derivative, AS. This preparation is obtained using extraction tubes that facilitate the subsequent coagulation process.

We examined the in vitro effects of each preparation on the growth, morphology, and cell-to-cell communication features of immortalized HCE cells, using concentrations of blood derivatives that are used commonly in clinical practice.

First, we determined the concentrations of several growth factors in these preparations to characterize their content. These factors can influence corneal healing and may explain the different effects of the blood derivatives. It has been demonstrated that EGF supports epithelial cell proliferation[38,39] and, as in all tissue repair, cell proliferation is a necessary step for corneal healing. Accordingly, we observed that EGF is critical for HCE cell proliferation because removal of EGF from the medium leads to almost 50% reduction in proliferation (data not shown). We found the highest concentrations of EGF in PRGF. Thus, the presence of high levels of EGF is likely to be one of the reasons for the robust proliferative activity of HCE cells in PRGF cultures. Consistent with this idea is the finding that in PRP, in which platelet activation has not occurred, EGF levels are lower than in other preparations and there is a weaker effect on corneal epithelial cell growth.

HGF has been shown to be implicated in the paracrine enhancement of angiogenesis by inducing VEGF expression. Thus, VEGF and HGF may be secreted or synthesized in the same local environmen.[40] Accordingly, the significantly higher concentrations of HGF in AS could induce angiogenic effects. However, despite these levels of VEGF and HGF, we did not detect neovascularization in any of the patients treated with AS.[28] This absence of neovascularization may be due to an inhibitory effect or to the existence of a balance between pro-angiogenic (VEGF, HGF, and so forth) and anti-angiogenic (thrombospodin-1, platelet factor-4, endostatin) factors in the preparations.

The mitogenic activity of PDGF has been demonstrated recently.[40,41] However, this activity is inhibited only modestly by an anti-PDGF antibody,[40] suggesting that other proteins may contribute to the total cell proliferation. Consistently, our data showed similar levels of PDGF in all three blood derivatives, which nevertheless exhibited very different cell proliferation patterns.

We detected high concentrations of fibronectin in the three preparations. During wound healing, fibronectin/fibrinogen receptors are upregulated on epithelial cells, which migrate over the bare wound.[42] Therefore, the high concentrations of fibronectin found in blood derivatives could favor cell migration. Furthermore, high levels of EGF receptor have been reported to be expressed in the cells migrating over the wound.[43] Thus, the relatively high EGF levels in PRGF also may contribute to improved wound healing.

AS and PRGF were found to induce robust proliferation in HCE cells. However, cultures incubated in the presence of PRGF did not exhibit the tendency to reduced viability exhibited by cells 24 hours after incubation with AS and PRP. These findings raise the possibility that PRGF may be tolerated better initially by the corneal epithelium than the other two treatments.

MTT assays showed that the proliferative response of cells exposed to AS and PRGF (but not PRP) was dose-dependent. On the other hand, HCE cells cultured with similar preparations produced by others have been shown to exhibit substantially reduced viability after 24 hours.[22] However, we showed here that the viability of cultures exposed to these three preparations does not decrease with respect to FBS cultures, following 24–72 hours of incubation, supporting the potential safety of these derivatives in clinical applications.

We also found that the three blood derivatives induced different morphologies in cultured cells. AS and PRP appeared to induce cell growth in quite homogeneous colonies of medium- or large-sized cells. In contrast, HCE cells cultured with PRGF exhibited a more heterogeneous morphology; they included a subpopulation of small cells organized in compact colonies. These may represent phenotypically less differentiated cells that may be a cellular source for the renewal of the epithelium. These cultures also exhibited another subpopulation of large and flattened cells, which likely represent a more differentiated phenotype. In agreement with these data, it has been reported that the differentiation of corneal cultured cells results in cell flattening and the formation of large epithelial sheets with increased intercellular communication.[44]

Corneal epithelial cells communicate with each other through gap junctions.[45–48] These gap junctions, which are made up of connexins, may contribute to the regulation of corneal epithelial cell functions, such as cell growth, differentiation, adhesion, and migration.[47,49] A number of connexins are known to be expressed in HCE cells. *Cx26* is expressed in the basolateral plasma membranes of basal cells.[44] A suprabasal distribution has been found for *Cx30*. In contrast, *Cx43* localizes to the apical surface in the basal layer and is found in all plasma membranes throughout the suprabasal layers. Finally, *Cx31.1* has been localized to the apical cell surface of basal cells.[44] However, neither *CX26*, *30*, *31.1*, nor *43* are expressed in the upper and most differentiated cells of epithelia; cells in the latter intensely express the late differentiation marker involucrin,[50] which is a structural protein found in the cytosol.

In this study, we detected the expression of *CX26*, *31.1*, and *43,* but not *CX30,* in cultured HCE cells. An increase in connexins expression in corneal epithelial cells is indicative of cell differentiation,[43,44,51] and the formation of a well-structured and organized epithelium. We found that the expression of *CX26, CX43*, and even involucrin are upregulated in PRGF-treated cultures, in comparison to those treated with AS and PRP. Consistently, at a morphologic level, we observed a subpopulation of flattened and differentiated cells in PRGF-treated cultures.

In response to corneal epithelium debridement, a dynamic modulation of gap junction expression occurs, accompanied by epithelial proliferation and differentiation.[52] In addition, migrating epithelial cells show reduced expression of Cx43, together with a different cellular distribution of this protein.[43,47,53,54] Here, we found that treatment of HCE cells with any of the three blood derivatives is associated with a lower expression of *CX43* in comparison to that found in FBS-treated cultures. Down-regulated *CX43* expression may reflect active migration processes under these culture conditions.

On the other hand, integrin α6 is an adhesion molecule that attaches cells to extracellular matrix proteins.[55] It is expressed mainly in less differentiated basal epithelial cells, as a component of hemidesmosomes. It also has been demonstrated that during regeneration of corneal epithelium, the hemidesmosomes relocate, allowing basal cells to migrate

towards denuded areas, and it probably is a prerequisite for the formation of new functional gap junctions.[43,56] In this context, we detected the highest levels of integrin α6 expression in PRGF-treated cultures, in which compact colonies of small and roundish cells could be observed. Cells with this more undifferentiated aspect were detected predominantly in PRGF-treated cultures. These cells are likely to be those that underlie the proliferation capacity of HCE cultures treated with PRGF, and may be the in vitro homologs that are responsible for the renewal of the epithelium.

Enhanced expression of involucrin and *CX31.1* was detected when supplements were removed from culture media. Increased *CX31.1* expression usually is associated with epithelial cell differentiation.[57,58] Consistently, we also observed a more differentiated phenotype of HCE cells and reduced proliferation under these culture conditions (not shown). In this sense, we observed that the proliferation of cultures incubated with FBS in the absence of supplements was reduced by 70%. However, this decrease was almost unappreciated in PRGF-treated cultures, possibly due to the presence of the aforementioned undifferentiated cells, which do not appear in cultures treated with other blood derivatives.

In summary, AS and PRGF induce dose-dependent cellular proliferation to a similar degree. However, PRGF exhibits a higher concentration of EGF, and upregulates the expression of several genes involved in communication and cell differentiation, in comparison with the other blood derivatives studied in our report. In addition, PRGF-incubated HCE cells present a heterogeneous morphology, exhibiting differentiated and nondifferentiated cell phenotypes, whereas AS- and PRP-incubated cells are more homogeneous. The clinical relevance of the present results resides in their support of the use of a novel serum derived from PRGF, which is produced in the absence of leucocytes, as a promising alternative treatment for ocular surface disorders. Although a well-characterized HCE cell line represents a very useful model to understand better and simulate in vivo situations, in vivo experiments will be necessary to confirm that our observations in culture correlate with biological outcomes.

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Biological Stability of Plasma Rich in Growth Factors Eye Drops After Storage of 3 Months

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Purpose: We evaluated whether plasma rich in growth factors eye drops maintain their composition and biological activity after storage for 3 months at −20°C and after storage at 4°C or room temperature (RT) for 24 hours, compared with samples obtained at time 0 (fresh samples).

Methods: Blood from 10 healthy donors was collected, centrifuged, and plasma rich in growth factors was prepared by avoiding the collection of the buffy coat. Eye drops were kept fresh or were stored at −20°C for 15, 30, and 90 days. For each time, 2 aliquots were kept at RT or at 4°C for 24 hours. Osmolarity, vitamin A, fibronectin, platelet-derived growth factor-AB, vascular endothelial growth factor, epithelial growth factor, and transforming growth factor-β1 were quantified. The proliferative and migratory potential of the eye drops was assayed on primary human keratocytes.

Results: Platelet-derived growth factor-AB, vascular endothelial growth factor, epithelial growth factor, and vitamin A levels remained constant for each time and for each storage condition, whereas fibronectin, transforming growth factor-β1, and osmolarity values were slightly modified after freezing. Cell proliferation and migration were significantly enhanced with the biological eye drops independently of the time and the storage condition. No microbial contamination was observed in any plasma rich in growth factors eye drops.

Conclusions: Plasma rich in growth factors eye drops can be stored for up to 3 months without any reduction of the main proteins involved in ocular surface healing. Their use during 24 hours either at 4°C or at RT did not alter the composition and the in vitro biological activity of the eye drops.

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Injuries of the ocular surface involve epithelial and stromal disorders caused by a variety of underlying diseases. Effective wound healing is essential and necessary to recover adequate functionality of the injured ocular tissue. These complex cellular and extracellular events are orchestrated by different protein mediators including growth factors.

Plasma rich in growth factors technology involves the use of the patient's own plasma enriched in platelets for therapeutic purposes.[1–5] The versatility, safety, and efficacy of this approach have been widely demonstrated in several medical fields.[1–5] Plasma rich in growth factors presents several distinguishing properties compared with other platelet-rich plasma products, and these include improved biosafety, an optimized platelet, and morphogen dose, which has been related to optimal biological benefits[6] and the absence of leukocytes in its composition. The latter release a number of proinflammatory agents such as metalloproteases and acid hydrolase content that may provoke negative tissue-destroying effects.[7]

Plasma rich in growth factors, also known as PRGF-Endoret, contains a pool of biologically active proteins and growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), and epithelial growth factor (EGF).[8,9] Recently, different types of platelet-rich plasmas have been successfully used in the treatment of several ocular surface diseases such as dry eyes and persistent corneal defects and ulcers.[10–13] Beneficial effects of platelet-rich plasma on ocular surface diseases could be attributed to biochemical and biophysical properties similar to those of natural tears, including pH, osmolarity, protein content, and growth factors involved in wound healing; and their antimicrobial effect.[13,14]

Ocular surface disorders are usually chronic diseases that demand medium or long-term treatments. Therefore, it is pivotal that the biological functionality and stability of the treatments are preserved for weeks so that they can be used on a daily basis for months. In the case of plasma rich in growth factors eye drops, although several reports have supported their safety and biological potential, there is a lack of studies demonstrating their long-term functionality at different storage conditions.

The main objective of this study was to investigate whether plasma rich in growth factors eye drops maintain their biological activity potency after storage at −20°C for 3

months compared with samples obtained at time 0 (fresh samples). In addition, if one assumes the daily use of an eye drop dispenser, it would also be necessary to determine whether biological eye drops preserve their properties during their 24-hour lifetime at both 4°C and room temperature (RT). To address this, the biophysical properties, concentration of wound-healing cytokines, microbial contamination, and biological potential of plasma rich in growth factors eye drops were analyzed and compared for each storage condition.

MATERIALS AND METHODS

Sample Preparation of Plasma Rich in Growth Factors (PRGF-Endoret)

Blood from 10 healthy young donors was collected after obtaining informed consent and was put into 9-mL tubes containing 3.8% (wt/vol) sodium citrate. The study was performed following the principles of the Declaration of Helsinki. The blood was separated using a centrifuge (BTI System IV, Vitoria, Spain) at 580*g* for 8 minutes at RT, and the whole plasma column was drawn off to avoid collecting the buffy coat containing the leukocytes. Platelet concentration was measured with a hematology analyzer (Micros 60; Horiba ABX, Montpelier, France). The collected platelet-rich plasma was incubated with PRGF activator (BTI Biotechnology Institute, SL, Miñano, Spain) at 37°C in glass tubes for 1 hour. The supernatants released were collected by aspiration after centrifugation at 1000*g* for 10 minutes. Finally, the supernatant volume was aliquoted under laminar air flow conditions to be either used at that precise moment (time 0) or stored at −20°C for 15, 30, and 90 days. Two aliquots from each donor were removed from the freezer, thawed, and stored the day before the end of each storage period. One of those aliquots was kept at 4°C (time + 4°C), whereas the other one was maintained at RT (time + RT) for 24 hours until its use (see Figure, Supplemental Digital Content 1, http://links.lww.com/ICO/A142).

Characterization of PRGF-Endoret Eye Drops

Several growth factors such as PDGF-AB, vascular endothelial growth factor (VEGF), EGF, and TGF-β1 (R&D Systems, Minneapolis, MN) and fibronectin protein (Takara, Shiga, Japan) were measured in the supernatants of the samples stored for each time and for each temperature of the study using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay kits. Vitamin A was quantified using ultraperformance liquid chromatography (Waters, Cerdanyola del Vallès, Spain), the pH was analyzed with a pH meter (Thermo scientific; Madrid, Spain), and the osmolarity was measured with an osmometer (Gonotec GmbH, Berlin, Germany).

Cell Culture

Primary human keratocytes (HK; ScienCell Research Laboratories, San Diego, CA) were cultured according to the manufacturer's instructions. Briefly, the cells were cultured at 37°C and 5% CO_2 atmosphere until confluence in fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories) and were then detached with animal origin–free trypsin-like enzyme (TrypLE Select; Gibco–Invitrogen, Grand Island, NY). Cell viability was assessed by means of trypan blue dye exclusion. Passage 3 to 6 cells were used in all the experiments.

Proliferation Assay

Keratocytes were seeded at a density of 10,000 cells per square centimeter on 96-well optical bottom black plates and maintained with serum-free medium for 48 hours. Then, the

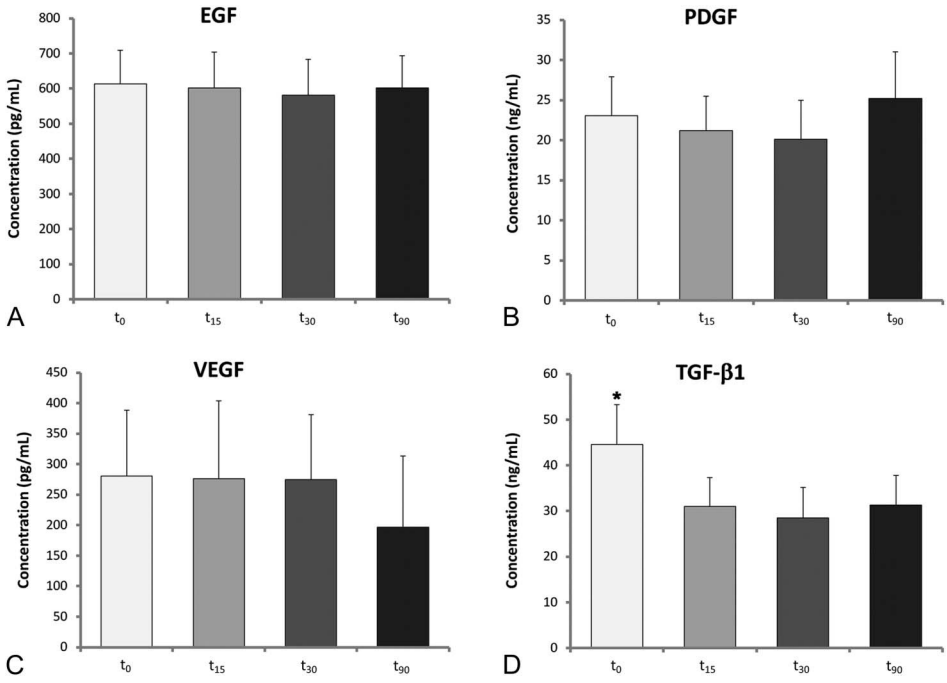

FIGURE 1. Growth factor concentration of fresh samples (t_0) of PRGF-Endoret eye drops and after storage at −20°C for 15, 30, and 90 days (t_{15}, t_{30}, and t_{90}, respectively; growth factor concentration values of storage at RT and at 4°C are not included). Levels of EGF (A), PDGF (B), VEGF (C), and TGF-β1 (D) in the different eye drops. *Significant differences ($P < 0.05$) in the growth factor concentration between PRGF-Endoret samples obtained at t_0 (fresh samples) compared with those stored at −20°C.

culture medium was replaced by serum-free medium supplemented with either the culture medium alone (FM) with 0.1% fetal bovine serum (FBS) as a control for nonstimulation (control) or with 20% (vol/vol) PRGF-Endoret for each time and for each temperature of storage. The study period was 48 hours. The density of cells in the culture was estimated using the CyQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA). Briefly, the medium was removed, and the wells were washed carefully with phosphate buffered saline (PBS). Then, the microplate was frozen at –80°C for efficient cell lysis in the CyQUANT assay. After thawing the plates at RT, the samples were incubated with RNase A (1.35 kU/mL) diluted in cell lysis buffer for 1 hour at RT. Then, 2× CyQUANT GR dye/cell lysis buffer was added to each sample well, mixed gently, and incubated for 5 minutes at RT, protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies). A DNA standard curve ranging from 7.8 to 1000 ng/mL was included in all fluorescence quantifications. As an index of cell number, calibration curves ranging from 2500 to 90,000 cells per square centimeter were established using the CyQUANT assay.

Migration Assay

To quantify the migratory potential of keratocytes, they were plated in culture inserts (Ibidi, GmbH, Martinsried, Germany) placed on a 24-well plate at a high density and were grown with complete FM until confluence. After carefully removing the inserts, 2 separated cell monolayers leaving a cell-free gap of approximately 500-μm thickness were created. The cells were washed with PBS and incubated with the same treatment as in the proliferation assay (0.1% FBS and 20% of PRGF-Endoret for each time and for each temperature of storage) in quintuplicate for 24 hours. After this period, the different culture media were removed, and the cells were incubated with 1/500 Hoechst 33,342 in PBS for 10 minutes. To quantify the number of migratory cells, phase contrast images of the central part of the septum before treatment and phase contrast and fluorescence photographs after the treatment time were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB; Leica Microsystems). The gap area and the migratory cells found in this gap after 24 hours of treatment were measured using the Image J Software (NIH, Bethesda, MD). The results were expressed as the number of cells migrated per square millimeter of area.

Sterility Analysis

One milliliter from each PRGF-Endoret sample stored for different time points and temperatures was collected to check the sterility. For this purpose, thioglycollate broth and tryptic soy broth were used for the qualitative determination of aerobic and facultative anaerobic microorganisms in the samples. After inoculation, culture vials were incubated at 32°C for thioglycollate broth and at 22°C for tryptic soy broth for 14 days and monitored for the growth of microorganisms. Increase in broth turbidity was considered as positive for microbial contamination.

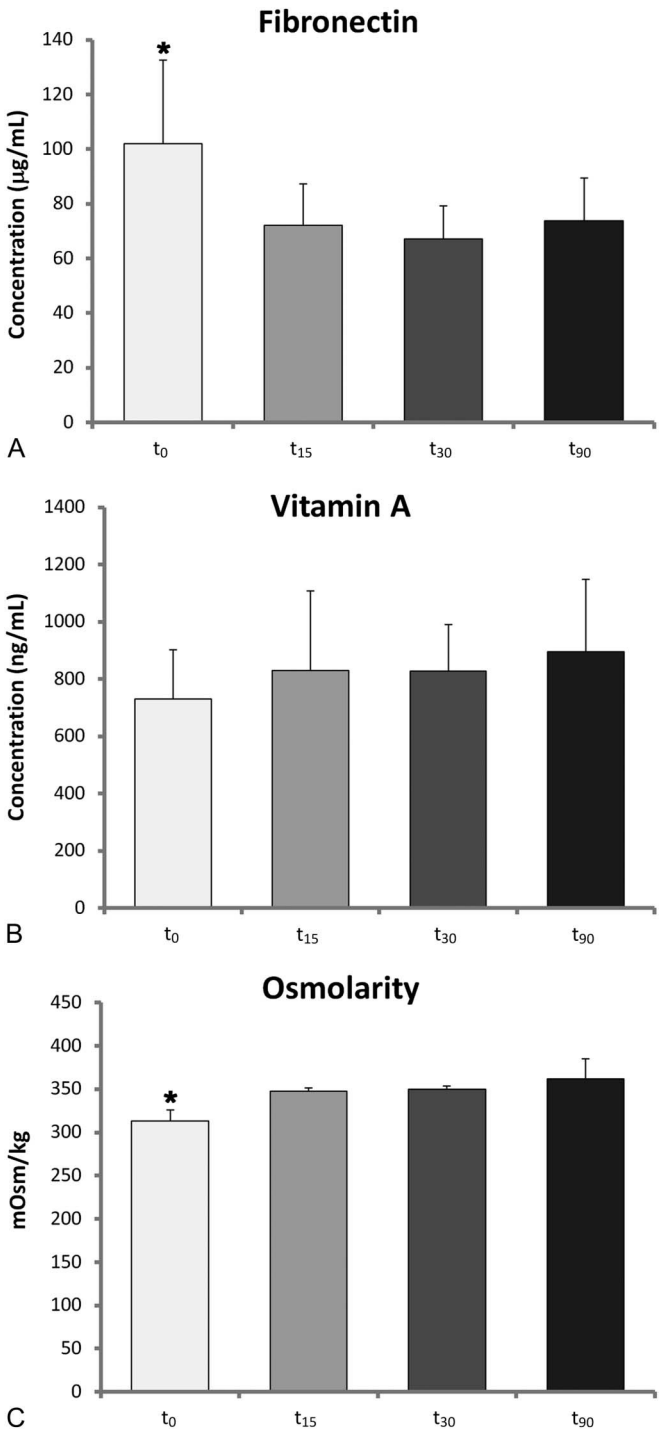

FIGURE 2. Characteristics of PRGF-Endoret eye drops for each time of the study [fresh samples (t_0) and after storage solely at −20°C for 15, 30, and 90 days]. Fibronectin concentration (A), vitamin A levels (B), and osmolarity (C). *Significant differences ($P < 0.05$) in the composition between PRGF-Endoret samples obtained at t_0 (fresh samples) compared with those stored at −20°C.

Statistical Analysis

Data are expressed as mean ± SD. After checking the normal distribution and homoscedasticity from groups, repeated measures analysis of variance was used to assess the differences between the variables at the 4 different times points (t_0, t_{15}, t_{30}, and t_{90}) and at different temperatures of storage (−20°C, RT, and 4°C). Significant differences were further investigated using the Scheffé test, with a level of significance set at $a = 0.05$. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc, Chicago, IL).

RESULTS

Characterization of Plasma Rich in Growth Factors Samples

Plasma rich in growth factors (PRGF-Endoret) eye drops were obtained from 10 donors with a mean age of 36 years, ranging from 23 to 44 years. Platelet enrichment of the PRGF-Endoret preparations was 1.96-fold over the baseline concentration in whole blood. None of the preparations contained detectable levels of leukocytes, a distinguishing property of this biological approach.

Eye Drop Characterization at Different Time Points

Characteristics of plasma rich in growth factors eye drops were analyzed on the day of the collection (fresh samples) and after storage at – 20°C for 15, 30, and 90 days. Growth factor concentration analyzed for each storage time is shown in Figure 1. The concentration of EGF, PDGF, and VEGF remained constant at all times. No significant differences were observed independently of the growth factor or the time point. In the case of TGF-β1 and fibronectin, although the concentration did not change during the storage time period, their values after freezing were significantly lower than those quantified in fresh eye drops (Figs. 1, 2). Interestingly, no variation of vitamin A concentration was observed during the study period (Fig. 2B). However, the storage conditions at –20°C slightly increased the osmolarity values with regard to fresh eye drops (Fig. 2C).

Effect of the 24-Hour Temperature Storage Conditions

The day before each study period (15, 30, and 90 days), 2 samples from each donor were taken from the freezer, 1 was stored at 4°C and the other was stored at RT for 24 hours. After that period, another sample from each donor was taken from the freezer and was thawed. The detailed characterization of each eye drop is given in table 1. With the exception of EGF, the remaining parameters measured, including osmolarity and PDGF, VEGF, TGF-β1, fibronectin, and vitamin A concentrations, remained similar independently of the 24-hour storage conditions, reinforcing the idea that PRGF-Endoret preserves its composition in its 24-hour lifetime.

Cell Proliferation

Representative images of keratocytes including those of the nontreated control group, cells treated with fresh plasma rich in growth factors eye drops (t_0), and cells cultured with plasma rich in growth factors eye drops frozen at −20°C for 30 and 90 days are shown in Figure 3. The proliferation of keratocytes significantly increased after treatment with all PRGF-Endoret preparations. No significant differences were observed among the different times and storage conditions. In fact, keratocytes treated with the fresh and frozen PRGF-Endoret eye drops at the different times and at storage conditions enhanced their proliferation 8- to 11-fold, with the mean being 9.8-fold, compared with the proliferation of the control group.

Migration Assay

PRGF-Endoret eye drops significantly ($P < 0.05$) stimulated the migration of HK cells (Fig. 4). No significant differences were observed among the different PRGF-Endoret eye drop preparations. Figures 4A to D show the fluorescence Hoechst images of HK cell migration after treatment or without treatment (control group) with either fresh or frozen PRGF-Endoret eye drop preparations. The biological eye drops increased the migratory capacity of the cells 3- to 4-fold

TABLE 1. Composition of the Different PRGF-Endoret Eye Drops

	EGF (pg/mL)	PDGF (ng/mL)	VEGF (pg/mL)	TGF-β1 (ng/mL)	Fibronectin (μg/mL)	Vitamin A (ng/mL)	Osmolarity (mOsm/kg)	pH
t_{15}								
−20°C	601 ± 103	21.17 ± 4.31	276 ± 128	30.99 ± 6.33	71.96 ± 15.29	829 ± 279	347 ± 4	7.96 ± 0.18
RT	546 ± 89*	21.65 ± 5.14	272 ± 105	31.71 ± 6.69	73.02 ± 15.34	889 ± 242	348 ± 5	7.97 ± 0.20
4°C	600 ± 109	21.07 ± 4.55	270 ± 105	31.17 ± 6.87	76.94 ± 18.75	891 ± 241	348 ± 5	7.96 ± 0.20
t_{30}								
−20°C	581 ± 101	20.13 ± 4.84	274 ± 107	28.44 ± 6.77	67.03 ± 12.12	827 ± 164	350 ± 4	8.28 ± 0.05
RT	533 ± 71*	20.78 ± 5.87	275 ± 107	29.19 ± 6.89	70.90 ± 11.98	779 ± 135	351 ± 5	8.33 ± 0.10
4°C	578 ± 91	20.63 ± 5.43	270 ± 104	29.23 ± 6.75	70.50 ± 19.40	834 ± 190	350 ± 5	8.29 ± 0.06
t_{90}								
−20°C	601 ± 92	25.22 ± 5.82	196 ± 116	31.29 ± 8.47	73.69 ± 15.66	894 ± 170	362 ± 13	8.27 ± 0.20
RT	562 ± 92*	25.48 ± 5.95	188 ± 108	31.60 ± 7.00	72.53 ± 13.61	909 ± 257	343 ± 3	8.30 ± 0.22
4°C	601 ± 107	25.13 ± 5.65	195 ± 110	31.29 ± 6.34	76.00 ± 16.98	854 ± 266	345 ± 4	8.26 ± 0.21

*Significant differences compared with fresh PRGF-Endoret eye drops.

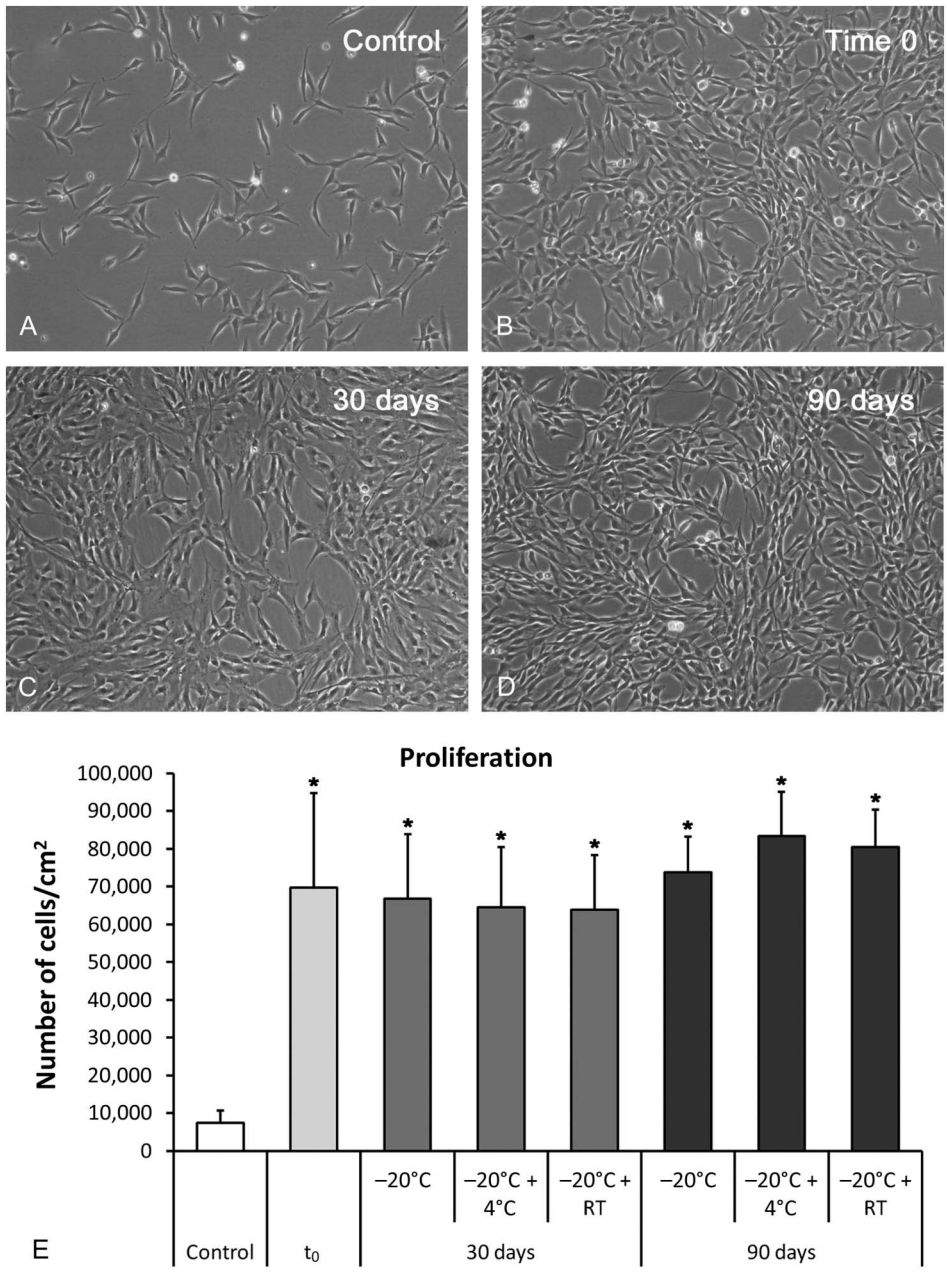

FIGURE 3. A–D, Phase contrast photomicrographs illustrating the proliferation rate of HK cells cultured with a control medium (A), with fresh PRGF-Endoret (B), or with PRGF-Endoret eye drops stored at −20°C for 30 (C) and 90 days (D). Proliferation of the HK cells after treatment with fresh or frozen PRGF-Endoret eye drops. Storage of PRGF-Endoret samples at different times or temperatures significantly increased (*$P < 0.05$) the proliferation rate of the HK cells compared with that in the control group, and no differences were found among the PRGF-Endoret (E).

compared with that in the control group, independently of the storage condition and frozen time period.

Sterility Analysis

Analysis performed on each eye drop sample stored at different times and at different temperatures showed no microbiological contamination. Furthermore, none of the cell cultures tested for proliferative and migration assays with PRGF-Endoret eye drops showed signs of contamination.

DISCUSSION

The tear film is a complex and dynamic solution composed mainly of growth factors and biologically active proteins involved in corneal tissue homeostasis and in ocular surface wound healing. Recent data suggest that plasma rich in growth factors (PRGF-Endoret) eye drops are safe and effective in the treatment of several ocular surface diseases.[12,13] However, a detailed evaluation of the biological stability of the formulation with the aim of showing that the autologous eye drop preserves its biological potential independently of when it is used and how it is stored is lacking.

Results from our study show that concentrations of some proteins and growth factors such as EGF, PDGF, and VEGF present in PRGF-Endoret eye drops are not modified after their storage for at least 3 months at −20°C. Furthermore, the vitamin A content in the eye drops after 3 months of storage at −20°C is similar to that in the fresh eye drops. On the

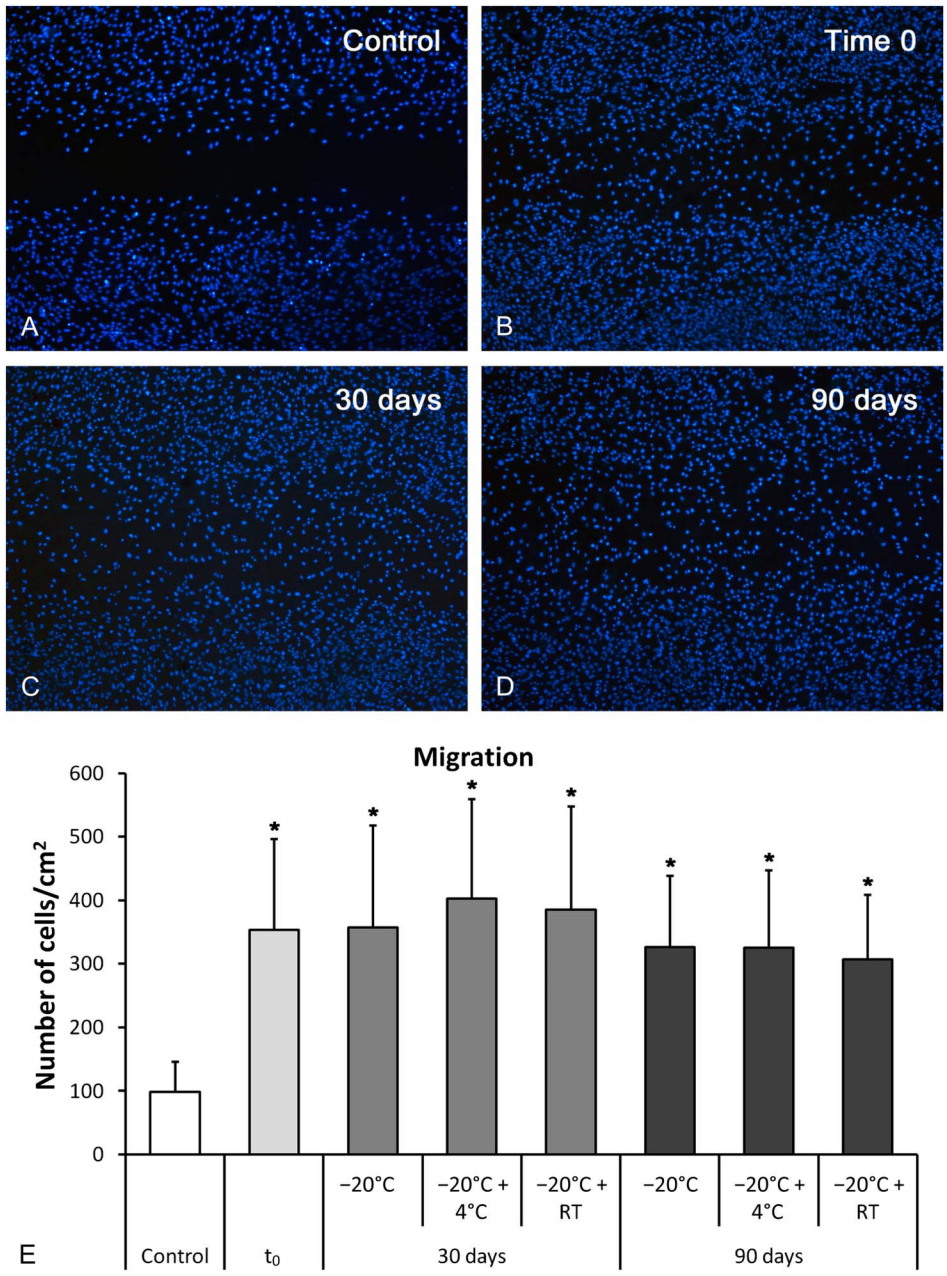

FIGURE 4. Migration assay in HK cells. (A–D) Images of Hoechst counterstaining of the migration assay in HK cells cultured with control medium (A), with fresh PRGF-Endoret (B), or with PRGF-Endoret eye drops stored at −20°C for 30 (C) and 90 days (D). Migration of the HK cells after treatment with fresh or frozen PRGF-Endoret eye drops increased significantly (*$P < 0.05$) compared with that in the control group, and no differences were found among the PRGF-Endoret samples (E).

contrary, TGF-β1, fibronectin, and osmolarity levels suffered variations after freezing storage conditions.

The osmolarity levels of normal tear fluid range between 300 and 310 mOsm/kg.[16,17] However, preclinical and clinical studies of hyperosmolarity in dry eyes have demonstrated that osmolarity levels >425 mOsm/kg are required to induce an inflammatory response on epithelial cells or to generate discomfort in patients with dry eyes.[18,19] Our results show that osmolarity levels remained <360 mOsm/kg during the entire study period, and was perfectly tolerated by the ocular tissues.[19,20]

In our study, fibronectin levels decreased after freezing compared with those of the fresh samples, suggesting that fibronectin may have been partially degraded after cryostorage.[22] However, the levels of fibronectin of the frozen and thawed PRGF-Endoret eye drops remained within the range found in normal ocular tears.[23]

The mean TGF-β1 concentration in fresh samples was 44.5 ng/mL, and although the levels were lower in the stored eye drops, they remained similar to those found in natural tears.[27] It has to be remarked that the overexpression of TGF-β1 in injured ocular tissue may be responsible for the development of tissue scarring.[28,29] In a previous study,[30] it was observed that PRGF-Endoret eye drop treatment protected keratocytes against myofibroblast transformation, minimizing fibrotic tissue development.

The minimal variation in some protein concentrations observed through the stability study did not change the biological activity of PRGF-Endoret eye drops. In fact, keratocytes treated

with the fresh and frozen PRGF-Endoret eye drops at different times and storage conditions enhanced their proliferation 8- to 11-fold and their migration 3- to 4-fold compared with that in the control group. No significant differences were observed among the different times and storage conditions.

With the exception of the EGF levels, no variations in protein levels and in biological effects were observed between the frozen PRGF-Endoret eye drops and the eyes drops stored at RT or 4°C for 24 hours. Finally, no microbial contamination was found in any of the PRGF-Endoret eye drops for any time or for any storage conditions.

In summary, the results obtained from this study demonstrate that plasma rich in growth factors (PRGF-Endoret) eye drops can be stored for up to 3 months without any reduction of the main proteins and growth factors involved in ocular surface wound healing. The storage of the eye drops at −20°C for up to 3 months and their use and preservation for 24 hours at either 4°C or RT did not alter the composition and the in vitro biological activity of the eye drops.

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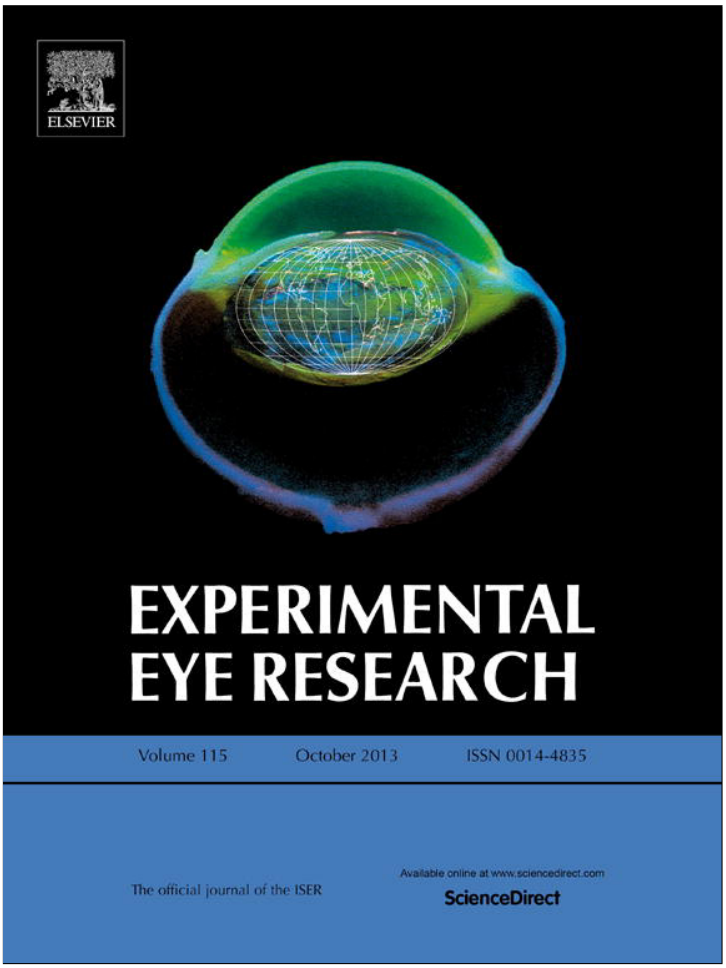

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Plasma rich in growth factors (PRGF-Endoret) stimulates corneal wound healing and reduces haze formation after PRK surgery

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ABSTRACT

This study evaluated the efficacy of Plasma rich in growth factors (PRGF-Endoret) on the corneal wound healing process after Photorefractive keratectomy (PRK). To address this, blood from three healthy donors was collected, centrifuged and, the whole plasma column (WP) and the plasma fraction with the highest platelet concentration (F3) were collected. The effects of F3 and WP on the proliferation and migration of human corneal epithelial cells (HCE) were analyzed. PRK was performed on C57BL/6 mice. Animals were divided in three treatment groups: Control, F3, and WP. Corneal wound healing and haze formation were evaluated macroscopically. Eyes were collected at 1, 2, 3, and 7 days after surgery, and were processed for histological studies. Immunofluorescence was used to assess cellular proliferation, apoptosis and myofibroblast transformation in the mouse cornea. Results showed a significant increased on proliferation and wound healing after F3 and WP treatment when compared with control group. In vivo studies showed significant reduction on haze formation in mice treated with both PRGF-Endoret formulations (F3 and WP). Histological studies showed an increase of epithelial cell proliferation in corneas of control group, promoting an epithelial hyperplasia. The number of SMA-positive cells (corresponding to myofibroblast differentiation) was significantly lower in the PRGF-Endoret group than in the control group, correlating with the higher transparence results observed macroscopically in both PRGF-Endoret groups. According to this, it can be concluded that PRGF-Endoret accelerates corneal tissue regeneration after PRK, reducing haze formation.

1. Introduction

The cornea is the transparent tissue of the eye that consists mainly in three layers: an outer layer containing an epithelium, a middle stromal layer composed by specialized cells called keratocytes, and an inner layer of endothelial cells. The homeostatic mechanisms regulating the normal physiological renewal of the corneal tissue involves proliferation, migration and differentiation of cells (Dua et al., 1994).

After corneal injury, epithelial cells from the basal epithelial layer and from limbus proliferate and migrate to cover the wound bed before differentiating into new multilayered epithelium (Dua et al., 1994). In contrast, keratocytes underlying the damage area undergo apoptosis (Wilson et al., 1996), and the quiescent keratocytes adjacent to the injury change into activated cells, enter into the cell cycle and subsequently migrate to the site of injury (Del Pero et al., 1990; Fini and Stramer, 2005). These new cells have the characteristics of fibroblasts, and some of them develop actin-myosin contractile elements, consistent with myofibroblasts (Garana et al., 1992; Zieske et al., 2001). During corneal repair, the transformed myofibroblasts are responsible for wound contraction and for extracellular matrix (ECM) deposition and organization (Jester et al., 1999b). After complete tissue repair, the new ECM formed takes over the mechanical load and myofibroblast cells disappear by apoptosis or by differentiation to keratocytes (Maltseva et al., 2001; Tomasek et al., 2002). Abnormal tissue regeneration process can lead to a corneal ulceration and scarring (Dupps and Wilson, 2006).

Abbreviations: PRGF-Endoret, Plasma Rich in Growth Factors; PRK, Photorefractive keratectomy; HCE, Corneal epithelial cells; SMA, Smooth muscle actin; ECM, Extracellular matrix; EGF, Epithelial growth factor; FGF, Fibroblast growth factor; PDGF, Platelet-derived growth factor; TGF, Transforming growth factor-beta; NGF, Nerve growth factor; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; PBS, Phosphate buffered saline; TUNEL assay, Terminal deoxyribonucleotidyl transferase-mediated dUTP-fluorescein Nick-End labeling assay.

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This biological process of wound healing is mediated by many proteins, including several growth factors like, epithelial growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factors (PDGF), among others (Imanishi et al., 2000; Klenkler and Sheardown, 2004; Wilson et al., 1994). These growth factors may infiltrate from the tear film to the superficial tissues of the eye (Wilson et al., 2001).

Artificial tears are the most widely used approaches for the conventional management of ocular surface damage with good outcome regarding lubrication and relieve the symptoms of dry eye. However, they lack the biological components of healthy tears. Furthermore, they often contain preservatives, stabilizers, and other additives that potentially may induce toxic or allergic reactions (Noecker, 2001; Tripathi and Tripathi, 1989).

One interesting alternative to artificial tears is the topical use of blood derivates. In particular, the use of plasma rich in growth factors may represent a potential therapeutic approach for ocular surface disorders as a tear substitute. Apart from its lubricating properties, the technology of plasma rich in growth factors (PRGF-Endoret) contains numerous of morphogens including EGF, transforming growth factor-beta (TGF-β), PDGF and nerve growth factor (NGF) among others (Blair and Flaumenhaft, 2009; Nurden et al., 2008). This biological approach consists on the elaboration and use of a platelet enriched plasma obtained from patient's own blood. After activation with calcium chloride, this approach allows the *in-situ* formation of a biodegradable fibrin scaffold and the release of a pool of biologically active proteins that influence and promote a range of biological processes including cell recruitment, growth and differentiation (Anitua et al., 2008, 2009).

Recently, we have observed that PRGF-Endoret significantly enhances proliferation and migration of both keratocytes and conjunctival fibroblasts. In addition, it prevents and inhibits TGF-β1-induced myofibroblast differentiation (Anitua et al., 2011). These interesting results suggest that PRGF-Endoret could have a promising role in myofibroblast modulation in the stroma of the ocular surface tissues.

In this study, concentrations of several factors considered to be important in corneal wound healing were measured in two different PRGF-Endoret-derived formulations, fraction 3 (F3: platelet enriched fraction) or whole plasma (WP) column (with lower amount of platelets than F3) obtained from PRGF-Endoret technology. The regenerative effects of these preparations on proliferation and migration of immortalized human corneal epithelial cells and on mice cornea after Photorefractive keratectomy surgery were evaluated.

2. Materials and methods

2.1. PRGF-Endoret preparations

The study was performed following the principles of the Declaration of Helsinki. Blood from three healthy young male donors was collected after informed consent into 9-mL tubes with 3.8% (wt/v) sodium citrate. Samples were centrifuged at 580 g for 8 min at room temperature in a PRGF-Endoret System centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Half of the tubes were used to separate the whole plasma column (WP) over the buffy coat and the other half to take the milliliter above the buffy coat called Fraction 3 (F3) – the platelet enriched fraction (see Fig. 1). In both cases, care was taken to avoid the buffy coat containing the leukocytes. Platelets and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France). Both preparations were incubated with PRGF-Endoret activator (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at 37 °C in glass tubes for 1 h. The growth factor enriched supernatants were collected by aspiration after centrifugation at 1000 g for 10 min. Finally, plasma obtained from WP and F3 was filtered with a filter pore size of 0.2 μm (Fisher Scientific, Madrid,

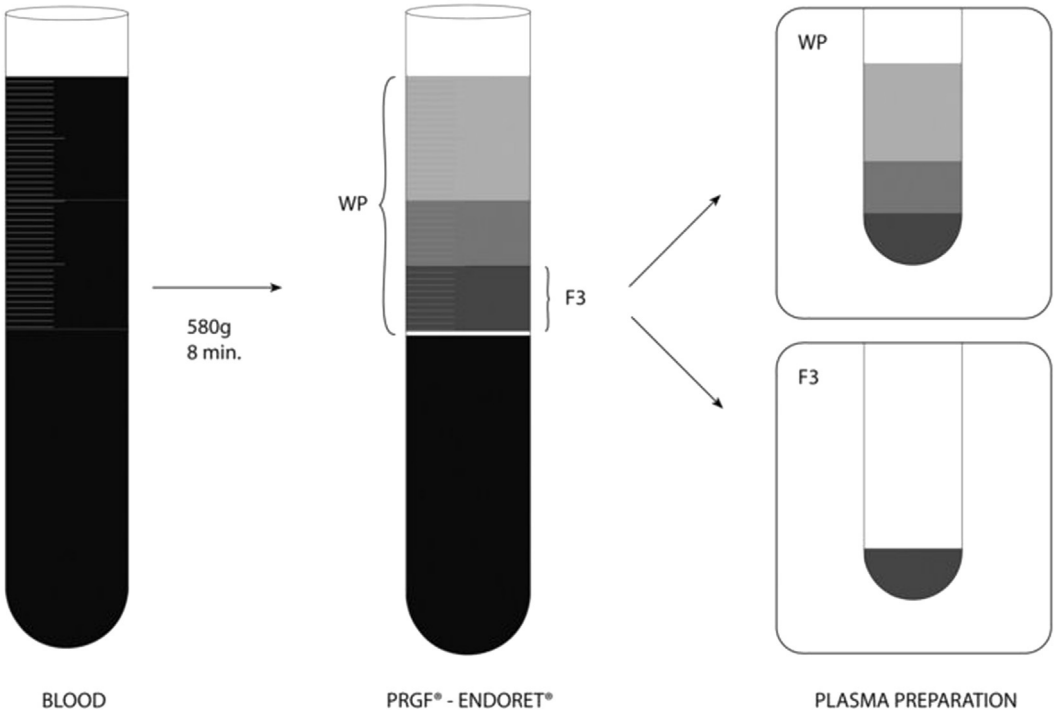

Fig. 1. Scheme of the different plasma formulations obtained with the PRGF-Endoret technology. WP: Whole Plasma column; and F3: fraction 3, the plasma fraction most enriched in platelet. In both plasma preparations, care was taken to avoid the buffy coat containing leukocytes.

Spain), aliquoted and stored at −80 °C until use. Several growth factors involved in the different tissue regeneration mechanism such as proliferation, migration, angiogenesis and inflammation (TGF-β1, PDGF-AB, VEGF, HGF, EGF, IGF-I and Endostatin) were measured in the supernatants using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

2.2. Cell culture

Human SV-40 immortalized corneal epithelial cells (termed HCE) (RCB1384: HCE-T, Riken Cell Bank, Ibaraki, Japan) were cultured at 5% CO_2 at 37 °C in DMEM/F12 medium (Invitrogen-Gibco, Grand Island, NY, USA), supplemented with 0.5 μg/mL insulin (Sigma–Aldrich, St Louis, MO, USA), 10 ng/mL EGF (Sigma–Aldrich, St Louis, MO, USA), 1% DMSO (Sigma–Aldrich, St Louis, MO, USA) and 7.5% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany). After confluence, cells were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion.

2.3. Proliferation assay

HCE cells were seeded at a density of 20,000 cells per cm^2 on 96 well optical bottom black plates and maintained with serum-free medium for 48 h. Then, culture medium was replaced by serum-free medium supplemented with either: (i) the culture medium alone (FM) with 0.1% FBS as a control of non-stimulation (Control) (ii) 20% (v/v) WP, or (iii) 20% (v/v) F3. Then the cultured cells were incubated at 5% CO_2 at 37 °C for 48 h. Density of cells in culture was estimated using the CYQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA). Briefly, medium was removed and wells were washed carefully with phosphate buffered saline (PBS). Then the microplate was frozen at −80 °C for efficient cell lysis in the CyQUANT assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Ku/ml) diluted in cell lysis buffer during 1 h at room temperature. Then 2× CyQUANT GR dye/cell lysis buffer was added to each sample well, mixed gently and incubated for 5 min at room temperature protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany). A DNA standard curve ranging from 7.8 to 1000 ng/ml was included in all fluorescence quantifications. As an index of cell number, calibration curves ranging from 10,000 to 100,000 cells per cm^2 were established using the Cyquant assay.

2.4. Wound healing assay

In order to quantify the wound healing potential of PRGF-Endoret on corneal epithelial cells, HCE cells were plated in culture inserts (Ibidi, GmbH, Martinsried, Germany) placed on a 24-well plate at high density and were grown until confluence. After carefully remove the inserts, two separated cell monolayers leaving a cell-free gap of approximately 900 μm thickness were created. The cells were washed with PBS and incubated with the same treatments as in the proliferation assay (Control, 20% WP or 20% F3) in quadruplicate for 8, 16 and 24 h. Then, the different culture mediums were removed and cells were incubated with 1:500 Hoechst 33342 in PBS for 10 min. To quantify the percentage area of wound healing, phase contrast images of the central part of the septum before treatment, and phase contrast and fluorescence photographs after each treatment time were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area was measured at 0, 8, 16 and 24 h from initial treatment using the ImageJ Software (NIH, Bethesda, MD, USA). The results were expressed as percentage area of wound healing.

2.5. Photorefractive keratectomy mouse model

Sixty male C57BL/6 mice aged between 16 and 20 weeks old were used in this study. All procedures were performed in accordance with the tenets established in the directive of the European Parliament and Council of the European Communities (2010/63/UE), of the Spanish legislation (RD 1201/2005 and Law 32/2007), and of the ARVO Statement for the Use of Animals.

Anesthesia was induced by intraperitoneal injection of ketamine hydrochloride (80 mg/kg, Imalgène 500, Merial, Lyon, France) and xylazine hydrochloride (5 mg/kg, Rompun, Bayer HealthCare, Kiel, Germany). In addition, topical anesthesia of tetracaine hydrochloride and oxibuprocaine hydrochloride (Alcon Cusí, Barcelona, Spain) was applied to each eye just before surgery.

One eye of each mouse was subjected to Photorefractive keratectomy (PRK) surgery with a 2 mm ablation zone on the central cornea and 45 μm of depth (including the epithelium) on an Allegretto WaveLight excimer laser (PR-020407, Wavelight GmbH – Alcon, Erlangen, Germany). The contralateral eye was used as unoperated control. Animals were divided in three groups: Control group, mice were treated with saline solution (Fresenius Kabi, Barcelona, Spain); F3 group, treated with fraction 3 of PRGF-Endoret; WP group, treated with the whole plasma fraction. The treatment was administrated topically applying one drop of compound six times per day along the first 3 days, and four times per day until the end of the study. Each group was analyzed at four time points: 1, 2, 3 and 7 days of treatment. These groups were selected because they include important events of the wound healing process.

2.6. Biomicroscopic analysis

Mice corneal wound healing evolution was examined macroscopically after fluorescein staining under a Leica S6D stereoscopic microscope (Leica Microsystems, Wetzlar, Germany) at 1, 2, 3 and 7 days from PRK surgery. The level of opacity (haze) in the cornea was graded according to Fantes et al., (1990) 34 (0–4: 0 = clear cornea, 4 = severely dense opacity). Two different investigators performed the clinical evaluations and all the observations were performed in a masked fashion. The morphometric analysis of wound healing evolution was carried out with the image analysis software ImageJ 1.45a (National Institutes of Health, Bethesda, MD, USA).

2.7. Tissue collection

At each time point of evaluation (1, 2, 3 and 7 days), mice were euthanized with an overdose of sodium pentobarbital (Dolethal, Vétoquinol, Lure, France) injected intraperitoneally. For histological analysis, eyes were enucleated and immersed in Somogyi's fixative solution without glutaraldehyde (4% paraformaldehyde and 0.2% picric acid in phosphate buffer) for 1 h at room temperature. Samples were stored in 30% sucrose solution for 12 h, embedded in OCT compound (Tissue-Teck, Sakura, Tokio, Japan), and snap frozen in liquid nitrogen. The frozen tissue blocks were maintained at −85 °C until sectioning.

Tissue blocks were placed in a cryostat sample holder and oriented in order to obtain transverse sections through the central region of the corneas. Five micron-thick sections were obtained with a Microm HM550 cryostat (Microm International GmbH, Walldorf, Germany), and collected on microscope slides (Superfrost Plus, Fisher).

2.8. Cell death assay

To detect DNA fragmentation associated with apoptosis, terminal deoxyribonucleotidyl transferase-mediated dUTP-fluorescein Nick-End labeling (TUNEL) was performed on frozen sections according to the manufacturer's instructions (Promega Corp., Madison, WI). Nuclei were counterstained with 4′, 6-diamino-2-phenylindole (DAPI) (Molecular Probes, Leiden, the Netherlands). All TUNEL-positive cells present in each sample were counted using ImageJ software.

2.9. Immunofluorescence

Immunofluorescent staining for alpha smooth muscle actin (SMA; Abcam, Cambridge, UK), a marker for myofibroblasts; and Ki-67 (Abcam), a proliferation marker, was performed using rabbit polyclonal antibodies. Tissue sections (5 microns) were incubated at room temperature with 0.13 M $NaBH_4$ (Sigma–Aldrich, St Louis, MO, USA) for 20 min to reduce tissue autofluorescence. Samples were then washed 3 times with 0.03% Triton-X 100 (Sigma–Aldrich, St Louis, MO, USA) in PBS for 10 min, and incubated overnight at 4 °C with the primary antibody diluted at 1:100 for SMA and 1:500 for Ki-67 in 5% normal goat serum and 0.03% Triton-X 100. After washing, slides were incubated with Alexa 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Leiden, the Netherlands) diluted at 1:500 in PBS for one hour. Slides were washed, counterstained with DAPI to allow nuclei visualization in tissue sections and mounted with DAKO fluorescent mounting medium (DAKO, Carpinteria, CA, USA). The sections were viewed and photographed with a Leica DM6000 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC310FX digital camera.

2.10. Quantification of immunofluorescence and TUNEL assay

Three sections (5 microns thick) from the central region of five corneas for each time point were used to quantify SMA-positive myofibroblasts, Ki-67 proliferative cells and TUNEL positive cells. Two independent observers counted every positive cell on five non-overlaping corneal regions corresponding to a 400× microscope field. Each microscope field comprises a column of central corneal tissue extending from the anterior epithelium to the posterior stromal surface.

2.11. Statistical analysis

Significant differences among defined groups were tested using the non-parametric Kruskal–Wallis procedure followed by Bonferroni to discriminate among the means. A difference at a level of $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

3. Results

Platelet enrichment of the PRGF-Endoret preparations were 2.6-fold for WP (481×10^6 platelets/ml) and 3.6-fold for F3 (663×10^6 platelets/ml) over the baseline concentration in whole blood. None of the preparations contained detectable concentrations of leukocytes. Table 1 shows the concentrations of several of the most important growth factors for each sample (WP and F3). No statistical differences were observed between both formulations.

3.1. Cell proliferation

Proliferation of human corneal epithelial cells significantly increased after 48 h of treatment with both PRGF-Endoret preparations (20%WP or 20% F3) as it is shown in Fig. 2. In fact, HCE density showed a significant increase of 1.59-fold with WP and 1.66-fold with F3 compared with non stimulated group (control). No significant differences were found between both PRGF-Endoret treatments.

3.2. In vitro wound healing assay

Fig. 3A shows fluorescence Hoechst images of HCE cells after 0, 8, 16 and 24 h of wound closure and highlights the potent stimulatory effect of WP and F3 over the treated cells. Both PRGF-Endoret formulations (WP and F3) stimulated the migratory capacity of HCE along the 24-h follow-up. WP and F3 showed a total closure of wound area at 24 h of treatment, while in the control group the wound area was closed minimally. Results from the in vitro wound healing study revealed that experimental area of 8.1 mm^2 was totally closed by the epithelial cells (100% of the damage area) in 24 h after treatment with PRGF-Endoret, while the control group was only partially closed (35% of total area). In particular, migration of HCE at 24 h of treatment increased 2.85-fold over the control group for WP and F3 (Fig. 3B). The percentage area of wound healing was significantly higher with plasma preparations in comparison to the control group at 16 and 24 h of treatment. No statistical differences were found between PRGF-Endoret treatments in the different periods of time.

3.3. Biomicroscopic analysis

Macroscopic analysis showed no adverse reactions of human PRGF-Endoret eye drops on mice ocular surface tissues, probably due to the homology between human and mice growth factors. Corneal wound healing evolution was studied under stereoscopic microscopy after fluorescein staining (Fig. 4A). At day 1 after surgery, PRGF-Endoret groups (F3 and WP) showed an improvement in the percentage of wound closure with regard to the control group, being these differences non-significant. Complete wound healing was observed in all groups 3 days after PRK surgery, showing no differences among the different groups (Fig. 4B).

Haze development on the PRK-treated corneas of three groups was evaluated at the 4 time points, showing different levels of clinical haze. The mouse corneas treated with PRGF-Endoret formulations (F3 or WP) showed lower grade of stromal haze than control group. At days 3 and 7 post-PRK surgery, WP and F3 groups presented significantly less haze formation ($p < 0.05$) than control

Table 1
Platelet and leukocyte counts and concentrations of several growth factors (mean ± SD) in the two different plasma preparations (WP and F3) of the blood's donor. No statistical differences were observed between both formulations.

Plasma preparation ($n = 3$)	Leukocyte count ($\times 10^6$/ml)	Platelet count ($\times 10^6$/ml)	Growth factor levels						
			TGF-β1 (ng/ml)	PDGF-AB (ng/ml)	IGF-I (ng/ml)	VEGF (pg/ml)	HGF (pg/ml)	EGF (pg/ml)	TSP-1 (μg/ml)
WP	<0.2	481 ± 114	63 ± 14	19 ± 6	83 ± 11	568 ± 203	400 ± 51	508 ± 140	29 ± 9
F3	<0.3	663 ± 156	81 ± 17	30 ± 10	86 ± 14	791 ± 275	491 ± 62	779 ± 171	50 ± 14

WP, Whole plasma column (enriched in platelets 2.6-fold over peripheral blood); F3, Fraction 3 (enriched in platelets 3.6-fold over peripheral blood). Peripheral blood contained 182×10^6 platelets/ml.

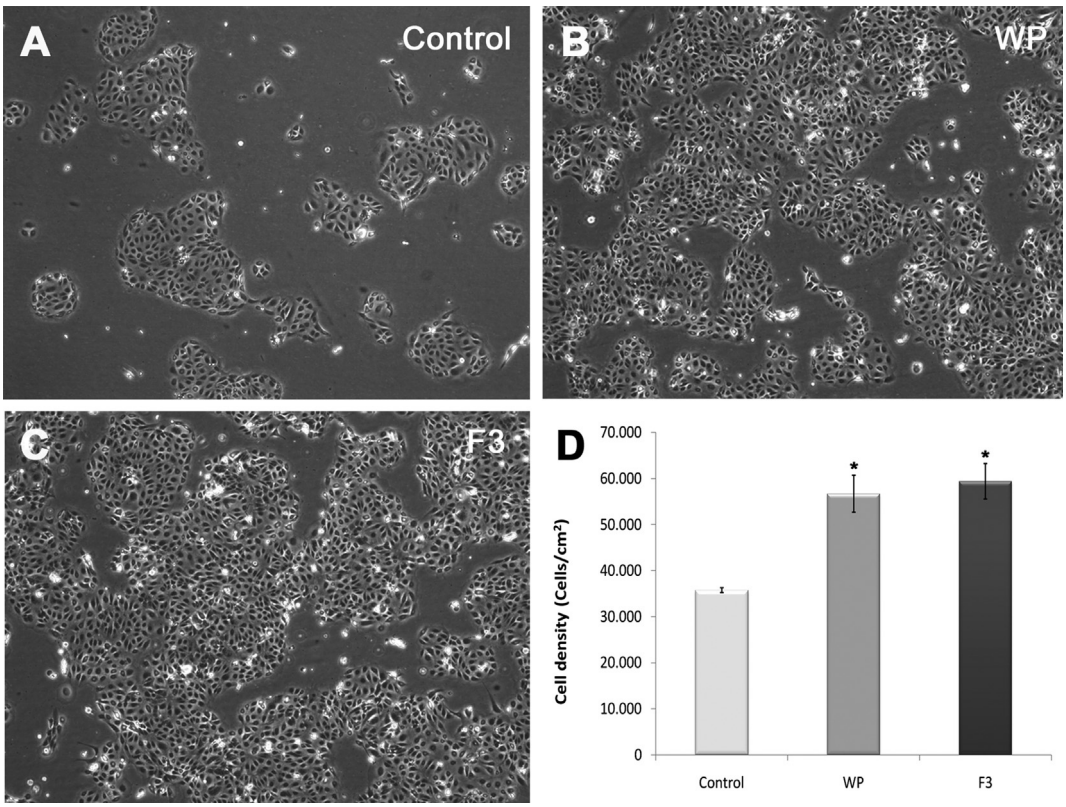

Fig. 2. Proliferative effect of PRGF-Endoret formulations on HCE culture cells. Representative images of HCE treated with Control (A), WP (B) and F3 (C). WP and F3 treatment showed a higher proliferation effect respect Control group. (D) WP and F3 increased significantly (*, $p < 0.05$) the proliferation of HCE compared with Control group, no differences were found between WP and F3 treatment group. Results are expressed as mean number of cells per square millimeter ± SD ($n = 3$).

group (Fig. 5). No significant differences were found between both PRGF-Endoret formulation groups at any time of treatment.

3.4. Histologic analysis

One day after surgery, epithelial defect was observed in the central zone of laser ablation in the corneas of control group, while mice treated with PRGF-Endoret formulations (F3 and WP) showed a completed simple layer of epithelial cells. Also, on day one, the corneal stroma underlying the PRK is occupied by granulation tissue, containing inflammatory cells and fibroblasts. No significant differences were shown in the amount of these cells among groups. Inflammatory cells undergo apoptosis at day two and they were no detectable in the remaining times. There was no blood vessel development in either treatment group.

Epithelial defects were completely closed 3 days post-surgery in all groups, independently of the treatment. At three days, the mice corneas belonging to the control group showed epithelial hyperplasia in the surgical region.

At day 7 post-surgery, corneal epithelium in the surgical region of the PRGF-Endoret treated animals showed similar appearance like healthy epithelium, while irregular hyperplasic epithelium was found in the control group (Fig. 6A–C).

3.5. Proliferation

At day 1 after surgery, Ki-67 positive cells were highly increased in all mice corneal epithelium near to limbal zones, showing a wave of Ki-67 positive elements from the periphery towards the center of the cornea. We found the greatest epithelial proliferation at day 2 post-surgery, decreasing progressively the number of Ki-67 positive cells until the end of the study.

No significant differences in the number of proliferative cells were observed at day 2 post-surgery among the different groups. However, at day 3 the number of Ki-67 positive cells decreased drastically in the epithelial corneas of mice treated with F3 and WP, being significantly lower ($p < 0.01$) than the control group. The number of proliferative cells progressively decreased in all groups until the end of the study (Fig. 6D).

3.6. Apoptosis

TUNEL-positive cells were detected at day 1 after surgery in mice stromal corneas, covering the outer surface of the damaged area and over the granulation tissue. The number of apoptotic cells decreased after 3 days of treatment. At 7 day, the number of TUNEL-positive cells decreased until basal levels, detecting only some positive cells in the upper part of the corneal epithelium. No differences were detected among the treatment groups in the number of apoptotic cells at each time of evaluation.

3.7. Immunofluorescence detection of myofibroblasts

The formation of haze in mice corneas was also confirmed by the immunofluorescence detection of α-SMA myofibroblasts. The number of SMA positive cells was increasing along the time of study in the corneas of the Control group, while the mice corneas treated with both PRGF-Endoret groups maintained the low number of SMA positive cells throughout the study period (Fig. 7). High number of SMA positive myofibroblasts were observed under the epithelial basal layer in control corneas at day 2 of treatment, showing significant differences ($p < 0.05$) with regard WP group. These significant differences were maintained in both PRGF-Endoret formulations compared with the control group at 3 and 7

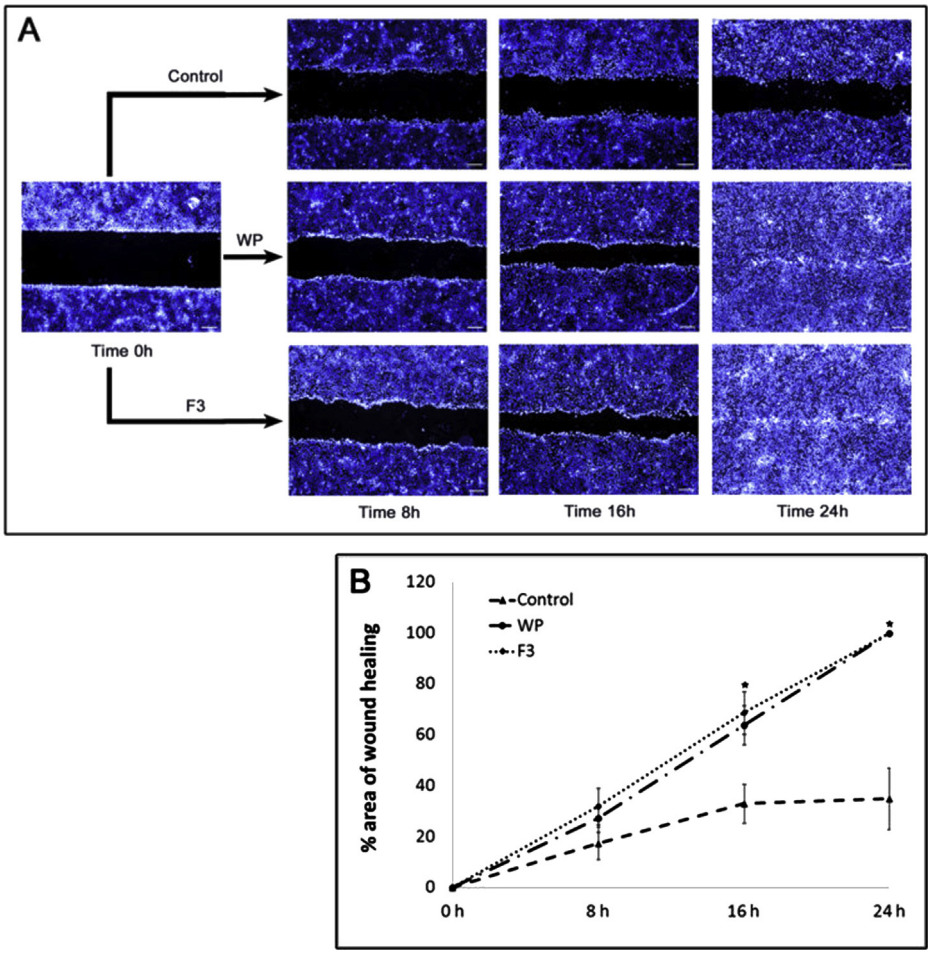

Fig. 3. Wound healing assay on HCE cells. (A) Hoechst counterstaining of wound healing at different times of treatments (8 h, 16 h and 24 h). PRGF-Endoret formulations showed a total closure of wound area at 24 h of treatment, in contrast to the control group. (B) Mean ± SD of the percentage area of wound healing of HCE culture cells after treatment with WP, F3 or control ($n = 3$) at different times of the study. HCE cells showed significant increased (*, $p < 0.05$) on wound healing after treatment with WP or F3 formulations regarding control group. (Scale bar: 200 μm).

days from PRK surgery. On the other hand, no differences were detected between the PRGF-Endoret groups at any time of the study in the number of SMA positive myofibroblasts.

4. Discussion

The cornea reacts to damage by releasing numerous substances, including cytokines, growth factors, proteases and neuropeptides with the aim of restoring anatomical integrity (re-epithelisation, stromal repair, and re-innervation). In chronological order, re-epithelisation is the first process that occurs during corneal tissue regeneration, through the stimulation of the proliferation, migration and differentiation of the adjacent epithelium. This process is initiated and controlled by the release of the soluble factors from the epithelium itself, keratocytes, and the lachrymal glands (Klenkler and Sheardown, 2004). The factors involved in corneal

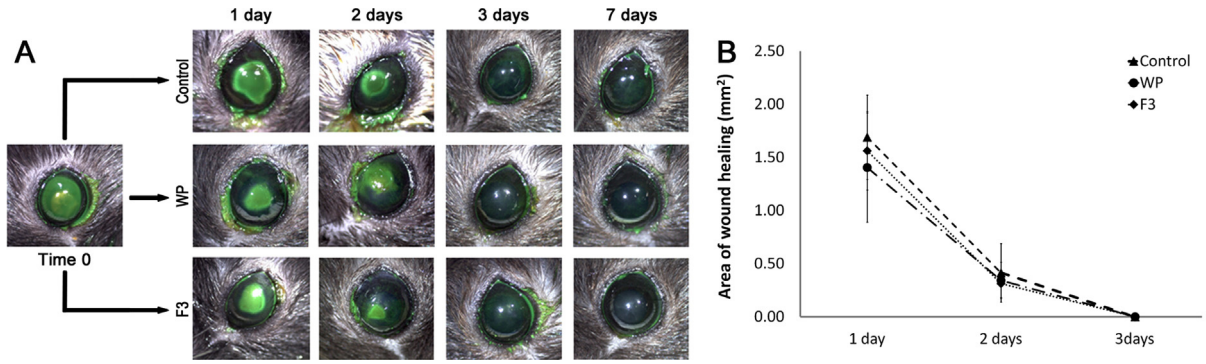

Fig. 4. Macroscopic analysis of corneal wound healing after PRK surgery. (A) Representative images of corneal wound healing of the different treatment groups ($n = 5$) at different times of the study. (B) Although, some differences in epithelial closure are appreciable at 1 day in the PRGF-Endoret groups, no differences were detected at the following times. Results have been expressed as a mean area of wound healing ± SD.

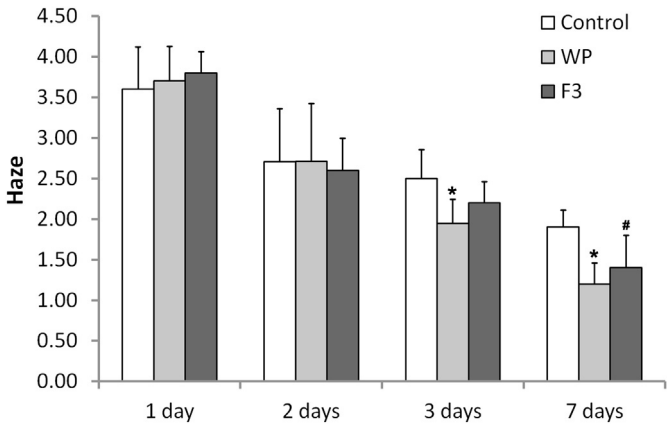

Fig. 5. Macroscopic evaluation of corneal haze formation ($n = 5$ per group and time of treatment). Corneal haze formation (mean ± SD) was significant lower in WP (*) and F3 (#) groups regarding control group at 3 and 7 days from PRK surgery.

wound healing include those with stimulatory effects such as fibronectin, TGF-β, PDGF, EGF, and those with inhibitory effects, such as hepatocyte growth factor (Imanishi et al., 2000).

Plasma rich in growth factors (PRGF-Endoret) is an autologous platelet rich plasma by which it is possible to obtain different growth factor-enriched formulations including a colirium, that can be used in the treatment of several disorders (Nurden et al., 2008). The effects of PRGF-Endoret on tissue regeneration have been deeply demonstrated in different areas of the medicine, such as dentistry, oral implantology, orthopedics, sports medicine and the treatment of skin disorders (Anitua et al., 2010).

In this study, we report the effects of PRGF-Endoret on the proliferation and wound healing of human corneal epithelial cells "in vitro", and on Photorefractive keratectomy mouse model. In vitro assays demonstrate that the different plasma formulations evaluated (WP and F3) enhance proliferation and accelerate wound healing of corneal epithelial cells, showing a rate of wound closure 2-fold higher in epithelial cells treated with PRGF-Endoret formulations. Interestingly, there are not significant differences between the effects induced by the platelet enriched plasma fraction (F3) and the whole plasma fraction (WP). Although preliminary, these results may help to understand the potential of the autologous formulations in corneal wound healing.

One of the greatest targets in corneal biology is the development of a methodology or a treatment that promote tissue repair via regeneration rather than fibrosis. It is highly describe in the literature that the choice between regeneration and fibrosis lies in the control of fibroblast activation (Anitua et al., 2010).

Myofibroblasts are thought to be one of the main contributors to the development of corneal opacity. Several treatments have been developed to regulate corneal myofibroblasts transformation minimizing haze formation (Jester et al., 1997; Maltseva et al., 2001; Singh et al., 2011; Thom et al., 1997), although the improvement in cornea wound healing after applying these treatment is unclear.

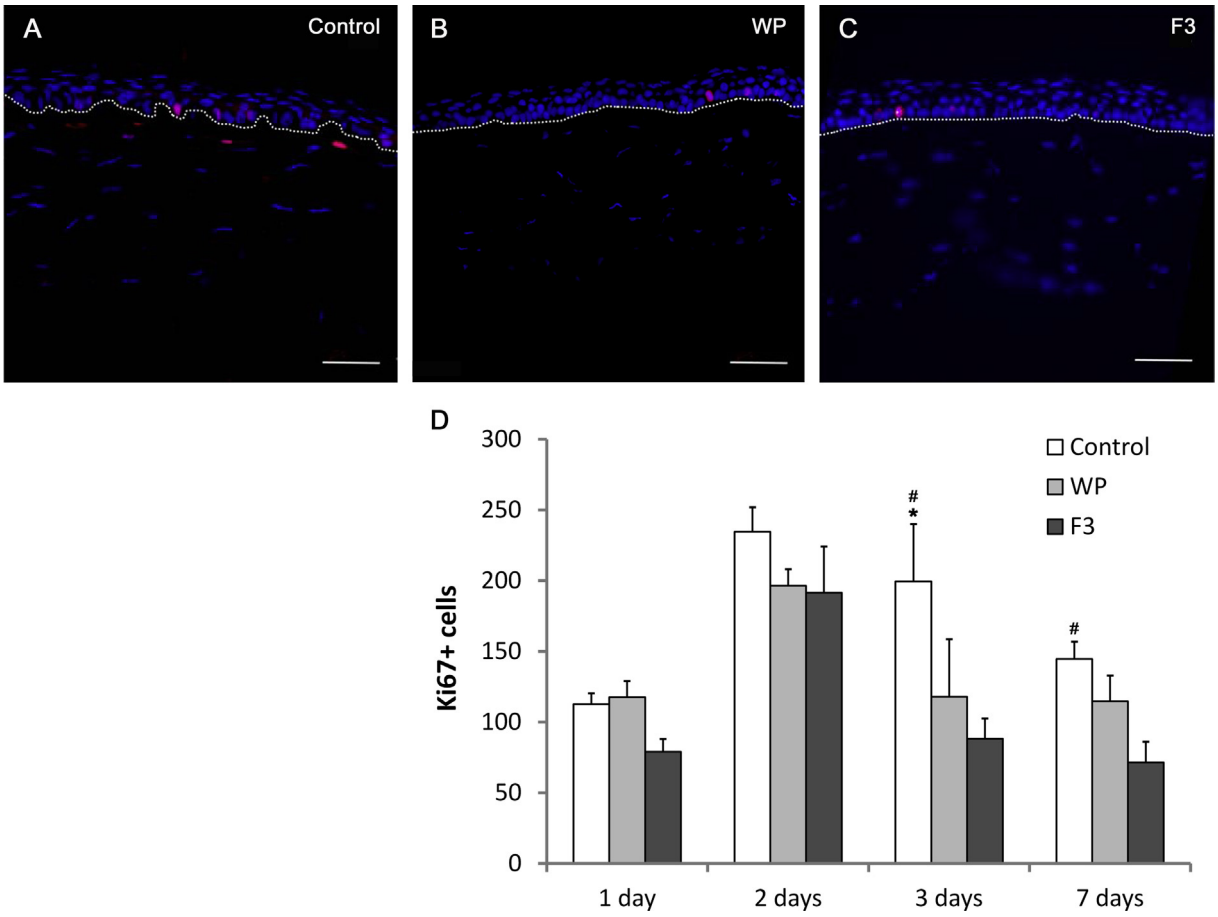

Fig. 6. Histological analysis of corneal epithelium and Ki-67 positive cells after PRK surgery. Representative images of the central area of the cornea at 7 day of treatment showing the hyperplasic and irregular (dotted line) epithelium observed in control group (A) regarding WP (B) and F3 (C) treated mice ($n = 5$). (D) Mice corneas treated with WP and F3 showed significant lower (* and # respectively) Ki-67 + cells (mean ± SD) at 3 and 7 days after PRK surgery respect the control group. Scale bar: 50 μm.

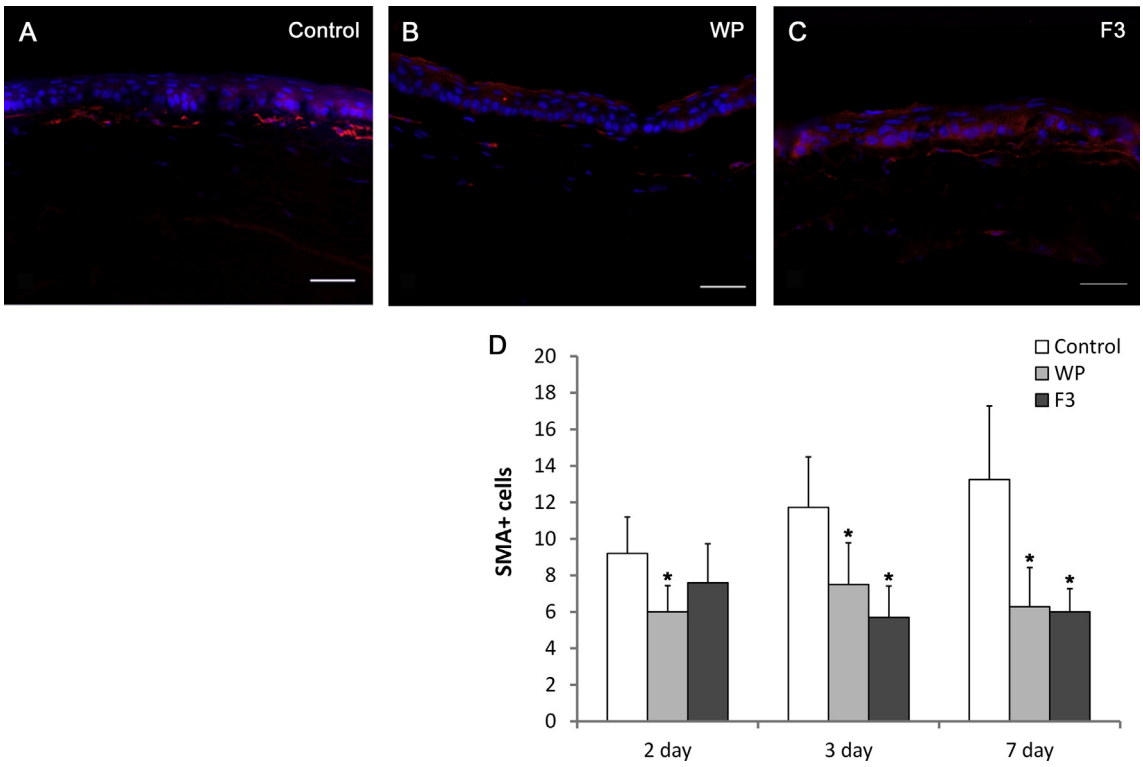

Fig. 7. Representative images of immunofluorescence for alpha smooth muscle actin (SMA) at 7 days after PRK surgery. Corneal stroma of control group (A) showed higher number of SMA positive cells at this time from PRK surgery regards WP (B) and F3 (C) treatment groups ($n = 5$). (D) PRGF-Endoret treatment groups reduce significantly ($p < 0.05$) the number of SMA positive cells at 2, 3 and 7 days after PRK surgery. Results are expressed as the mean number of SMA positive cells ± SD per group and time of treatment. Scale bar: 40 μm.

Previous studies have shown the potential of PRGF-Endoret technology in protecting fibroblast transformation to myofibroblasts, while different cellular processes involved in wound healing are enhanced (Anitua et al., 2011, 2012).

The in vivo PRK model was perform in this study to assess the potential of PRGF-Endoret technology on cornea scarless tissue regeneration. PRGF-Endoret formulations showed a reduction of re-epithelisation time at the first hours after treatment, as shown the histological analysis at 1 day after PRK surgery, where a simple epithelial cell layer were observed in both PRGF-Endoret groups compared with control group. This simple epithelial layer is not enough to avoid fluorescein staining, as it was observed on biomicroscopic analysis.

Three days after surgery, no differences were observed among the different groups of treatment, showing a complete epithelial closure. The absence of differences in the re-epithelisation level between control and PRGF-Endoret treatment groups at 3 days after surgery, may be due to the increase number of proliferating cells (Ki-67 +) in the control group. However, this proliferative cellular increase in the epithelium of the control group may be related with the epithelia hyperplasia observed at 3 and 7 after surgery, suggesting a possible myopic regression in control group after PRK surgery (Gauthier et al., 1996; Weber et al., 2001). In contrast, the epithelium of the PRGF-Endoret-treated groups is formed by five to six cell layers, closely resembling the native corneal epithelium.

In addition, detection of SMA + cells on mice corneas of control group from second to seven day of treatment demonstrated the presence of myofibroblasts. This appearance of myofibroblasts in control eyes may be a key factor in the development of corneal haze (Jester et al., 1999a; Maltseva et al., 2001) that is correlated by biomicroscopic analysis in control eyes at the same time points after surgery. The number of SMA positive cells was significantly lower ($p < 0.05$) on stromal corneas treated with PRGF-Endoret colirium compared with the control group at 2, 3 and 7 days of treatment. These results suggest that PRGF-Endoret treatment after corneal injury enhances corneal wound healing without haze formation, reducing the possibility of scar formation if myofibroblast presence on stromal cornea persists along the time (Wilson, 2012).

TUNEL assay showed no differences among the different groups of treatment, suggesting that apoptosis process is not influenced by the type of treatment. In each group of treatment, the number of TUNEL positive cells at each time of study follows in the footsteps of apoptosis process after PRK surgery described previously (Mohan et al., 2003).

Another important conclusion from this study is the in vivo corroboration of results observed previously (Anitua et al., 2011), where it is showed that WP and F3 formulations exert similar biological effects. The data presented herein may modify the current clinical protocols as WP shows similar biological effects to F3 but represents an improvement in the yield of 400%. In fact, while only 1 mL of F3 can be obtained from 9 mL of blood, almost 4 mL of WP can be obtained from the same blood volume.

5. Conclusions

In summary, our results indicate that different formulations of PRGF-Endoret enhance proliferation and wound healing of epithelial corneal cells, and enhance wound healing after excimer laser photoablation, reducing corneal haze formation. Although further studies are needed to determine the exact mechanisms underlying the effects of this autologous technology, results from this study suggest that the different PRGF-Endoret formulations (WP and F3) could improve the wound healing in ocular surface.

Acknowledgments

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Corneal Wound Healing Promoted by 3 Blood Derivatives: An In Vitro and In Vivo Comparative Study

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Purpose: The aim of this study was to compare the effect on corneal wound healing of 3 differently manufactured blood derivatives [autologous serum (AS), platelet-rich plasma, and serum derived from plasma rich in growth factors (s-PRGF)].

Methods: Scratch wound-healing assays were performed on rabbit primary corneal epithelial cultures and human corneal epithelial cells. Additionally, mechanical debridement of rabbit corneal epithelium was performed. Wound-healing progression was assessed by measuring the denuded areas remaining over time after treatment with each of the 3 blood derivatives or a control treatment.

Results: In vitro data show statistically significant differences in the healing process with all the derivatives compared with the control, but 2 of them (AS and s-PRGF) induced markedly faster wound healing. In contrast, although the mean time required to complete in vivo reepithelization was similar to that of AS and s-PRGF treatment, only wounds treated with s-PRGF were significantly smaller in size from 2.5 days onward with respect to the control treatment.

Conclusions: All 3 blood derivatives studied are promoters of corneal reepithelization. However, the corneal wound-healing progresses differently with each derivative, being faster in vitro under AS and s-PRGF treatment and producing in vivo the greatest decrease in wound size under s-PRGF treatment. These findings highlight that the manufacturing process of the blood derivatives may modulate the efficacy of the final product.

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Corneal epithelial integrity is a critical requirement for normal visual function. After an injury, ocular surface health is altered and corneal epithelial disorders can appear.[1] Corneal epithelial wound healing is a complex mechanism that involves cell proliferation, migration, reattachment of the epithelium to its extracellular matrix, and cell differentiation.[2] These processes are sustained by glucose, vitamins, and growth factors.[3] In the cornea, because of its avascular nature, these requirements are provided by tear film, aqueous humor, and limbal blood vessels.[4,5] It has been observed that natural tears and serum have similar pH, osmolarity, and biomechanical properties,[6] as well as adhesion proteins, proteases, antimicrobial proteins, cytokines, lipids, vitamins, and growth factors.[7] In fact, alpha granules of platelets have been identified as a major source of these growth factors,[8] which have significant potential to repair and regenerate damaged tissue.[9] All these common features between serum and tear film have consolidated the role of serum derivatives as tear film substitutes and promoters of corneal wound healing.

Several different blood derivatives have been used for the treatment of various corneal ophthalmological disorders.[7–16] Our group originally adapted a plasma preparation, which was first used in maxillofacial surgery and trauma,[17] to produce serum derived from plasma rich in growth factors (s-PRGF). Then, we applied s-PRGF eye drops as a novel treatment of persistent epithelial defects[15] and dry eye syndrome.[16] More recently, we have adapted the methodology to obtain s-PRGF to be used for the first time in a rabbit animal model[18] as a step toward translation of the technology from research to clinical practice.

There is no standardized protocol for the clinical use of blood-derived preparations. In addition, there have been very few in vivo studies comparing their efficacy.[19] Thus, the aim of this study was to explore whether the formulation of blood derivatives influences their efficacy as treatment of corneal wounds. To that end, we have compared the effects of s-PRGF preparation with other previously used blood derivatives, autologous serum (AS) and platelet-rich plasma (PRP), in cultures of rabbit and human corneal epithelial (HCE) cells and, as a further step, in an in vivo model of rabbit corneal epithelial wound healing.

MATERIALS AND METHODS

Preparation of Blood Derivatives

Informed consent was obtained from all human volunteers, in accordance with the Declaration of Helsinki. All rabbits were cared for in accordance with the procedures and experimental designs approved by the animal experimentation ethics committee of the University of the Basque Country (UPV/EHU). Blood samples were collected from human volunteers and New Zealand white rabbits. A blood sample from each individual was divided and processed by 3 previously described methods[18,20] to obtain the blood derivatives AS, PRP, and s-PRGF.

For in vitro assays, we pooled samples from several individuals. For in vivo assays, we used autologous preparations. All preparations were stored at −20°C until use.

Isolation and Expansion of Rabbit Primary Corneal Epithelial Cultures

To obtain rabbit primary corneal epithelial (RPCE) cultures, the central cornea button from 3 New Zealand rabbit eyes was removed, keeping the limbal zone intact. The rings of tissue obtained were digested with 25 IU/mL dispase II (BD Biosciences—Discovery Labware, Bedford, MA) in HBSS (Sigma, St Louis, MO) for 16 hours at 4°C. The epithelial cell sheets were collected, centrifuged, and incubated with 0.5% trypsin–0.2% ethylenediaminetetraacetic acid (Sigma) for 30 minutes at 37°C to obtain single-cell suspensions. Finally, the cells obtained were cultured at 37°C in 5% CO_2 in DMEM:Ham's F12 (1:1 mix) (Lonza, Verviers, Belgium) with 2 mM L-glutamine (Lonza) and 1% penicillin-streptomycin (Lonza), together with 10% fetal bovine serum (FBS) (Lonza). This culture medium was also supplemented with: 10 ng/mL epithermal growth factor (EGF) (Sigma), 5 μg/mL insulin (Sigma), and 0.1 μg/mL cholera toxin (Gentaur Molecular Products, Brussels, Belgium).

Human Corneal Epithelial Cell Line Culture

SV-40 immortalized HCE cells were kindly provided by Araki-Sasaki et al.[21] These cells were cultured at 37°C in 5% CO_2 in DMEM:Ham's F12 with 2 mM L-glutamine and 1% penicillin-streptomycin, together with 10% FBS, 10 ng/mL EGF, 5 μg/mL insulin, 0.1 μg/mL cholera toxin, and 0.5% DMSO (Sigma).

In Vitro Scratch Wound Healing Assays

These assays were performed on RPCE and HCE cultures. Cells were seeded at 20,000 cells per well in 96-well plates and left to form monolayers. After that, overnight, the culture medium containing FBS was replaced by a medium with 1% bovine serum albumin. Then, a rounded area in the cell monolayer of each well was scraped off with a pipette tip, and cultures were maintained with culture medium without FBS but containing 50% of one of the blood derivatives (AS, PRP, or s-PRGF) or 1% bovine serum albumin as control. Areas from which cells had been scraped away were photographed every 12 hours with a phase contrast microscope (Nikon Eclipse TS 100; Nikon, Tokyo, Japan), and images were acquired with the ProgRes CapturePro 2.6 software. The size of the denuded areas was quantified using ImageJ software (developed by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD). The closure rate was described in terms of the mean remaining denuded area ± SD in square millimeters. All experiments were performed at least in sextuplicate (6 wells) and repeated in 3 different HCE or RPCE cultures.

Rabbit Corneal Reepithelization Assays

Sixteen female New Zealand white rabbits were included in the study. Initially, each rabbit underwent surgery in its right eye, and the left eye was operated on 2 to 3 weeks after the right eye had recovered. The surgery was performed under general anesthesia (1 mL/kg of Ketolar 50 mg/mL; Pfizer, Madrid, Spain, and 0.3 mL/kg of Xilagesic 20 mg/mL; Laboratorios Calier SA, Barcelona, Spain). The epithelium inside a 9-mm corneal trephine circular mark was scraped off with an ophthalmic blade.

Postoperatively, until the epithelial closure was complete (maximum of a week), every rabbit was treated with a topical dexamethasone and chloramphenicol ointment (Deicol ophthalmic ointment; Alcon laboratories, Barcelona, Spain) and diclofenac drops (Voltaren drops; Allergan Inc, Irvine, CA), twice a day. In addition, the rabbit eyes were treated topically 4 times a day with one of the treatments under study. For this, rabbits were randomized for each surgical intervention into 1 of the following 4 groups: group (1) 100% AS drops (n = 8), group (2) 100% PRP drops (n = 8), group (3) 100% s-PRGF drops (n = 8), and group (4) artificial tears (Oculotect; Alcon) as the control group (n = 8).

Rabbit eyes were photographed with and without fluorescein twice a day. The wounded areas were measured using ImageJ software, and the results were expressed as mean wound area ± SD in square millimeters.

Histochemical Analysis

After both eyes of each animal had been operated on and followed up, the corneas were removed and processed for hematoxylin and eosin staining. Stained sections were observed with a phase contrast microscope (Nikon Eclipse TS 100), and images were acquired with the ProgRes CapturePro 2.6 software. We evaluated the structural integrity and histological characteristics of the cornea, as well as the regeneration of the epithelium and cell infiltration.

Statistical Analysis

IBM SPSS Statistics 18 software (SPSS, Chicago, IL) was used to calculate mean and SDs and to assess the statistical significance of differences between treatments with the Mann–Whitney U and χ^2 tests, as well as a time-dependently multiple comparison with the generalize linear model method.

TABLE 1. Closure Over Time of Remaining Denuded Areas in RPCE Cultures Incubated With 50% of Each of the 3 Blood Derivatives

	Time, h			
Treatment	**0**	**12**	**24**	**36**
AS*	0.63 ± 0.13	0.06† ± 0.07	0.01† ± 0.03	Completely closed†
PRP	0.63 ± 0.13	0.20 ± 0.12	0.10 ± 0.11	0.03† ± 0.08
s-PRGF*	0.63 ± 0.15	0.09† ± 0.13	0.02† ± 0.04	Completely closed†
Control	0.63 ± 0.14	0.32 ± 0.14	0.14 ± 0.12	0.13 ± 0.06

The results are expressed as mean remaining denuded area ± SD in square millimeter.
*Statistically significant differences with respect to control ($P \leq 0.01$) (time-dependently multiple comparison, generalize linear model method).
†Statistically significant differences with respect to control ($P \leq 0.05$) (Mann–Whitney U test).

RESULTS

In Vitro Scratch Wound-Healing Assays

The wound-healing assays were performed on RPCE and HCE cultures to test the ability of the 3 blood-derived preparations to promote in vitro migration and reepithelization. We found that 2 of the 3 preparations (AS and s-PRGF) notably accelerated the recovery of the monolayer in both RPCE and HCE cultures with respect to that in controls (see Figure, Supplemental Digital Content 1, http://links.lww.com/ICO/A215).

When analyzing the closure rate in RPCE cultures, cells treated with AS and s-PRGF covered the denuded areas faster than those receiving the control treatment, with statistically significant differences at all time points (Table 1). In contrast, the closure rate in cultures treated with PRP was relatively similar to that in control cultures. Specifically, the mean remaining denuded area (in square millimeters) in cultures treated with AS and s-PRGF was already very small after 12 hours (0.06 ± 0.09 and 0.09 ± 0.13 mm^2, respectively) though the cells did not completely cover the denuded area in the monolayer until 36 hours (Table 1).

As for the percentage of wells in which the defect in the monolayer had completely resolved at 36 hours, this was much higher with AS and s-PRGF (almost 100%) than PRP (60%) or control (20%) treatments (Fig. 1A). However, by this time point (36 hours), all 3 blood derivatives produced statistically significant differences in the number of wells in which the denuded area had been completely covered compared with the control treatment.

The pattern of the reepithelization process in the HCE cell line was very similar to that in RPCE cultures, but somewhat slower. That is, AS and s-PRGF promoted faster resolution of the defect in the monolayer, but the denuded areas were not all covered until 48 hours (Table 2). Furthermore, in the HCE cultures, we also found statistically significant differences in the mean remaining denuded area (in square millimeters) between cells treated with AS or s-PRGF and control cells. Nevertheless, these differences between treatments were not as marked in the HCE as in RPCE cultures, especially at 12 hours.

With respect to resolution of defects in the HCE cultures by 48 hours, 100% of defects had resolved with AS and s-PRGF treatments and only 20% with PRP, whereas none of the denuded areas had completely closed in control cultures (Fig. 1B).

In Vivo Corneal Reepithelization Assay in a Rabbit Animal Model

Assays performed on surgically induced epithelial defects confirmed that AS and s-PRGF promoted faster corneal

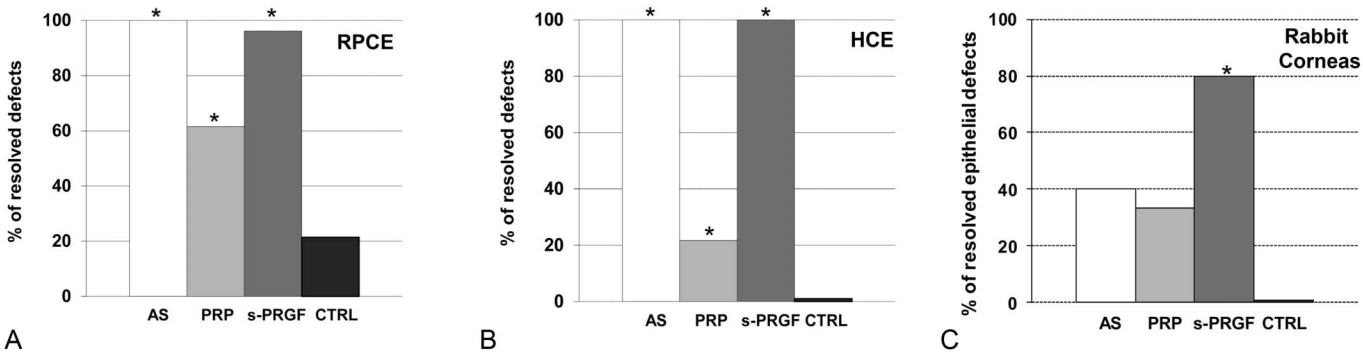

FIGURE 1. Percentage of wells in which the defect in the monolayer had completely resolved in RPCE cultures at 36 hours (A) and HCE cultures at 48 hours (B). By the end of these periods, almost all cell monolayers treated with AS or s-PRGF had recovered. Percentage of resolved epithelial defects in rabbit corneas by 3.5 days after surgery (C). Treatment with s-PRGF resulted in the highest rate of healing of the corneal epithelial defects. *Statistically significant differences with respect to the control treatment ($P \leq 0.05$) (χ^2 test).

TABLE 2. Closure Over Time of Remaining Denuded Areas in HCE Cultures Incubated With 50% of Each of the 3 Blood Derivatives

	Time, h				
Treatment	**0**	**12**	**24**	**36**	**48**
AS*	0.72 ± 0.19	0.43† ± 0.14	0.10† ± 0.11	0.02† ± 0.04	Completely closed†
PRP	0.76 ± 0.23	0.53 ± 0.15	0.34† ± 0.13	0.21† ± 0.12	0.11† ± 0.11
s-PRGF*	0.75 ± 0.22	0.38† ± 0.15	0.10† ± 0.09	0.01† ± 0.03	Completely closed†
Control	0.72 ± 0.12	0.48 ± 0.09	0.39 ± 0.06	0.30 ± 0.07	0.22 ± 0.10

The results are expressed as mean remaining denuded area ± SD in square millimeter.
*Statistically significant differences with respect to control ($P \leq 0.01$) (time-dependently multiple comparison, generalize linear model method).
†Statistically significant differences with respect to control ($P \leq 0.05$) (Mann–Whitney U test).

wound healing than PRP and control treatments (Fig. 2; Table 3). Specifically, the mean time to complete closure of the epithelial defect in control and PRP groups was 4.75 ± 0.76 and 4.42 ± 0.97 days, respectively, whereas it was shorter for eyes treated with AS or s-PRGF, 3.80 ± 0.27 and 3.50 ± 0.35 days, respectively.

Analyzing the wound-healing progression, the results showed that all treatments produce a similar initial phase of wound healing, with no statistically significant differences being detected. From the second day onward, however, the wound-healing process was accelerated by AS and s-PRGF compared with PRP and control treatments. Furthermore, although the length of time to complete wound healing was almost the same in AS and s-PRGF treatments, the smallest epithelial defect sizes were measured for s-PRGF at all time points (Table 3). In addition, s-PRGF was the only treatment that produced statistically significant differences in erosion sizes with respect to the control treatment from 2.5 days onward.

Focusing on 3.5 days after surgery, most of the corneal defects under s-PRGF treatment had already reepithelialized, whereas only 35% to 40% of them had healed in the AS and PRP groups (Fig. 1C) and in the control group, none of the defects had closed by this time. Furthermore, at this time point (3.5 days), s-PRGF was the only treatment with which the percentage of resolved corneal defects was significantly higher than with the control treatment.

No adverse events, such as corneal inflammation or neovascularization, were observed in any eyes in any of the study groups, either during the healing process or after resolution of the epithelial defects (Fig. 2).

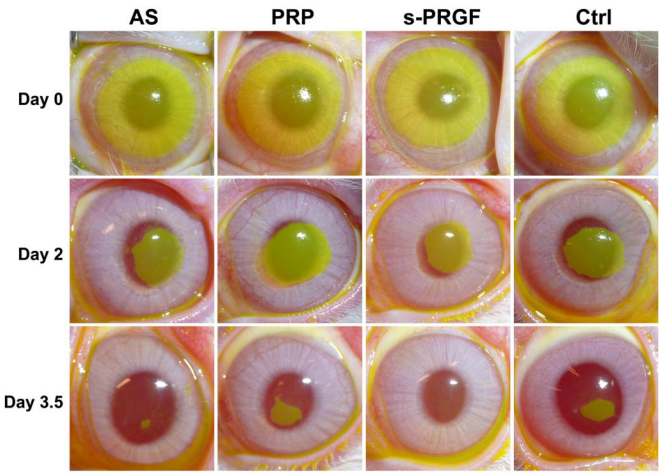

FIGURE 2. Evolution of the epithelial defect monitored with fluorescein staining in rabbit eyes treated with each of the 3 blood derivatives or artificial tears. The images illustrate that treatment with s-PRGF resulted in the fastest complete corneal reepithelization.

Histopathology Analysis

By the end of the study, the corneal epithelium had successfully healed in all rabbit eyes. Epithelial regeneration was normal with a multi-stratified, non-keratinized, and well-organized epithelium, with no notable differences between the 4 study groups (see Figure, Supplemental Digital Content 2, http://links.lww.com/ICO/A216). Nevertheless, histological sections revealed focal hyperplasia of one or two more layers of epithelial cells in all reepithelialized corneas, regardless of the treatment used compared with the histological sections of a healthy cornea (data not shown).

DISCUSSION

Corneal epithelial healing involves migration of epithelial cells to cover the denuded wound area, as well as cell proliferation and differentiation.[2] An important determinant of the healing potential of tissues is their ability to increase blood supply through physiological angiogenesis.[2] In the case of corneal injury, topical treatment with blood derivatives is used to compensate for the lack of physiological angiogenesis of this avascular tissue. Blood-derived preparations contain growth factors, cytokines, and other signaling molecules, that are essential for cell turnover in epithelial and stromal tissue in corneal wound healing.[5] Furthermore, these molecules may suppress inflammation in the case of impairment of epithelization and also have antimicrobial effects.[22]

In this context, this study sets out to determine, both in vitro and in vivo, the influence of the manufacturing of 3 blood derivatives on their efficacy to promote corneal epithelial healing. The main differences in the production of these preparations include the activation of the coagulation process and the presence or absence of leukocytes during coagulation. In the case of AS and s-PRGF, coagulation involves the activation of platelets, which release large

TABLE 3. Wound Healing Progression in Rabbit Eyes Treated With 100% of Each of the 3 Blood Derivatives or Artificial Tears

	Time, d					
Treatment	**0**	**2**	**2.5**	**3**	**3.5**	**4**
AS	73.65 ± 4.08	16.87 ± 2.89	9.07 ± 3.74	2.26* ± 1.36	0.45 ± 0.49	Completely closed*
PRP	73.33 ± 3.31	17.89 ± 6.49	11.89 ± 5.24	4.25 ± 3.31	2.59 ± 2.17	1.24 ± 0.80
s-PRGF†	73.58 ± 2.33	17.02 ± 1.82	7.78* ± 2.39	0.82* ± 1.02	0.03* ± 0.08	Completely closed*
Control	73.50 ± 2.36	18.23 ± 3.86	11.04 ± 2.77	6.09 ± 3.66	3.05 ± 2.38	1.76 ± 2.52

The results are expressed as mean wound area ± SD in square millimeter.
*Statistically significant differences with respect to control ($P \leq 0.05$) (Mann–Whitney U test).
†Statistically significant differences with respect to control ($P \leq 0.05$) (time-dependently multiple comparison, generalize linear model method).

amounts of growth factors. However, the coagulation process in AS occurs spontaneously and in the presence of leukocytes; whereas in s-PRGF, coagulation is induced after the elimination of leukocytes and red cells. This is a controversial topic,[23,24] with some authors asserting that the presence of leukocytes can be highly beneficial because of their antimicrobial properties[25,26]; whereas others suggest that leukocytes should be avoided to prevent proinflammatory effects.[27–29] Unlike in AS and s-PRGF, coagulation does not occur in the production of PRP. Platelets are neither activated nor removed. Some authors claim that the coagulation process in PRP is activated in the site of application, which results in a slower release of growth factors.[22]

In wound healing, epithelial cells distal to the wound area are stimulated to migrate toward the wound to restore corneal epithelial function.[30] In vitro scratch wound-healing assays are usually used to determine the migration ability of cultured cells under given treatments.[31–33] Our analysis using rabbit primary cultures and a human cell line indicates that all of the 3 blood derivatives studied significantly improve the migratory ability of cells compared with that in controls. However, AS and s-PRGF induce markedly faster wound healing. The capability of blood derivatives to improve cell migration has been associated with their high content of fibronectin.[34,35] In a previous study,[20] we quantified higher concentrations of fibronectin in these 3 blood derivatives (see Table, Supplemental Digital Content 3, http://links.lww.com/ICO/A217) in comparison with preparations used by other authors.[12] Furthermore, some authors suggest that in the presence of fibronectin, platelet-derived growth factor stimulates the migration of corneal epithelial cells in corneal wound healing.[5] Another factor that can affect cell migration involves connexin 43 (Cx-43), a component of gap junctions. Lower expression of Cx-43 has been related to a greater ability of cells to migrate.[36] Consistent with this, we observed in a previous study[20] a lower level of expression of Cx-43 in cells treated with any of the 3 blood derivatives with respect to those under the control treatment, and furthermore, in this study, a greater ability to migrate in cells under those same treatments.

Because a high content of fibronectin and platelet-derived growth factor and lower Cx-43 expression are detected with all the 3 blood derivatives, these factors do not seem to be responsible for the greater migratory ability of cells treated with AS or s-PRGF with respect to those given PRP or the control treatment. This discrepant behavior could be explained by other biochemical differences between the blood derivatives, such as EGF content. It has been described that EGF has an important role in epithelial cell proliferation and enhances migration by promoting actin filament rearrangement.[37] Furthermore, high levels of the EGF receptor have been found to be expressed in cells migrating over wounds.[38] Thus, previously quantified concentrations of EGF (see Table, Supplemental Digital Content 3, http://links.lww.com/ICO/A217) being higher in AS and s-PRGF than in PRP could explain not only the faster proliferation rate in HCE cells[20] but also the greater migratory ability of cells in RPCE and HCE cultures. Furthermore, the lowest concentrations of EGF and weakest effect on corneal epithelial cell growth and migration were observed with PRP, in which platelet activation has not occurred. These results agree with data previously published.[31]

Although in vitro culture systems are useful tools to simulate in vivo situations, they are simplified environments, which cannot reproduce the complex physiological and molecular interactions between tear film and the ocular surface.[37] Probably for this reason, we found that though AS and s-PRGF behave similarly in vitro, there are differences in their effect in vivo. In rabbit corneas, although the mean time to complete reepithelization was similar with AS and s-PRGF treatments, most of the rabbit defects (80%) in the s-PRGF group were already reepithelialized by 3.5 days after surgery, whereas only less than half (40%) were in the AS group. In addition, s-PRGF formulation is the only treatment with which from 2.5 days onward, the wound size was significantly smaller than in eyes receiving only the unpreserved tear substitute. It should be underlined that a decrease in the size of epithelial defects is usually associated with reductions in the risk of infection, as well as in patient pain and discomfort. Furthermore, it has been suggested that prolonged episodes of corneal deepithelization may lead to increased production of collagenases in the corneal stroma, and in turn to corneal perforation.[39]

The concentrations and frequency of administration of blood derivatives may influence the wound-healing process.[37] Several studies have reported the effectiveness of undiluted concentrations of blood derivatives in the epithelial healing process of mechanical corneal wounds[2,18,40] and corneal alkali burns,[41] whereas other studies have shown no significant effects when using 20% dilutions.[18,42] For this reason, we

used undiluted concentrations of blood derivatives in our in vivo experiments, which were applied 4 times a day. Unfortunately, there have been few in vivo trials comparing the dosage and efficacy of different blood derivatives, which makes this an interesting area for further investigation.

To sum up, we have seen that all 3 blood derivatives studied (AS, PRP, and s-PRGF) are promoters of corneal epithelization. However, some of our previous[20] and present results taken together support the view that the manufacturing process of blood derivatives leads to variations in the concentrations of certain molecules and, therefore, strongly influences the efficacy of these preparations in corneal wound healing. Notably, both AS and s-PRGF promote faster wound healing in vitro. However, the latter, the derivative with the highest content in EGF, induces considerably earlier reepithelization of in vivo corneal wounds, and hence it could even be effective in diminishing the risk of infection and increasing patient comfort. We believe that it would be interesting to confirm the outcomes of this study in a clinical trial comparing the effect of different blood derivatives.

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Preservation of Biological Activity of Plasma and Platelet-Derived Eye Drops After Their Different Time and Temperature Conditions of Storage

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Purpose: To analyze whether plasma rich in growth factors (PRGF) eye drops preserve their biological characteristics and activity after storage for 3 and 6 months at −20°C, at 4°C, and at room temperature for 72 hours, compared with fresh samples (t_0).

Methods: Blood from 6 healthy donors was harvested and centrifuged to obtain PRGF free of leukocytes. Resulting PRGF eye drops were stored for 3 and 6 months at −20°C. At each time, 2 aliquots were maintained at room temperature or at 4°C for 72 hours. Platelet-derived growth factor-AB, transforming growth factor-β1, vascular endothelial growth factor, epidermal growth factor, insulin-like growth factor-1, angiopoietin-1, and thrombospondin-1 were quantified at each time and temperature of storage. Also, the effect of PRGF eye drops on proliferation of primary human keratocytes was evaluated.

Results: All the analyzed growth factor levels remained constant at each time and storage condition. No differences were observed in the proliferative activity of keratocytes after treatment with PRGF eye drops at any studied time or temperature. Finally, there was no microbial contamination in any of the PRGF eye drops.

Conclusions: The preservation of the PRGF eye drops at −20°C for up to 3 and 6 months does not mean reduction of the main growth factors and proteins implicated in ocular surface wound healing. Eye drop characteristics and in vitro biological activity were not affected by their usage and conservation for 72 hours at 4°C or at room temperature.

Key Words: plasma rich in growth factors, stability, growth factors, eye drops, platelet-rich plasma

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Ocular surface disorders including dry eye and persistent epithelial defects of the cornea are serious circumstances caused by a variety of underlying diseases and can result in severe visual impairments. Efficient wound healing is necessary and essential to regenerate the injured ocular tissues, and thus recover their adequate functionality. It is widely known that growth factors and cytokines are strongly involved in the orchestration of these cellular and extracellular mechanisms.[1]

Plasma rich in growth factors (PRGF) is an autologous platelet-rich plasma based on the use of the patient's own blood, and the resulting therapeutic formulations derived from this technology contain a wide variety of growth factors, proteins, and biomaterials involved in tissue regeneration. The effectiveness, significant clinical advantages, and safety of this approach have been deeply demonstrated in several medical fields.[2–5] In fact, PRGF provides a pool of biologically active proteins and growth factors, including platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), thrombospondin-1 (TSP-1), and angiopoietin-1 (ANG-1) among others. These proteins are involved in a range of biological processes, including cell recruitment, growth, and differentiation.[6,7]

Ocular surface disorders usually represent chronic diseases, often establishing their treatment on a long-term basis. As a consequence, therapies used for the management of these pathologies should preserve their functionality and stability for several weeks to be used daily during months.

Recently, we have demonstrated that PRGF eye drops can be stored for up to 3 months without reduction of the main proteins and growth factors implicated in ocular surface wound healing.[8] Our data showed that the autologous eye drops preserved their composition and in vitro biological activity both at 4°C and at room temperature during 24 hours. Extending the time of storage up to 6 months and maintaining the dispenser eye drops in use at room temperature for several days may significantly improve safety, cost-effectiveness, and comfort. This fact facilitates the daily use of the autologous eye drops, avoiding the need for keeping them under freezing storage (avoiding the cold chain).

The purpose of this study was to analyze whether PRGF eye drops conserved their composition in proteins and growth factors as well as their biological potential after preservation at −20°C for 3 and 6 months compared with the

freshly prepared eye drops. Also, the integrity of PRGF eye drops after 72 hours both at 4°C and at room temperature was determined. To address this, PRGF eye drops were characterized at each storage condition by measuring the concentration of wound-healing cytokines and by testing and comparing the proliferative potential and the microbial contamination against the freshly obtained eye drops.

MATERIALS AND METHODS

PRGF Sample Preparation

PRGF eye drops from each donor were obtained using the Endoret-Ophthalmology kit (BTI Biotechnology Institute, SL, Miñano, Álava, Spain). Briefly, blood from 6 healthy donors was collected after they provided informed consent. The study was performed following the principles of the Declaration of Helsinki. After blood centrifugation, the whole plasma column was drawn off avoiding collecting the buffy coat containing the leukocytes. The platelet concentration was measured with a hematology analyzer (Micros 60; Horiba ABX, Montpelier, France). The collected platelet-rich plasma was incubated at 37°C for 1 hour until complete clot retraction. Finally, the supernatant volume was filtered and aliquoted to be used as fresh samples (t_0) or to be stored at –20°C for 3 (t_3) months and 6 (t_6) months. Two aliquots from each donor and each storage time under freezing conditions (t_3 and t_6) were removed from the freezer, thawed, and stored 72 hours before the end of each storage period. One of those aliquots was kept at 4°C (4°C), and the other was maintained at room temperature (RT) for 72 hours until its use.

Characterization of Autologous Eye Drops

To check the stability of PRGF eye drops at each time and storage temperature, several growth factors involved in ocular surface tissue regeneration such as PDGF-AB, EGF, VEGF, TGF-β1, insulin-like growth factor (IGF-1), ANG-1, and TSP-1 were measured using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. All enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Minneapolis, MN).

Cell Culture

Primary human keratocytes (ScienCell Research Laboratories, San Diego, CA) were cultured according to the manufacturer's instructions. Briefly, cells were cultured at 37°C and 5% CO_2 atmosphere in a fibroblast medium supplemented with a fibroblast growth supplement (ScienCell Research Laboratories), 2% fetal bovine serum, and antibiotics until confluence. Then, they were detached with an animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY). Cell viability was assessed by the trypan blue dye exclusion method, based on the principle that live (viable) cells do not take up certain dyes, whereas dead (nonviable) cells do. Finally, these cells in passage 3 were used in proliferation assays.

Cell Proliferation

Cells were seeded at a density of 5000 cells per square centimeter on 96-well optical bottom black microplates in a serum-free medium supplemented with 20% (vol/vol) of PRGF from each donor and for each time of storage including fresh samples as t_0 hours. The study period was 72 hours. Density of cells in the culture was estimated using the CyQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA). Briefly, the medium was removed, and wells were washed carefully with phosphate-buffered saline. Then, microplates were frozen at −80°C for efficient cell lysis in the CyQUANT assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.4 Kunitz/mL) diluted in cell lysis buffer during 1 hour at room temperature. Then 2× CyQUANT GR dye/cell lysis buffer was added to each sample well and mixed gently and incubated for 5 minutes at room temperature but protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies). A DNA standard curve ranging from 7.8 to 500 ng/mL was included in all fluorescence quantifications to correspond fluorescence units with the DNA amount.

Sterility Analysis

One milliliter from each PRGF sample stored at different time points and temperatures was collected to check the sterility. For this purpose, thioglycollate broth and tryptic soy broth were used for qualitative determination of aerobic and facultative anaerobic microorganisms in the samples. After inoculation, culture vials were incubated at 32°C for thioglycollate broth and at 22°C for tryptic soy broth for 14 days and monitored for the growth of microorganisms. An increase in broth turbidity is considered as a positive indicator of microbial contamination.

Statistical Analysis

Data are expressed as mean ± SD. After the analysis of the normal distribution and homoscedasticity of the results, repeated-measures analysis of variance was used to assess the differences between the variables at the 3 different time points (t_0, t_3, and t_6) and at distinct temperatures of storage (−20°C, RT, and 4°C). The level of significance was set at $P = 0.05$. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc, Chicago, IL).

RESULTS

Characterization of PRGF Specimens

PRGF eye drops were obtained from 6 healthy donors with a mean age of 41 years (range, 26–59 years). Platelet and leukocyte counts in the peripheral blood and in PRGF are shown in Table 1. Autologous eye drop samples showed a 2-fold increase in the platelet concentration over peripheral

TABLE 1. Platelet and Leukocyte Count

			Peripheral Blood		PRGF		
	n	Age, yrs	Platelets ($\times 10^6$/mL)	Leukocytes ($\times 10^6$/mL)	Platelets ($\times 10^6$/mL)	Leukocytes ($\times 10^6$/mL)	Platelet Enrichment
Donors	6	41 ± 12	185 ± 5	6.3 ± 0.5	367 ± 18	0.3 ± 0.1	2.0 ± 0.1
Range (min–max)		26–59	136–226	3.3–9.4	258–497	0.1–1.0	1.4–3.1

Data represent mean ± SD and minimum (min) and maximum (max) levels in the peripheral blood and in PRGF preparations from the 6 donors.

blood. Furthermore, no detectable levels of leukocytes were observed.

Autologous Eye Drops Growth Factor Levels at Different Time and Temperature Storages

Concentrations of growth factors within eye drops were measured on the day of collection (fresh samples, t_0) and after storage at –20°C for 3 (t_3) and 6 (t_6) months. The different growth factor concentrations measured at each storage time are represented in Table 2. None of the growth factors analyzed in the study showed a significant change ($P > 0.05$) within the follow-up period of 6 months. In fact, the concentration of each growth factors remained constant at all studied times.

At the end point of each study period (3 and 6 months), 2 samples from each donor were taken from the freezer; one was stored at 4°C and the other at RT for 72 hours. Another eye drop aliquot from each donor was taken from the freezer and thawed, corresponding to the −20°C sample. The concentration levels of some relevant growth factors (PDGF-AB, TGF-β1, VEGF, EGF, IGF-1, ANG-1, and TSP-1) present in the eye drops stored during 72 hours at 4°C or RT are illustrated in Table 2. Results showed no significant differences ($P > 0.05$) in the content of growth factors.

Effect on Cell Proliferation

Representative images of cells treated with freshly prepared PRGF eye drops (t_0) and with PRGF eye drops stored at −20°C for 3 and 6 months are shown in Figure 1. Keratocytes showed no significant differences ($P > 0.05$) in their proliferation rate, measured as the DNA amount, after treatment with the autologous eye drops conserved at distinct time and storage conditions.

Sterility Analysis

No microbiological contamination was detected in any of the PRGF eye drops. Moreover, there was no sign of microorganism growth in any of the cell cultures assayed for proliferation with the eye drops.

DISCUSSION

The liquid layer bathing the cornea and conjunctiva is called the tear film, and it is a complex solution composed mainly of a wide range of growth factors and biologically active proteins that are involved in corneal tissue homeostasis and ocular surface wound healing. Frequently, ocular injuries do not respond to conventional treatments such as artificial tears, because they do not mimic the characteristics and properties of natural tears.[9,10]

Patient's own autologous serum has been used for decades with controversial results because the way this serum is prepared, diluted, and applied varies significantly from one clinical center to another. More recently, a new type of blood-derived product with an improved biological effect has been launched.[6,7] PRGF provides an autologous eye drop rich in plasma and especially platelet-derived proteins and growth factors. These blood-based formulations have been used for the treatment of several ocular surface pathologies including dry eye or persistent epithelial defects.[11–13]

Recent data provide evidence of the potential of the autologous eye drops in the treatment of various ocular surface disorders.[11,14,15] However, more studies are needed to

TABLE 2. Concentrations of Several Growth Factors in the Different PRGF Eye Drops

	PDGF-AB, ng/mL	TGF-β1, ng/mL	VEGF, pg/mL	EGF, pg/mL	IGF-1, ng/mL	ANG-1, ng/mL	TSP-1, ng/mL
t_0	13.4 ± 4.6	30.8 ± 10.8	215 ± 148	569 ± 141	106 ± 29	29.4 ± 10.8	22.4 ± 9.2
t_3							
−20°C	15.5 ± 3.6	34.6 ± 6.7	188 ± 87	454 ± 95	111 ± 31	26.8 ± 8.5	19.3 ± 6.0
4°C	10.6 ± 4.0	31.9 ± 9.4	185 ± 77	474 ± 111	113 ± 31	25.8 ± 7.2	20.2 ± 4.5
RT	14.4 ± 3.0	30.7 ± 7.3	157 ± 59	428 ± 106	123 ± 37	25.8 ± 7.2	21.2 ± 3.8
t_6							
−20°C	13.4 ± 4.7	29.7 ± 9.1	251 ± 111	475 ± 113	117 ± 33	30.3 ± 10.9	20.2 ± 5.0
4°C	11.5 ± 4.4	33.1 ± 13.4	224 ± 122	513 ± 265	118 ± 35	25.2 ± 6.8	20.6 ± 3.8
RT	10.4 ± 3.7	25.4 ± 10.7	191 ± 112	430 ± 126	125 ± 41	24.1 ± 6.5	20.8 ± 3.8

No significant differences ($P > 0.05$) were observed among the distinct time and storage conditions of the growth factors analyzed in the autologous eye drops.

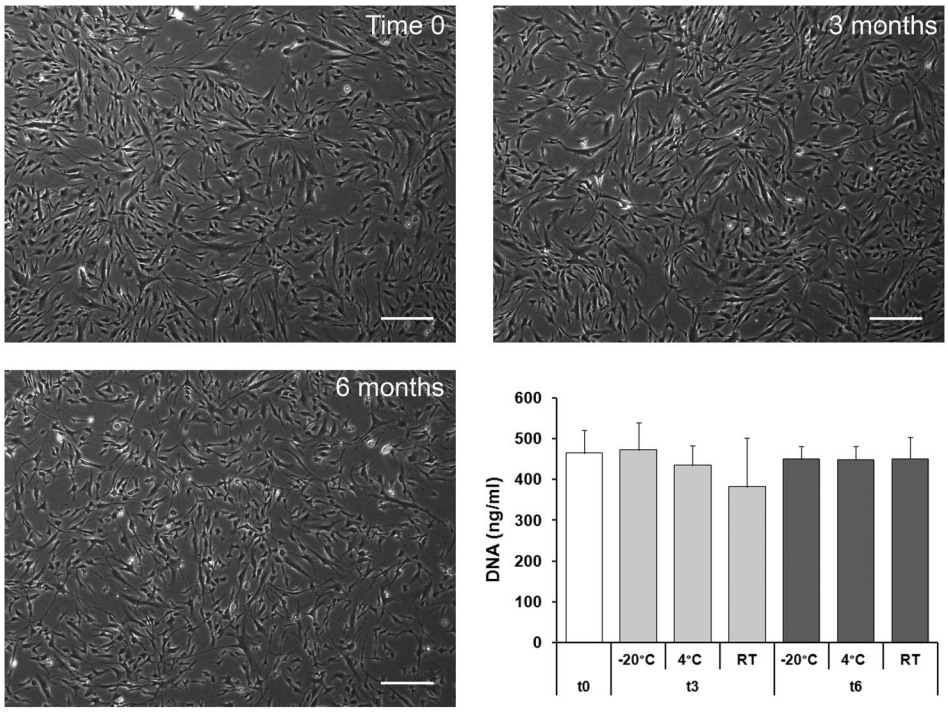

FIGURE 1. Phase contrast photomicrographs showing the proliferation of human primary keratocytes cultured with fresh PRGF-Endoret samples (Time 0, t_0), PRGF-Endoret eye drops after storage at −20°C for 3 months and 6 months. There was no difference ($P > 0.05$) between the proliferation induced by PRGF eye drops at any time and condition of storage (scale bar, 300 μm).

characterize the biological stability of the formulation and to demonstrate whether PRGF preserves its biological activity regardless of when it is used or how it is stored. Assuming a future clinical dosage based on a daily use of the eye drops (from 2 to 4 instillations) and up to 6 months of treatment,[14,16] we decided to evaluate the composition and biological activity of PRGF eye drops after different storage conditions. Storage conditions of the autologous eye drops were designed to minimize protein degradation from baseline levels and microbial contamination.

Our results show that some levels of growth factors and cytokines implicated in corneal wound healing such as TGF-β1, EGF, VEGF, PDGF-AB, IGF-1, TSP-1, and ANG-1 maintain their concentrations after their storage both at 3 and 6 months at −20°C. Moreover, the levels of these proteins remain similar to those of fresh PRGF eye drops.

Bradley et al analyzed the stability of several growth factors such as EGF, substance P, calcitonin gene-related peptide, and neurotrophic growth factor at different storage temperatures (−15, 4, 25, 37, and 42°C) for up to 24 hours in human autologous serum eye drops. Some of them including substance P, neurotrophic growth factor, and calcitonin gene-related peptide showed a reduction in their concentration levels after storage at room temperature or 4°C.[17] However, in our study, no differences were observed between the main protein levels of the frozen PRGF eye drops and the eye drops stored at RT or 4°C for 72 hours for 3 and 6 months. Furthermore, there were no significant differences in keratocyte proliferation after treating the cells either with the PRGF stored up to 3 and 6 months and maintained at 4°C or room temperature for 72 hours. The latter is particularly relevant, as it drastically improves the dosage of autologous eye drops, allowing the use of a freshly opened PRGF eye drop dispenser up to 72 hours without cold storage.

Blood-derived eye drops are commonly used without preservatives to avoid the risk of chemical toxicity.[18] Although these products have natural antimicrobial properties,[19,20] there is a general concern about potential microbial contamination related to the long-term use of the eye drop dispenser.[21,22] As a consequence, it is generally recommended to preserve the eye drop dispenser at 4°C for 5 to 7 days.[23] Despite this recommendation, 7% to 25% of the eye drop containers analyzed in several studies were positive for microbial contamination.[18,24,25] In this study, we show the absence of microbial contamination in each of the PRGF eye drops maintained at 4°C or room temperature for 72 hours.

In summary, this study reveals that storage of PRGF eye drops for up to 3 and 6 months does not mean reduction of the main growth factors and proteins implicated in ocular surface wound healing. The characteristics and in vitro biological activity of the eye drops were not affected by their usage and conservation for 72 hours at 4°C or at room temperature.

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Review Article

Autologous serum and plasma rich in growth factors in ophthalmology: preclinical and clinical studies

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ABSTRACT.

The use of blood derivatives represents an alternative therapeutic approach that is gaining interest in regenerative medicine due to its potential to stimulate and accelerate tissue healing. Autologous serum eye drops and platelet-enriched plasma eye drops are being used in the treatment of different ophthalmological disorders. In this review, we summarize the different blood-derived formulations used in the treatment and care of ocular surface disorders. The biological basis and use of autologous serum and plasma rich in growth factors are deeply evaluated as well as the challenges to be addressed in the future in this new generation of blood-derived therapies.

Key words: autologous serum – cornea – dry eye – eye – ocular surface – plasma rich in growth factors – platelet-rich plasma

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Introduction

Ocular surface may suffer from several disorders including dry eye syndrome, persistent epithelial defects (PEDs), neurotropic ulcerations, limbal deficiency and corneal dystrophies among others. Dry eye syndrome is the most common disorder, and its prevalence has tripled in the last decade. These disorders are characterized by impaired tissue repair process. A reduction in epitheliotrophic factors compromises the integrity of the surface epithelia, leading to the formation of epithelial defects that may persist and progress as a result of the compromised wound-healing process (Quinto et al. 2008). Conventional therapeutic options include intensive artificial tear supplements, punctual occlusion, therapeutic contact lenses and appropriate management of adnexal disease. Surgical procedures, as keratoplasty or amniotic membrane transplantation, are used every day to restore the ocular surface but they are no suitable for all patients.

The integrity of the corneal epithelium and the process of re-epithelialization are dependent on many factors. Although underlying mechanisms have not been fully elucidated, it is likely that trophic factors exert a pivotal role in corneal epithelium integrity. For example, nerve growth factor (NGF) is being investigated as a potential treatment in neurotrophic keratopathy (Werner & Grose 2003). Topical NGF shows encouraging results in clinical trials, but its use is limited as still recombinant growth factors are not cost efficient. Heparan sulphate derivates are potential alternatives. ReGeneraTing Agent (RGTA, Cacicol20®, OTR3, Paris, France) is a new type of matrix therapy agent. The latter is based on large polymers, which replace destroyed heparan sulphate molecules, creating a suitable cellular environment that promotes healing. RGTA has been reported to show encouraging results in the treatment of corneal ulcers and dystrophies of various aetiologies, and it is currently being evaluated by means of clinical trials (Aifa et al. 2012).

Several studies have shown that cyclosporin improves corneal epithelium in dry eye patients, including those with graft-versus-host disease (GVHD). Improvements in the ocular surface and tear functions resulted presumably from the decreased inflammation and the increased goblet cell density. However, the use of topical cyclosporin eye drops is correlated with several side-effects such as strong irritation, which limits its use (Wang et al. 2008; Toker & Asfuroglu 2010). Another approach consists of using topical anti-inflammatory agents like

corticosteroids. Although these drugs improve patient′s symptoms, they are also associated with damaging long-term side-effects including cataracts and increased intra-ocular pressure (Dinning 1976; Renfro & Snow 1992). As a consequence, the use of artificial tears, that increase ocular surface humidity and provide additional lubrication, remains as the most widely use treatment. The emergence of presentations without preservatives represents a turning point, as preservatives may induce ocular toxicity, irritation and provokes epithelial damage (Geerling et al. 2001; Noecker 2001). The new types of formulations including hypotonic solutions, tears that contain lipids to prevent evaporation, substances with bioadhesive properties to increase water retention, and formulations that contain protective substances of the cell stress caused by the hypertonicity of the tear represent an advance in the field (Aragona et al. 2013). However, all of them are far from having all the properties of the human tear, as they lack biologically active molecules and growth factors (Klenkler et al. 2007; Dogru & Tsubota 2011).

Blood Derivatives in Ophthalmology

It widely known that natural tears have three main objectives: provide a smooth surface that allows the regular refraction of light, keep the metabolism of the ocular surface and lubricate the eye surface to facilitate blinking. They also have a complex composition, being the main component water (98.3%), followed by salts (1%), proteins and glycoproteins (0.7%), and minor hydrocarbons, lipids and other fractions. Teardrop brings regulatory enzymes and nutrients such as glucose, oxygen, water and electrolytes necessary for the metabolism of the corneal epithelium. It contains numerous active or functional proteins such as growth factors, vitamins, immunoglobulins and neuropeptides that regulate the processes of proliferation, migration and differentiation of the cells of the corneal epithelium and conjunctiva. Furthermore, it also presents antimicrobial properties, not only because of its barrier effect and washing, but because it contains lymphocytes, macrophages and enzymes such as arisulfatase A, peroxidase, lactoferrin and lysozyme with bacteriostatic and bactericide effects (Geerling et al. 2004). Tears thus have lubricating, mechanical and antimicrobial effects, but also epitheliotrophic properties.

Historically, there has been an interest in developing 'natural-tear'-like agents that may be used in the treatment of eye surface diseases. The use of blood and blood derivatives is not new, and their rationale in ophthalmology is based mainly on their potential properties including lubrication, mechanical actions and antimicrobial effects. The Ebers Papyrus, 1534 BC, is the very first reference in history to the implementation of a blood derivate at eye level. It was in 1975 when autologous serum (AS) was initially applied for dry eye (Ralph et al. 1975). The beneficial effect of AS in the treatment of dry eye syndrome was later described by Fox et al. (1984a). But it was at the end of the 1990s of the last century when began its use of a more extended form (Tsubota et al. 1999a,b); since then, AS eye drops have become more popular for treating ocular surface disease.

Through these decades, fetal bovine serum, allogeneic serum and umbilical cord serum have been used for this purpose, but they are heterologous products, with a higher risk of allergic reactions and infectious disease transmission, and their use is possible only in some specialized centres (Yoon et al. 2007a, 2011, 2013; Sharma et al. 2011; Harritshoj et al. 2014). The composition of serum is very similar to that of tears and most concentrations are the same, with the exception of more vitamin A, lysozyme, transforming growth factor-β (TGF-β) and fibronectin, and less IgA, epidermal growth factor (EGF) and vitamin C in serum than in tears (Pan et al. 2013; Table 1). Several studies have shown that AS eye drops contain growth factors such as EGF, vitamin A, TGF-β, fibronectin, substance P (SP), insulin-like growth factor 1 (IGF-1), NGF and other cytokines that are essential for the proliferation, differentiation and maturation of the normal ocular surface epithelium. Some of the main roles of these proteins found in plasma and platelets are summarized below and resumed in Table 1.

Epidermal growth factor

Epidermal growth factor plays an important role in corneal epithelial migration and proliferation that improves and accelerates the wound healing process. EGF stimulates DNA synthesis of epithelial cells and stromal fibroblasts in culture and synthesis of fibronectin by epithelial cells and is chemotactic for human epithelial and stromal cells. It has an anti-apoptotic effect and has been linked with the production of mucin 1 by the Goblet cells of the conjunctiva (López García et al. 2011).

Transforming growth factor-β

Transforming growth factor-β1 has been detected in corneal epithelium, stroma and endothelium. In the epithelium, the TGF-β1 levels are higher during the stromal repair processes. TGF-β is secreted by a large number of cells such as platelets, endothelial cells, lymphocytes and macrophages. In the cornea, TGF-β1 decreases the keratocytes migration, but favours the chemotaxis of fibroblasts as well as the production of extracellular matrix by a dual mechanism: by stimulating the production of collagen, fibronectin and proteoglycans and diminishing its degradation by inhibiting the metalloproteases and other proteolytic enzymes. In a synergistic mechanism with platelet-derived growth factor (PDGF) and integrins, TGF-β promotes the differentiation of myofibroblasts and exerts important anti-inflammatory effects (López García et al. 2011).

Vitamin A

Vitamin A is one of the main epithelium-trophic factors in AS, being its concentration 100 times higher than that found in the tear. It seems to prevent processes of squamous metaplasia of the epithelia (Geerling et al. 2004).

Platelet-derived growth factor

Platelet-derived growth factor was one of the first growth factors

Table 1. Main growth factors present in blood and their roles in ocular surface regeneration.

Proteins	Roles	References
EGF	• Induces corneal epithelial migration and proliferation • Stimulates DNA synthesis of epithelial cells and stromal fibroblasts • Stimulates synthesis of fibronectin by epithelial cells • Chemotactic effect for human epithelial and stromal cells • Anti-apoptotic effect • Induces production of mucin 1 by the Goblet cells	López García & del Castillo (2011)
TGF-β	• Decreases keratocyte migration • Favours chemotaxis of fibroblasts • Induces the production of extracellular matrix by stimulating the production of collagen, fibronectin, and proteoglycans and diminishing its degradation by inhibiting the metalloproteases and other proteolytic enzymes • Promotes the differentiation of myofibroblasts • Anti-inflammatory effect	López García & del Castillo (2011)
Vitamin A	• Prevent processes of squamous metaplasia of the epithelia	Geerling et al. (2004)
PDGF	• Chemotactic effect for monocytes, macrophages and fibroblasts • Synergistic effect with TGF-β to promote myofibroblasts differentiation	
Fibronectin	• Promotes wound healing and phagocytosis • Important role on cell migration during the repair process of corneal epithelium	Phan et al. (1987) and Gordon et al. (1995)
Annexin A5	• Stimulates the secretion of the plasminogen activator-type urokinase facilitating cell migration	
Albumin	• Reduces degradation of cytokines and growth factors	Tsubota et al. (1999b), Shimmura et al. (2003) and Unterlauft et al. (2009)
α2 macroglobulin	• Neutralizes the proteolytic enzymes	Tsubota et al. (1999a) and Poon et al. (2001)
bFGF	• Promotes corneal wound healing increasing cell proliferation and motility	Andresen et al. (1997)
IGF-I	• Acts synergistically with substance P to promote corneal epithelial migration	Yamada et al. (2004)
NGF	• Induces neurite sprouting by neural cells • Restores the function of injured neurons • Induces SP and calcitonin gene-related peptide production in the central and peripheral nervous system enhancing epithelial proliferation • Increases epithelial cell proliferation and differentiation • Promotes fibroblast cell growth	Matsumoto et al. (2004)

EGF = epidermal growth factor, TGF-β = transforming growth factor beta, PDGF = platelet-derived growth factor, bFGF = fibroblast growth factor b, IGF-I = insulin-like growth factor 1, NGF = nerve growth factor.

characterized. It is chemotactic for monocytes, macrophages and fibroblasts and stimulates the expression of other factors such as TGF-β.

Fibronectin

It is a soluble protein that promotes wound healing and phagocytosis. On the ocular surface, fibronectin is one of the most important factors in the phase of cell migration during the repair of corneal epithelium (Phan et al. 1987; Gordon et al. 1995). Topical fibronectin has been used in the treatment of persistent corneal epithelial defects and trophic ulcers with different results.

Annexin A5

Annexin A5 is being investigated as an alternative to the fibronectin eye drops, which interacts with the domain kinase of some integrins mimicking its effect. It also stimulates the secretion of the plasminogen activator-type urokinase, whose expression is increased in epithelial defects, facilitating cell migration.

Albumin

Albumin is one of the most important proteins in blood. It reduces the natural degradation of cytokines and growth factors in the areas of tissue injury and shows anti-apoptotic activity. The wound healing effect of albumin eye drops has already been demonstrated *in vitro* and *in vivo* (Tsubota et al. 1999b; Shimmura et al. 2003; Unterlauft et al. 2009).

α2 macroglobulin

Its main function is to neutralize the proteolytic enzymes. It is useful in ocular burns and marginal ulcers (Tsubota et al. 1999a; Poon et al. 2001).

Fibroblast growth factor b

Fibroblast growth factor promotes corneal wound healing, not only by increasing cell proliferation, but also through increased motility (Andresen et al. 1997).

Insulin-like growth factor-I

Insulin-like growth factor-I acts synergistically with SP to promote corneal epithelial migration (Yamada et al. 2004). The use of IGF-I-containing eye drops in patients with neurotrophic keratopathy leads to successful results (Chikama et al. 1998; Yamada et al. 2004). Patients with seasonal allergic conjunctivitis and VKC have showed significant elevation of SP in tears,

which suggests that SP may contribute to the pathogenesis and severity of ocular allergic diseases (Matsumoto et al. 2004).

Nerve growth factor

Nerve growth factor is the best-known neurotrophin. Some reports state the efficacy of the novel use of NGF in resurfacing corneal ulcers resulting from neurotrophic keratopathy. It is well known that NGF induces neurite sprouting by neural cells and restores the function of injured neurons. NGF also has been shown to induce the production of SP and calcitonin gene-related peptide in the central and peripheral nervous system. The biological effects of NGF on the ocular surface are known to be mediated by specific receptors localized on corneal and conjunctival epithelial cells and immune cells (Matsumoto et al. 2004).

Autologous Serum

In general, dry eye treatments attempt to manage symptoms, including burning, irritation and foreign body sensation but fail to repair injured tissues. One potential reason for this is that artificial tears have variable osmolarity, viscosity, electrolyte composition and sometimes the presence of preservative (Lemp 2008). The use of preserved artificial tears may cause toxicity and allergic reactions. Furthermore, artificial tears do not contain the proper mixture of growth factors, neuropeptides and vitamins present in the healthy tear film. As serum composition resembles that of tears (Table 2), it has been proposed as a substitute to treat ocular surface injuries. As early as 1975, Ralph et al. showed the successful use of serum eye drops in treating advanced ocular surface dysfunction (Ralph et al. 1975). After the initial report, Fox et al. and Tsubota et al. elaborated on the use of AS tears in Sjögren dry eye patients, demonstrating that there was not only subjective improvement in symptoms (Fox et al. 1984b), but that there was also a direct effect on the ocular surface epithelium (Tsubota et al. 1999a). Since then, it has been discovered that artificial tears do not maintain intracellular ATP levels and epithelial cell membrane integrity compared to AS eye drops (Poon et al. 2001). Key factors that maintain a healthy ocular surface, such as epithelial growth factor (Pastor & Calonge 1992; van Setten et al. 1992), Vitamin A, SP and insulin-like growth factor albumin, a2-macroglobulin and immunoglobulins, which have a bacteriostatic effect, are present in the AS (Poon et al. 2001; Lopez-Garcia et al. 2007). However, AS eye drops contain also the pro-inflammatory cytokines expressed by the leucocytes and the monocytes. The latter together with the uncontrolled presence of immunoglobulins and complement may be deleterious for many patients, especially those suffering from immunological alterations. In addition, there is not one single approach for preparing the AS eye drops since this product still remains like a 'home-made' approach whose predictability, reproducibility and the molecular mechanisms driving their roles still remains unknown. The preparation and storage of AS is a matter of debate as each clinical centre produces its own AS eye drop and provides different preparation and storage protocols (Geerling et al. 2004; Lopez-Garcia et al. 2014).

Table 2. Comparison of human tears and serum.

	Tears	Serum	References
pH	7.4	7.4	Tsubota et al. (1999b), Geerling et al. (2004), López García & del Castillo (2011) and Pan et al. (2013)
Osmolarity	298	296	
EGF (ng/ml)	0.2–0.3 1.9–9.7	0.5	
TGF-β (ng/ml)	2–10	6–33	
Vit A (mg/ml)	0.02	46	
Fibronectin (μg/ml)	21	205	
Lysozyme (mg/ml)	1.4	6	
SIgA (μg/ml)	1190	2	
IGF-I (ng/ml)	157		
SP (ng/ml)	0.157	0.071	
NGF (pg/ml)	468	54	

EGF = epidermal growth factor, TGF-β = transforming growth factor beta, SIgA = surface immunoglobulin A, IGF-I = insulin-like growth factor 1, SP = substance P, NGF = nerve growth factor.

Plasma Rich in Growth Factors

Platelets: from the knowledge to the technology

Platelets contribute to haemostasis by preventing blood loss at sites of vascular injury and contain a large number of growth factors and cytokines that have a key role in tissue regeneration. In the past two decades, an increased understanding of the physiological roles of platelets in wound healing and after tissue injury has led to the idea of using and concentrating platelets as therapeutic tools. Indeed, after fibrin glue was introduced in the early 1990s as a biomaterial with haemostatic and adhesive properties, the strategic modification of the fibrin to include platelets was reported (Gibble & Ness 1990). The initial rationale of platelet-rich products was to replace the blood clot with a preparation enriched in platelets which could, once activated, secrete a large pool of proteins and factors including PDGF, TGF-b, VEGF, IGF-I, hepatocyte growth factor (HGF), angiopoietins, platelet factor-4 (PF-4) and thrombospondin among others to the local milieu, driving the tissue regeneration mechanism.

The proteins and other substances that are provided by platelets and which can participate in tissue repair and healing are mainly stored in their alpha-granules. The proteins contained in these granules are secreted by exocytosis by the formation of secretory vesicles that fuse with the plasma membrane allowing the release of their contents to the milieu (Reed et al. 2000). Interestingly, by getting rid of erythrocytes and leucocytes, the preparation would take full advantage of the plasma and the concentrated platelets and the stored growth factors, without the presence of pro-inflammatory agents released by the leucocytes.

In the last decade, several technologies have translated the power of the plasma and platelet-derived proteins and growth factors to the clinics by means of a predictable technology that can be easily prepared and used. The

technology of plasma rich in growth factors (PRGF) consists of a limited volume of plasma enriched in platelets, which is obtained from the patient. Once the platelet concentrate is activated, a three-dimensional and biocompatible fibrin scaffold is formed, and a myriad of growth factors and proteins are released, progressively, to the local environment, contributing to the acceleration of wound healing and tissue repair (Anitua et al. 2004). Furthermore, the autologous origin of this preparation eliminates concerns about immunogenic reactions and disease transmission (Ogino et al. 2005). 'PRGF is an autologous 100% platelet-rich plasma with some specific and particular characteristics. Unfortunately, it is almost impossible to reach an agreement about a definition for platelet-rich plasma. There are more than 30 different protocols and platelet-rich plasma products reported in the literature, and many of them are also commercialized. The variability of composition, protein content, platelet enrichment, presence or absence of leucocytes, application protocols, etc. have lead to different scientific results using platelet-rich plasmas in both efficacy and safety. PRGF follows a predictable protocol by which platelets are concentrated twofold in the plasma of the patients. Red cells and white cells including leucocytes are discarded and therefore are not present within PRGF. Furthermore, for ophthalmological purposes, the new PRGF eye drops are not diluted as usually happens with AS. Last but not least, the medical devices needed to prepare PRGF are EU and FDA accepted.'

As it has been previously reported, the use of AS eye drops was the first hemoderivative product used in the ophthalmology field (Tsubota et al. 1999a; Geerling et al. 2004; Matsumoto et al. 2004). Their use in the treatment of ocular surface tissue regeneration was attributed to its content in growth factors, which are mainly provided by blood platelet content (Nurden et al. 2008; Blair & Flaumenhaft 2009). However, the presence of leucocytes during AS preparation procedure increases the level of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α, involved in the inflammatory process of some ocular diseases (Pflugfelder et al. 1999; Yoon et al. 2007b).

The pioneering use of PRGF in ophthalmology is mainly based on improving and overcoming some of the main limitations that AS products show. As PRGF does not contain leucocytes but doubles the concentration of platelets, it is expected to have more growth factors and neurotrophic factors but without pro-inflammatory cytokines (Fig. 1). The controlled activation process enables the preparation of different therapeutic formulations ranging from an autologous eye drop to a three-dimensional fibrin scaffold (Fig. 2).

Preclinical studies

Plasma rich in growth factors technology has been successfully used in several medical fields like dentistry and oral implantology, orthopaedics, sport medicine and ulcer treatment among others for promoting wound healing and tissue regeneration (Anitua 1999; Sanchez et al. 2007; Anitua et al. 2008, 2010; Torres et al. 2009; Orcajo et al. 2011).

The use of PRGF in ophthalmology begun with a cost-effective study in which it was observed that it was possible to obtain almost the 4.5 mL of PRGF from every 9 mL of blood. The latter significantly reduces the blood volume needed to prepare the month-to-month medication to the patient (Anitua et al. 2011, 2013a). Recently, Freire et al. (2012)

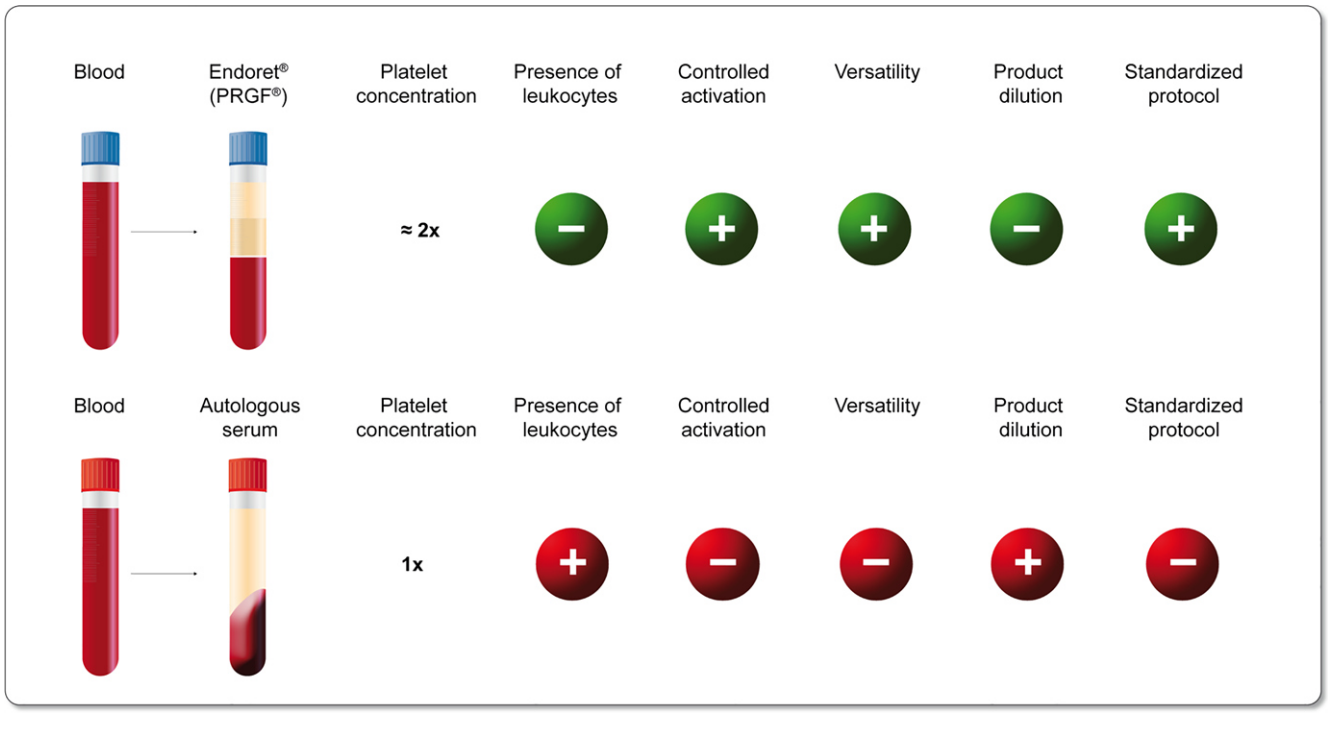

Fig. 1. Differential characteristics of plasma rich in growth factors versus autologous serum. Green ball represents the advantage of one technology against the other.

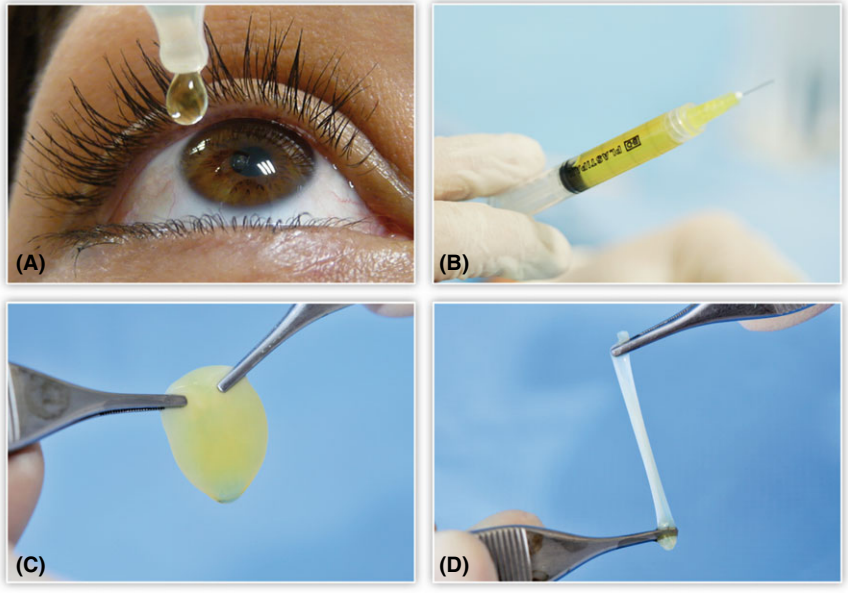

Fig. 2. Different therapeutic fomulations obtained with plasma rich in growth factors (PRGF) technology. (A) PRGF eye drops, (B) injectable PRGF, (C) fibrin scaffold and (D) fibrin membrane.

showed that PRGF eye drops enhance the proliferation of corneal epithelial cells (HCE) compared with other non-activated platelet-based products, suggesting that platelet degranulation is a critical step to enrich the formulation in growth factors and proteins. In this study, it was observed that PRGF upregulated the expression of several genes involved in communication and cell differentiation and significantly improved the biological activity of human corneal epithelial cells compared with AS (Liu et al. 2006; Freire et al. 2012). In another study, it was concluded that PRGF eye drop protects ocular surface tissues against scar formation, reducing the number of SMA-positive cells (myofibroblasts) after TGF-β1 induction (Figs 3 and 4; Anitua et al. 2011). These results were correlated with in '*in vivo*' studies, where mice underwent PRK surgery showing a reduction in scar and haze formation after treatment with PRGF eye drops (Anitua et al. 2013a).

Of particular importance is the dosage and duration of these blood derivatives. Assuming that ocular surface diseases are usually chronic diseases that demand long-term treatments, it is necessary that the biological functionality and stability of the treatments are preserved for weeks or months. PRGF eye drops maintain their protein content and their biological activity potential at least for 3 months after storage at −20 celsius. Moreover, PRGF eye drops can be preserved for their daily use at 4 celsius or room temperature maintaining their composition and biological activity (Anitua et al. 2013b).

Another exciting property of PRGF technology is its versatility. In fact, it is not only an autologous eye drop but also a biocompatible and biodegradable fibrin membrane (Anitua et al. 2007). The fibrin scaffold and membrane can be used in ophthalmology as an autologous sealant or biomaterial to regenerate deep wounds in ocular surface (Anitua et al. 2012).

The promising *in vitro* results obtained with PRGF eye drops have stimulated its use in several animal models of ocular surface diseases. PRGF stimulates ocular surface wound healing, reducing corneal haze formation in mice subjected to PRK surgery. PRGF reduces myofibroblast-transformed cells in the corneal stroma (Anitua et al. 2013a). Tanidir et al. (2010) found similar results in corneal re-epithelization in rabbits. In the same way, successful results were found in a rabbit model of corneal ulcers, showing an acceleration in corneal regeneration, a reduction in ocular inflammation and an improvement in collagen fibre arrangement in the corneal stroma compared to the control group (Khaksar et al. 2013). Last but not least, the fibrin membrane has also been used as a bio-adhesive in preclinical studies, showing successful results in attaching the corneal flap in the lamellar keratoplasty (Luengo Gimeno et al. 2006, 2010).

Clinical application of PRGF eye drops

The positive results obtained with PRGF *in vitro* stimulated the evaluation of the approach in different ocular surface injuries, including dry eye, GVHD and corneal ulcers, among others.

Dry eye

Although dry eye is seen in young adults, its prevalence increases with age. About 15% of adults over the age of 40 have dry eye, and the prevalence of dry eye increases above 19% in people over 80 years old. Women are affected about 1.5 times more frequently than men. Dry eye is a disorder of the tear film caused by an alteration in the amount of tears and/or the composition of it. Artificial tears are the first standard treatment for this disease, but often it is not plenty for the proper management of this pathology. Ideal artificial tears should emulate the physicochemical properties of natural tears, in addition to having the highest possible retention capacity. They should also allow the addition of biological agents, which may be necessary for the metabolism of the cells of the ocular surface.

Over recent years, the biological outcomes of PRGF eye drops on dry eye syndrome treatment have been evaluated in two prospective observational studies (Alio et al. 2007b; Lopez-Plandolit et al. 2011). A total of 34 patients who presented a moderate or severe dry eye syndrome were included in these studies. PRGF technology was applied topically 4–6 times a day per eye for 1–3 months. After PRGF treatment, a total of 82% of the patients showed relevant improvement or full disappearance of symptoms. No cases of poor tolerance or undesirable effects that could be attributed to the PRGF use were observed. On the contrary, it was observed that PRGF eye drops reduced significantly the inflammation in 89% of cases, and fluorescein staining improved significantly in 72% of the cases, indicating an improvement

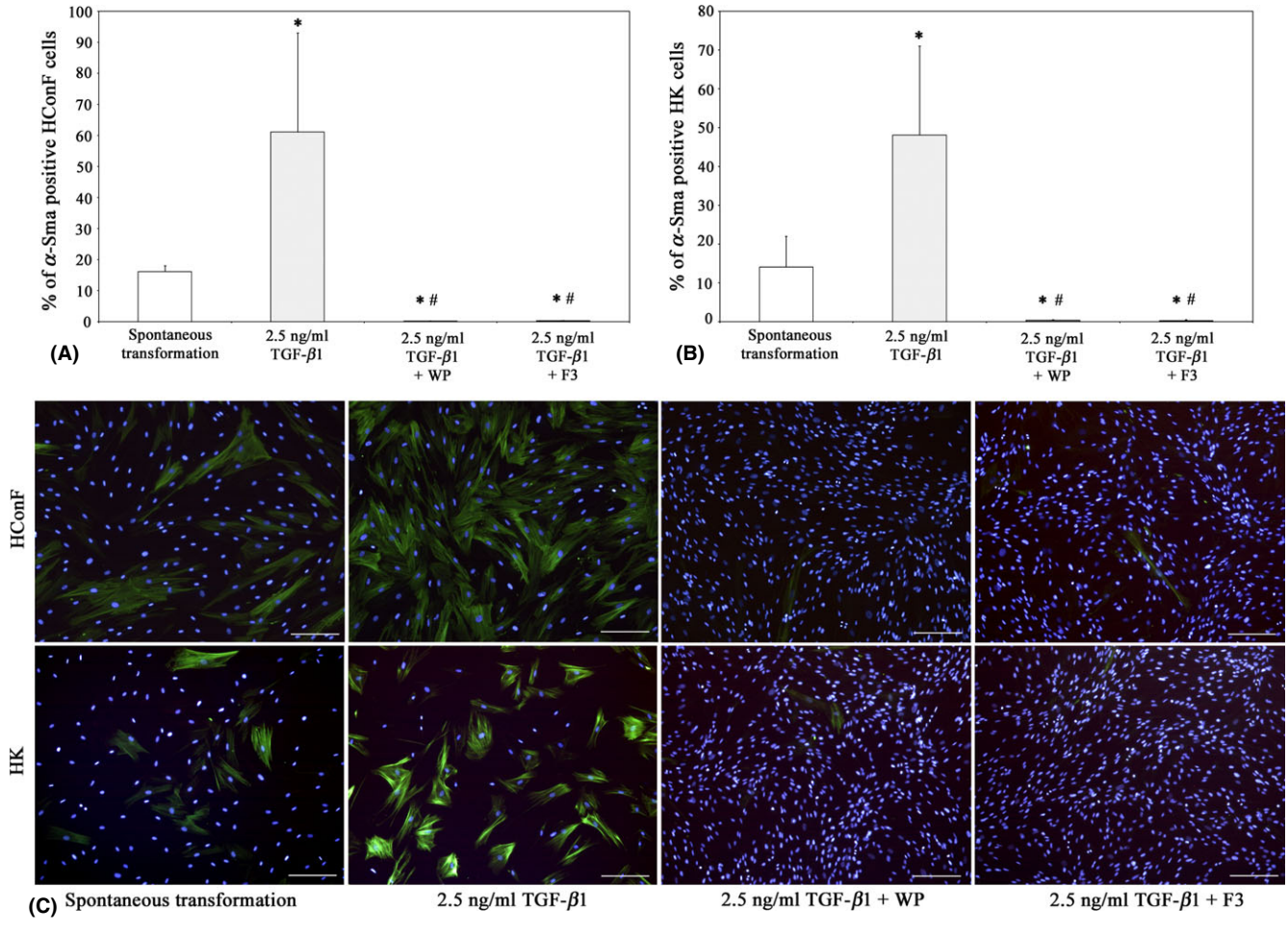

Fig. 3. Protective effect of plasma rich in growth factors (PRGF) against myofibroblast transformation of (A) conjunctival fibroblasts (HConF) and (B) keratocytes (HK). The number of α-SMA-positive cells was significantly lower compared with the TGF-β1 treatment group. (C) Immunofluorescence for the detection of α-SMA protein in HConF- and HK-cultured cells, showing a significant reduction of positive staining in cells treated with PRGF compared to TGF-β1. WP: the whole column of PRGF, which is the final dosage used to prepare the eye drops; F3: the lowest millilitre of PRGF over the buffy coat, which in general contains more platelets. No differences were observed between both PRGF formulations (Anitua et al. 2011). *Statistically significances with regard to spontaneous transformation ($p < 0.05$). # Statistically significances with respect to 2.5 ng/ml TGF-β1 ($p < 0.05$).

in punctate keratitis (Alio et al. 2007b).

Graft-versus-host disease

Ocular GVHD affects approximately 60–80% of patients with cGVHD (Franklin et al. 1983; Arocker-Mettinger et al. 1991). Keratoconjunctivitis sicca (KCS), or dry eye associated with cGVHD, is one of the main complications after allo stem cell transplantation. KCS has a significant impact on patient's life quality and can lead to blindness. Although several therapies including artificial tears, therapeutic contact lenses, punctual plugs, topical or systemic corticosteroids, and other immunosuppressive drugs have been used to minimize the symptoms of dry eyes associated with cGVHD, an effective treatment has not been established (Rocha et al. 2000; Ogawa et al. 2001).

Previous results have shown that PRGF can be applied to the treatment of severe dry eye in patients from different aetiopathologies like Sjögren's syndrome (Lopez-Plandolit et al. 2011). To test its effects in ocular disorders from GVHD patients, Pezzotta et al. (2012) treated a total of 23 patients with refractory GVHD (grade II–IV) unresponsive to conventional treatments. The results showed that 74% of patients (17 of 23) were classified as responders, showing an improvement in the dry eye symptoms. Photophobia was the best symptom improvement (82.6% of patients). Clinical manifestations also improved significantly, showing an improvement in the tear break-up time and fluorescein corneal staining of 86.9% and 69.6%, respectively. These results suggest that this autologous approach may be considered an alternative to the treatment of ocular surface disorders in GVHD patients.

Persistent epithelial defects

Persistent epithelial defects are defined as lesions that measure more than 2 mm in diameter and persist for more than 2 weeks, and are resistant to conventional treatments (Tsubota et al. 1999b). Tear surface and neurogenic dysfunctions are the two main causes of PED, although its etiopathology is very variable (Lopez-Garcia et al. 2007). Other situations that may provoke a PED include burns, immunological factors, infections, dystrophies of the epithelium, metabolic alterations and trauma (Tsubota et al. 1999b; Vajpayee et al. 2003; Lopez-Garcia et al. 2007). Unfortunately, conventional treatments including

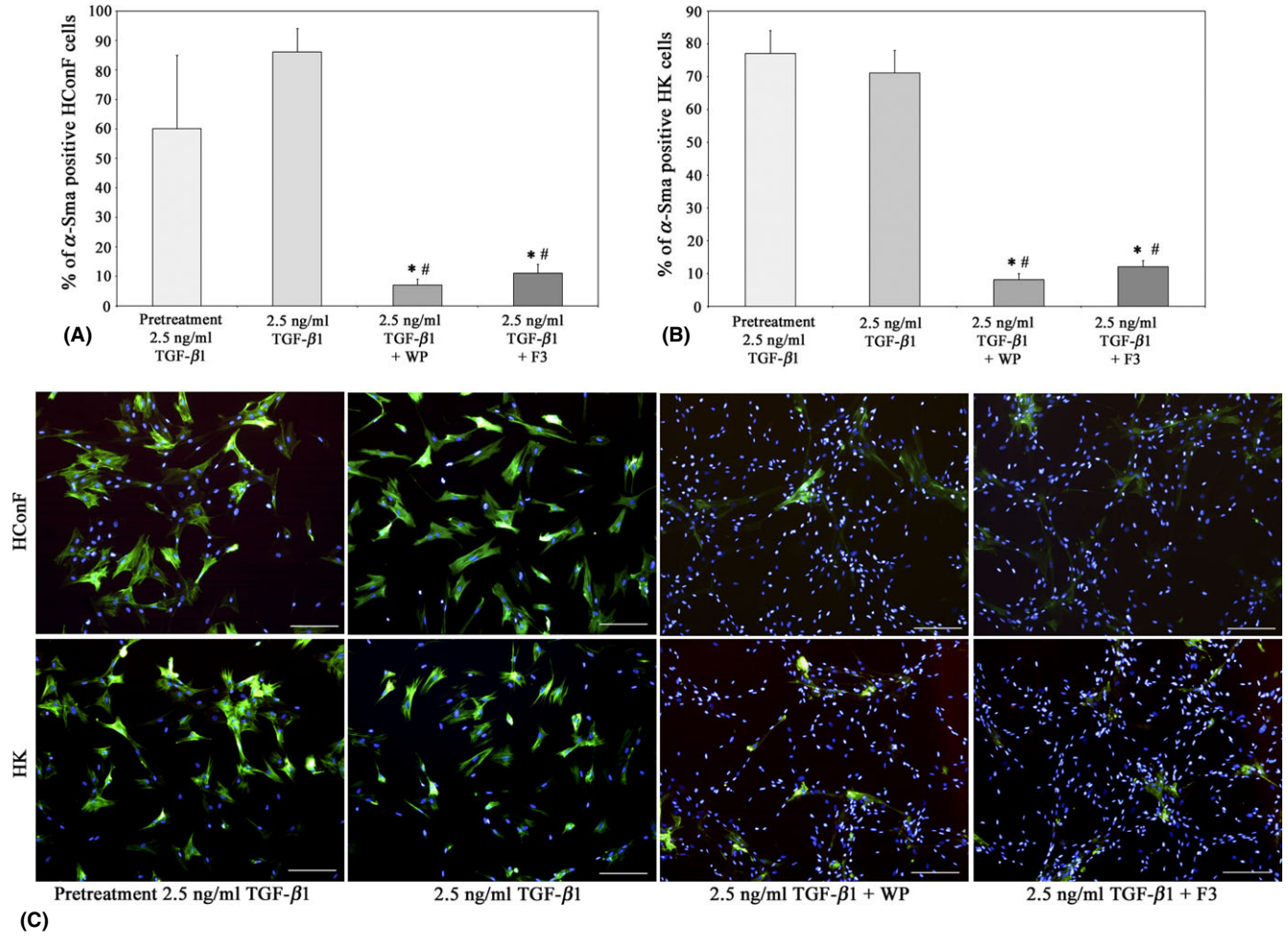

Fig. 4. Plasma rich in growth factors (PRGF) reverts the myofibroblastic phenotype. HConF (A) and HK (B) cells were treated with 2.5 ng/ml TGF-β1 for 3 days to promote differentiation to myofibroblasts. Significant differences were found between the responses induced by PRGF treatment with respect to starting point (2.5 ng/ml). (C) Representative images of α-SMA immunofluorescence showing positive cells before and after treatment with PRGF. No differences were observed between WP and F3 PRGF formulations (Anitua et al. 2011). *Statistically significances with regard to pretreatment with 2.5 ng/ml TGF-β1 ($p < 0.05$). # Statistically significances with respect to 2.5 ng/ml TGF-β1 ($p < 0.05$).

artificial tears, therapeutic contact lenses, tarsorrhaphy, anti-inflammatory agents and oral antibiotics do not improve PED symptomatology and resistant PEDs could degenerate, leading to progressive stromal lysis and subsequent perforation.

The effect of PRGF on PEDs was evaluated by means of a prospective study in 18 eyes (Lopez-Plandolit et al. 2010). Results showed full recovery of the epithelial defect in 85% of cases (17 of 20 eyes). The tolerance to PRGF eye drops treatment was good in 95% of cases (19 of 20). Only one case showed discomfort to PRGF treatment showing redness and itching. No other complications associated with its use were detected. In another retrospective and non-randomized comparative study in patients with PED after infectious keratitis, Kim et al. (2012) reported similar successful results after using PRGF eye drops. In fact, all patients treated with PRGF achieved complete re-epithelialization, while only 77% of patients completed the corneal re-epithelialization after treatment with AS.

Corneal ulcers

A corneal ulcer can be defined as an erosion of the outer layer of the ocular surface, which is often caused by infection, but also by foreign bodies, abrasions, severe dryness and allergic or inflammatory eye conditions. Depending on its aetiology, initial treatment of ocular ulcers relies on accelerating tissue regeneration and reducing the risk of infection and the formation of scar tissue that impairs vision.

The most severe eye ulcers are usually treated with amniotic membranes, which give support to those cells that will colonize the ulcer, and are also treated with growth factors that will promote corneal regeneration. The main disadvantage of the amniotic membrane is that it has heterologous origin and therefore may present biosafety risks. The use of an autologous tissue would be highly desirable for the treatment of corneal ulcers, mainly on corneal perforations. PRGF provides also a fibrin scaffold that can be use as membrane in ocular ulcers (Marquez De Aracena Del Cid & Montero De Espinosa Escoriaza 2009; Geremicca et al. 2010; Panda et al. 2012). In general, the use of PRGF is associated with a reduced healing and epithelization time of the cornea and conjunctiva, with better corneal clarity and best-corrected visual acuity (Marquez De Aracena Del Cid & Montero De Espinosa Escoriaza 2009). A recent study ($n = 38$) showed that 92% of patients with dormant corneal ulcers

improved significantly, reduced inflammation and decreased ocular pain after PRGF treatment (Alio et al. 2007a).

Several studies have evaluated the potential benefits of PRGF-derived fibrin membrane alone (Alio et al. 2013b) or in combination with other membranes like amniotic membrane (Alio et al. 2007a) or Tutopach (Alio et al. 2013a). In all studies, a stable closure of corneal perforation was observed in all patients treated with PRGF fibrin. Furthermore, no evidence of infection, inflammation or pain was observed in any patients treated with PRGF membranes.

Other applications

The use of PRGF in the ophthalmology field has been successfully extended to other ocular surface disorders including the treatment of ocular surface syndrome (Alio et al. 2007c) and flap necrosis (Rocha et al. 2007) after LASIK surgery. A recent study observed that administration of plasma- and platelet-derived proteins adjacent to the lacrimal gland restored the lacrimal function in all patients (Avila 2014). In addition, significant improvement in lacrimal volume, an increase in tear break-up time and a decrease in ocular staining after PRGF treatment were observed.

Future trends and perspectives

Initial studies evaluating blood-based derivatives show efficacy and safety in the treatment of many different ocular surface disorders. However, it is necessary to carry out randomized clinical trials to evaluate and compare properly the potential of these technologies. To succeed, it is mandatory to provide standardized technologies that enable us to fabricate the same type of blood formulations. The development and optimization of PRGF could provide some light on this topic. Furthermore, a more intense basic research focused on the mechanisms of actions of these blood therapies may also help us to better understand how they work and how we can optimize their therapeutic use.

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Autologous Plasma Rich in Growth Factors Eyedrops in Refractory Cases of Ocular Surface Disorders

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Key Words
Ocular surface disorders · Plasma rich in growth factors · Platelet-rich plasma

Abstract
Purpose: Preliminary information about the safety and efficacy of plasma rich in growth factors (PRGF) eyedrops in the treatment of refractory cases of diverse ocular surface disorders (OSDs) is presented here. ***Material and Methods:*** This retrospective cohort study included cases with OSDs refractory to previous treatment with conventional treatments or autologous serum or cyclosporine, and treated with PRGF eyedrops. The signs and symptoms of ocular surface disorders [using the ocular surface disease index (OSDI), best-corrected visual acuity (BCVA), visual analog scale (VAS) frequency and VAS severity] were evaluated before and after treatment with PRGF. A safety assessment was also performed reporting all adverse events or complications. ***Results:*** Forty-one patients with a total of 80 treated eyes were evaluated. Statistically significant reductions in the OSDI scale (39.27%), VAS frequency (38.9%) and VAS severity (40.3%), and a significant improvement in BCVA (54.86%) were all observed ($p < 0.05$). The results were stratified according to the identified potential effect modifiers. There were only two adverse events (eye redness and eyelid inflammation), which were reported as mild and resolved in a few days. ***Conclusions:*** PRGF eyedrops could be a safe and effective treatment option for refractory cases of OSDs. When treating patients the possible influence on the results of some clinical variables must be taken into account.

Introduction

The ocular surface covers the cornea, conjunctiva and limbus with a stratified, squamous, nonkeratinizing epithelium joined to an underlying connective tissue stroma. Its proper functioning depends on a number of different systems that contribute to their physiological integrity. The ocular surface is very vulnerable to possible external aggravations by the nature of its duties and its anatomical location on the eye [1].

The ocular surface requires regular cleansing with tears to provide an optimum comfort and a clear vision.

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Any alteration or reduction in the production of any component of the tear film, especially in the lipid layer, or any interference with the tear recovering process, can negatively affect the physiological functions and structure of the ocular surface.

Ocular surface disorders (OSDs) include abnormal lid anatomy or function, abnormal or altered tear production or composition, and related symptoms and clinical or subclinical signs of damage to their structure. These pathologies may lead to noticeable irritation, a reduction of visual function, and even chronic tissue changes. Additional consequences of chronic compromise to the ocular surface include a risk of infection and chronic inflammation that may not respond to conventional treatments. OSDs can severely affect the quality of life of patients and their evaluation must include a carefully detailed patient history, assessment of associated risk factors and examination of the anterior ocular structures and their functions.

Even though artificial tears are used as the conventional treatment for OSDs through the provision of additional lubrication [2], they fail to mimic the composition of natural tears, which contain a complex composition of water, salts, hydrocarbons and proteins. Ocular inserts, lacrimal drainage point occlusion and anti-inflammatory drugs are other therapeutic alternatives. Topical corticosteroids have been shown to improve symptoms [3], but their use is limited due to long-term side effects, including cataracts and increased intraocular pressure [4, 5]. Cyclosporine A solution has also been recently proposed as an ocular therapeutic option for OSDs [6]. Its use to treat ocular conditions has been met with somewhat mixed results in the treatment of nonspecific, noninfectious inflammatory disease [6], and its use is not exempt from some adverse events, predominantly ocular burning (16%), but also stinging, itching, conjunctival hyperemia, photophobia, blurred vision, headache, eyelid edema and pain (1–3%).

Autologous serum (AS) eyedrops have been reported to be effective for the treatment of a variety of ocular surface diseases [5, 7–10]. Due to significant variations in elaboration procedures, production and storage regimens and treatment protocols, the efficacy of AS eyedrops in OSDs has varied substantially between studies. The critical steps in the production of AS, such as clotting time, centrifugation and dilution can influence the biochemical properties of AS and may lead to variable efficacy and treatment outcomes [5]. Although cyclosporine and AS are commonly used in these pathologies, they are not effective in a considerable percentage of patients without a specific cause.

Recently, a new type of standardized autologous plasma and platelet-based technology has been reported. Plasma rich in growth factors (PRGF) represents a number of formulations, including an eyedrop that is rich in proteins and growth factors and has a long-term biological stability [11–14]. PRGF contains a large number of biologically active agents, including EGF, PDGF, IGF, fibronectin and vitamin A [14], and a comfortable dosage that allows PRGF eyedrop dispensers to be used for 72 h at room temperature, and the solution for a total of 3 months.

Our hypothesis is that, due its biological composition and properties, PRGF could be an effective therapeutic alternative in OSD cases that do not respond to conventional treatment. The present retrospective study aims to provide preliminary information about the safety and efficacy of PRGF eyedrops in the treatment of patients suffering from OSDs that did not previously respond to AS or cyclosporine treatment, and to analyze the influence of certain variables on treatment outcomes.

Material and Methods

A retrospective design was used in this study and all the principles of the Declaration of Helsinki were fulfilled. The study included patients sequentially diagnosed at Ophthalmological Institute Fernández-Vega (Oviedo, Spain) with OSDs from various etiologies (table 1).

The following criteria need to be met for the diagnosis of OSD: Schirmer test less than 5 mm (the measurement of tear secretion requires about 5 min, allowing for natural blinking), tear film breakup time less than 5 s, and the relevant severity of associated subjective symptoms. To be included in the study, patients with OSDs must have met the following criteria: diagnosis of a severe OSD that did not respond (with poor or no response) to conventional treatment (artificial tears, topical antibiotics and steroids, oral antibiotics or antiviral drugs, therapeutic contact lenses, occlusion tear knit caps), or AS and/or cyclosporine.

All patients signed an informed consent before beginning PRGF therapy. The included patients were treated between May, 2011, and September, 2013, with one initial cycle of 6 consecutive weeks of treatment with PRGF eyedrops. In cases where a poor or no initial response was observed, an additional 2–4 cycles of treatments were administered. Patients were encouraged to instill one PRGF drop in the eye conjunctival sac, four times a day. Both the demographic (gender, age) and clinical variables of patients (systemic diseases, previous and concomitant treatments, etc.) were collected. All necessary data were obtained from patient clinical records.

PRGF Preparation

PRGF eyedrops were obtained using the Endoret (PRGF) ophthalmology kit (BTI Biotechnology Institute SL, Vitoria, Spain). Briefly, blood was collected into 9-ml tubes and centrifuged at 580 *g* for 8 min, the whole plasma column was drawn off avoiding

Table 1. Patient demographics and baseline characteristics of cases

		n (%)
Gender (n = 41 patients; 100%)	Male	32 (78.0)
	Female	9 (22.0)
Age group (n = 41 patients; 100%)	18–65 years	25 (61.0)
	>65 years	16 (39.0)
OSD (n = 80 eyes; 100%)	Evaporative dry eye	22 (27.5)
	Sjögren-associated dry eye	18 (22.5)
	Traumatic glaucoma	6 (7.5)
	Ocular rosacea	6 (7.5)
	Neurodeprivative dry eye	6 (7.5)
	Herpes keratitis	5 (6.3)
	Adenovirus keratitis	4 (5.0)
	Psoriasis dry eye	4 (5.0)
	Rheumatoid arthritis dry eye	2 (2.5)
	Production deficit dry eye	2 (2.5)
	Exposition due to dry eye	2 (2.5)
	Corneal dystrophy	1 (1.3)
	Adenovirus sequels	1 (1.3)
	Recurrent corneal ulcer	1 (1.3)

the buffy coat containing the leukocytes and incubated at 37°C for 1 h; finally, the released supernatants were collected by aspiration, filtered, aliquoted and stored at –80°C until use.

All procedures were performed under highly sterile conditions, operating inside a laminar flow hood. Before initiating the treatment, the patients were instructed to keep the bottles at –20°C for a maximum of 3 months; the bottle in use was to be stored at 4°C and used for 5–7 days. The solution was to be applied 4 times per day.

Outcome Measures

The ophthalmologic evaluation was conducted before treatment with PRGF, in successive visits to the center (ranging from 2 to 6 weeks) and at the end of the treatment. Treatment response was determined by a combination of clinical examination (slit lamp) and subjective OSD scales [ocular surface disease index (OSDI), visual analog scale (VAS)], and the best-corrected visual acuity (BCVA) was additionally determined using a Snellen optotype (conversion to logMAR scale-logarithm of the minimum angle of resolution). The evaluation of ocular surface symptoms was performed using the OSDI scale, which estimates the severity of dry-eye symptoms in a range from 0 to 100, and with the VAS, which is a 100-point scale in which 0 = no discomfort and 100 = maximal discomfort (dryness, burning/stinging, photophobia, foreign body sensation, blurred vision, itching and pain), both in the frequency and severity of symptoms. All measurements were recorded as baseline values and after treatment with PRGF. A safety assessment was performed recording and evaluating any adverse event or complication that occurred during therapy with PRGF.

Statistical Analysis

Descriptive statistics were performed using absolute and relative frequency distributions for qualitative variables, and mean values and standard deviations for quantitative variables. Different normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) were performed on each variable sample.

Variables that showed differences among them in the baseline values and the variables with a potentially modified effect on outcome measures were identified by the statistical test required on each case. These variables were taken into account when analyzing the results before and after treatment with PRGF, stratifying them accordingly. Any potential difference observed between baseline and after PRGF treatment was analyzed using the nonparametric Wilcoxon statistical test. The level of statistical significance was set at $p < 0.05$. The statistical software package SPSS v15.0 for Windows (SPSS Inc., Chicago, Ill., USA) was used for all statistical analyses.

Results

Forty-one patients with a total of 80 eyes were included and evaluated in the present study; 32 were women (78.0%) and 9 were men (22.0%). The mean age of the patients was 55.71 years (SD 15.90, range 25–90 years). Most of the cases were bilateral (39 of 41 patients), while 2 patients were treated unilaterally since the pathology only affected one of their eyes. Of the 80 eyes treated, a total of 41 (51.3%) were right eyes and 39 (48.8%) were left eyes. The most frequent ocular surface pathologies were evaporative dry eye, with 22 treated cases (27.5%), and dry eye associated with Sjögren syndrome (18 cases, 22.5%; table 1).

The majority of patients (63.8%) received one or two cycles of PRGF (1 cycle = 6 weeks of treatment), while 36.3% received 3 or 4 cycles of PRGF. High blood pressure (HBP) was recorded in 23.8% of the cases, and in 8 cases rheumatoid arthritis was present (10.0%). Sjögren syndrome was the cause of the pathology in 22.5% of the cases.

Thirty-nine cases had been treated previously with AS (48.8%), 51 (63.8%) with cyclosporine and 44 (55.0%) with corticosteroids, all without improvement. In addition, 11 cases (13.8%) had been treated previously with both AS and cyclosporine. A total of 46 (57.5%) cases received corticosteroids (dexamethasone 1 mg/ml, or fluorometholone 1 mg/ml, eyedrops, 2–3 times a day) concomitantly with PRGF due to disease severity.

The results of the primary outcome measures for the entire study population are summarized in figure 1. A significant reduction in the OSDI scale was observed from a baseline value of 49.58 (SD 18.04) to a final post-

Table 2. Results stratified by groups of BCVA outcome measure before and after treatment with PRGF

		BCVA baseline	BCVA final	BCVA change	p value	n (eyes)
Gender	male	0.24±0.37	0.13±0.22	–33.16*	0.008	17
	female	0.28±0.39	0.18±0.41	–60.72	0.000	63
Age	18–60 years	0.21±0.32	0.12±0.29	–59.33	0.000	49
	>60 years	0.36±0.45	0.24±0.49	–47.80	0.000	31
HBP	yes	0.30±0.37	0.17±0.30	–37.06*	0.001	19
	no	0.26±0.39	0.16±0.41	–60.41	0.000	61
Sjögren syndrome	yes	0.25±0.24	0.10±0.14	–65.83	0.001	16
	no	0.27±0.41	0.18±0.42	–51.12	0.000	64
Previous treatment corticosteroids	yes	0.31±0.42	0.19±0.43	–47.36	0.000	44
	no	0.22±0.33	0.13±0.31	–64.04	0.000	36
Previous treatment autologous serum	yes	0.35±0.47	0.26±0.51	–53.11	0.000	39
	no	0.19±0.27	0.08±0.14	–56.54	0.000	41
Previous treatment cyclosporine	yes	0.20±0.29	0.09±0.22	–62.31	0.000	51
	no	0.39±0.49	0.29±0.55	–41.76	0.002	29
Concomitant treatment corticosteroids	yes	0.26±0.41	0.15±0.41	–54.49	0.000	46
	no	0.28±0.35	0.18±0.33	–55.37	0.000	34

* Statistically significant difference between groups ($p < 0.05$).

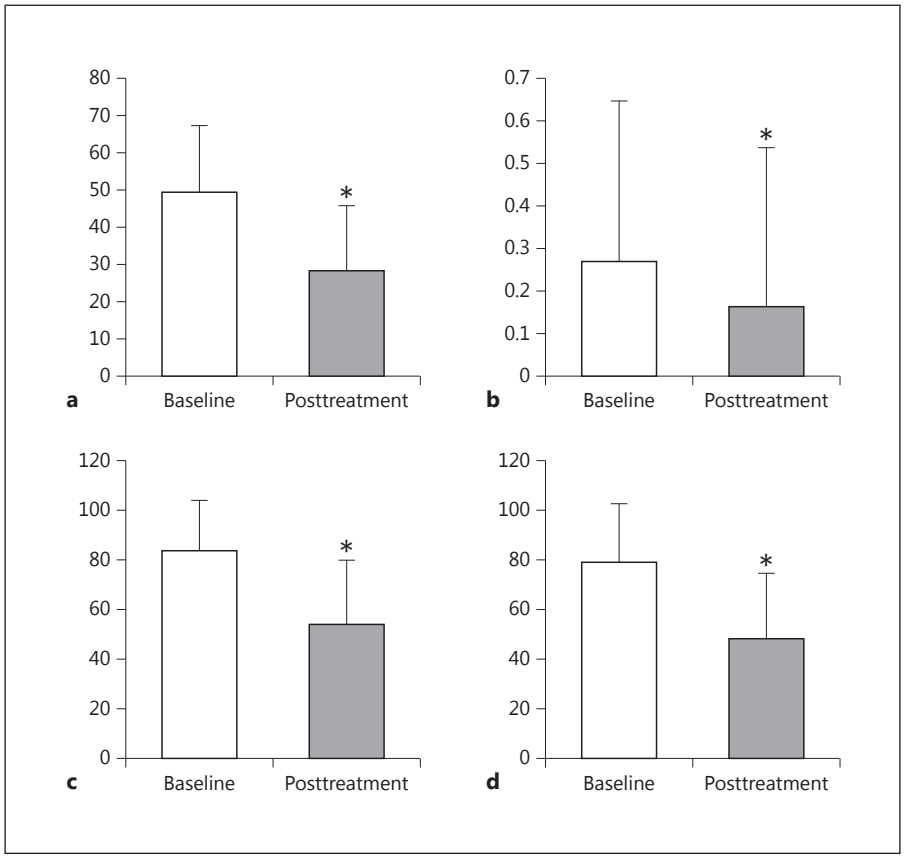

Fig. 1. Results of the primary outcome measures for the entire study population: OSDI (**a**), BCVA (**b**), VAS frequency (**c**) and VAS severity (**d**). * Statistically significant ($p < 0.05$).

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Table 3. Results stratified by groups of VAS frequency outcome measure before and after treatment with PRGF

		VAS frequency baseline	VAS frequency final	VAS frequency change	p value	n (eyes)
Gender	male	66.5±27.0	40.6±30.1	–46.1	0.000	17
	female	88.4±16.0	56.8±25.6	–37.0	0.000	63
Age	18–60 years	81.6±23.4	50.0±28.9	–42.6	0.000	49
	>60 years	87.1±15.5	58.7±23.9	–33.0	0.000	31
HBP	yes	90.3±25.0	65.8±33.0	–29.0*	0.001	19
	no	81.7±19.1	49.5±24.2	–42.0	0.000	61
Sjögren syndrome	yes	92.5±8.6	54.38±27.0	–42.6	0.001	16
	no	81.6±22.3	53.1±27.5	–38.0	0.000	64
Previous treatment corticosteroids	yes	82.7±23.6	48.9±29.5	–44.5*	0.000	44
	no	85.0±16.9	58.9±23.6	–32.1	0.000	36
Previous treatment autologous serum	yes	90.5±15.8	61.7±23.4	–32.7*	0.000	39
	no	77.3±23.0	45.49±28.5	–44.8	0.000	41
Previous treatment cyclosporine	yes	80.7±21.9	49.8±29.5	–41.7	0.000	51
	no	89.1±17.8	59.7±21.8	–34.0	0.000	29
Concomitant treatment corticosteroids	yes	86.3±18.4	54.4±25.1	–39.1	0.000	46
	no	80.3±23.5	52.1±30.1	–38.7	0.000	34

* Statistically significant difference between groups ($p < 0.05$).

treatment value of 28.32 (SD 17.76). The latter represents a total reduction of 39.27%, which was statistically significant ($p < 0.05$; fig. 1a). The BCVA was improved from a baseline value of 0.27 (SD 0.38) to a final posttreatment value of 0.16 (SD 0.38), representing an increase of 54.86% ($p < 0.05$; fig. 1b).

Regarding the OSD symptoms (discomfort, dryness, burning/stinging, photophobia, foreign body sensation, blurred vision, itching and pain) measured by VAS score frequency, a significant decrease was measured ranging from a baseline value of 83.75% (SD 20.77) to a final posttreatment value of 53.38% (SD 27.21). This represented a reduction of more than 38% and was statistically significant ($p < 0.05$; fig. 1c). The VAS score of symptom severity resulted in a relevant and significant decrease of more than 40%, from a baseline value of 79.0% (SD 23.57) to a final posttreatment value of 47.88% (SD 26.76; fig. 1d).

Results from the primary outcome measures were stratified according to the variables identified as potential effect modifiers. These variables include gender, age, HBP (defined as BP ≥140/90 mm Hg), presence of Sjögren syndrome, previous treatment with cyclosporine, AS or corticosteroids, and concomitant treatment with corticosteroids.

As it is shown in table 2, in the case of BCVA, significantly better results ($p < 0.05$) were observed in female patients and in patients without HBP. Interestingly, stratification of the VAS scale both in frequency and severity led to improved outcomes ($p < 0.05$) also in those cases without HBP, patients treated previously with corticosteroids and in cases not previously treated with AS (tables 3, 4). Finally, regarding the OSDI scale, significantly better results were observed in patients with Sjögren syndrome and in patients receiving treatment with corticosteroids (table 5).

Two adverse events (2.5%) were reported during the study, as shown in table 6. These two events (eye redness and eyelid inflammation) were registered as mild and were resolved in a few days.

Discussion

A considerable percentage of OSD cases do not respond to standard treatment, and there are currently no truly effective therapeutic alternatives for these patients. Blood-derived eyedrops are well known in ophthalmology and have been used in the treatment of several pa-

Table 4. Results stratified by groups of VAS severity outcome measure before and after treatment with PRGF

		VAS severity baseline	VAS severity final	VAS severity change	p value	n (eyes)
Gender	male	61.2±28.2	35.3±27.9	–46.6	0.000	17
	female	83.8±19.8	51.3±25.6	–38.6	0.000	63
Age	18–60 years	76.9±23.4	45.7±27.4	–43.4	0.000	49
	>60 years	82.3±23.9	51.3±25.8	–35.4	0.000	31
HBP	yes	87.6±25.1	57.9±33.0	–36.0	0.001	19
	no	76.3±22.6	44.8±24.0	–41.6	0.000	61
Sjögren syndrome	yes	87.5±13.4	51.9±26.3	–41.1	0.001	16
	no	76.9±25.1	46.9±27.0	–40.1	0.000	64
Previous treatment corticosteroids	yes	80.0±24.8	42.1±29.1	–50.0*	0.000	44
	no	77.8±22.3	55.0±22.0	–28.4	0.000	36
Previous treatment autologous serum	yes	83.3±23.3	54.8±26.9	–34.1*	0.000	39
	no	74.8±23.3	41.3±25.3	–46.1	0.000	41
Previous treatment cyclosporine	yes	78.7±22.5	46.7±27.1	–42.7	0.000	51
	no	79.5±25.8	50.0±26.6	–36.1	0.001	29

* Statistically significant difference between groups ($p < 0.05$).

Table 5. Results stratified by groups of OSDI scale outcome measure before and after treatment with PRGF

		OSDI baseline	OSDI final	OSDI change	p value	n (eyes)
Gender	male	45.3±18.0	21.5±12.7	–50.4	0.000	17
	female	50.7±18.0	30.17±18.6	–36.2	0.000	63
Age	18–60 years	52.7±20.0	31.2±20.6	–35.5	0.000	49
	>60 years	44.6±13.3	23.8±10.8	–45.2	0.000	31
HBP	yes	42.2±20.9	30.0±27.0	–19.1	0.019	19
	no	51.9±16.6	27.8±14.0	–45.6	0.000	61
Sjögren syndrome	yes	57.1±12.6	26.3±9.6	–54.1*	0.000	16
	no	47.7±18.8	28.8±19.3	–35.6	0.000	64
Previous treatment corticosteroids	yes	48.15±16.5	26.7±18.1	–38.2	0.000	44
	no	51.3±19.9	30.3±17.4	–40.5	0.000	36
Previous treatment autologous serum	yes	54.7±18.4	32.6±19.0	–35.9	0.000	39
	no	44.7±16.5	24.3±15.7	–42.5	0.000	41
Previous treatment cyclosporine	yes	47.7±18.0	26.1±16.1	–45.9	0.000	51
	no	52.8±17.9	32.1±20.1	–27.6	0.001	29
Concomitant treatment corticosteroids	yes	50.4±14.9	23.4±9.7	–51.9*	0.000	46
	no	48.5±21.8	35.0±23.4	–22.2	0.000	34

* Statistically significant difference between groups ($p < 0.05$).

Table 6. Adverse events

Adverse event	Frequency, cases	%
Eye redness	1	2.4
Eyelid inflammation	1	2.4
Total	2	4.8

thologies of the ocular surface. Optimal and consistent clinical results are directly related to the preparation and composition of these products [8], as well as the assurance of their biological stability after preparation. The poor standardization of preparation protocols, and consequently of blood-based eyedrops, including AS has resulted in inconclusive and controversial results [15].

PRGF represents a new technology that uses autologous proteins, growth factors and biomaterials as therapeutic formulations for different regenerative purposes. Under strict pharmaceutical development, it is possible to create biologically stable eyedrops that can be used for the treatment of several diseases of the ocular surface [11–14].

Recent data suggest that the pool of molecules playing a role in PRGF eyedrops is extensive, including trophic factors [16], anti-inflammatory agents [17] and bacteriostatic/bactericide molecules [18, 19]. It has been reported that PRGF significantly enhances the proliferation and migration of both keratocytes and conjunctival fibroblasts. In addition, it prevents and inhibits TGFβ1-induced myofibroblast differentiation [11], suggesting that it may have a role in myofibroblast modulation in the stroma of the ocular surface tissues. In a mouse model of corneal lesions, PRGF promoted wound healing after excimer laser photoablation, reducing corneal haze formation [12]. In another in vitro study, the biological outcomes of AS eyedrops versus PRGF eyedrops on corneal stromal keratocytes and conjunctival fibroblasts were evaluated and compared [20]. The potential of PRGF and AS in promoting wound healing was tested by means of proliferation and migration assay, and the ability of each to prevent and inhibit TGFβ1-induced differentiation was also evaluated. The results showed significantly higher levels of all growth factors in PRGF eyedrops compared to AS. Moreover, PRGF eyedrops significantly enhanced the biological outcomes of both corneal stromal keratocytes and conjunctival fibroblasts, and reduced TGFβ1-induced myofibroblast differentiation in contrast to AS eyedrops.

AS may contain circulating antibodies that can create deposits of immune complexes in the cornea and increase inflammatory mechanisms in autoimmune diseases [8]. Other problems exist with ASA, such as stability, risk of infection, standardization in production, lack of quality control and storage problems.

Results from the present retrospective study indicate that the use of PRGF eyedrops in patients suffering with OSDs who were unsatisfactorily treated with AS or cyclosporine led to a clinically significant improvement in all the efficacy outcome measures. These effects were observed for both OSDs related to autoimmune diseases as well as for inflammatory or infectious pathologies.

VAS data for both frequency and severity suggest that PRGF eyedrops may moderate pain perception in patients. The hypothesis that this biological therapy exerts an 'analgesic' effect is not new. In fact, it has been shown that plasma and platelet-based formulations show a potent antinociceptive activity linked, at least in part, to their endocannabinoids and related compound content, and to their ability to elevate the levels of these lipid mediators in cells [21]. Improvement in pain perception and in life quality has been reported in several randomized clinical trials, such as pain associated with knee osteoarthritis [22–24], postoperative pain associated with molar tooth extractions [25], and in some oral surgery procedures such as elevation of the maxillary sinus [26]. Moreover, this 'analgesic' effect in OSDs may also be related to the NF-kB pathway-mediated anti-inflammatory effects of PRGF [17, 27].

Analyzing the results observed in this study, and taking into account the modifying effects identified in certain variables, it is noteworthy that in female patients without HBP and without pretreatment with topical corticosteroids or concomitant treatment with cyclosporine, PRGF provides a significant improvement in BCVA ($p < 0.05$). Surprisingly, patients with Sjögren syndrome showed a greater improvement in OSDI scale than the other subjects ($p < 0.05$), even with associated HBP, which is a very frequent associated pathology in these patients [28]. This may be due to the absence of circulating antibodies in PRGF that could interfere with the mechanism of inflammatory disease, which may occur using AS. Concomitant treatment with corticosteroids seems also to improve OSDI values significantly after PRGF treatment, possibly by a synergic effect.

A further interesting observation was that women generally responded better to treatment with PRGF (although not statistically significantly) compared to men. This could also be explained by the increased presence of au-

toimmune diseases in women as a cause of OSD, which seem to respond better to treatment with PRGF for the above-stated reasons. It could also be related to a greater adherence to treatment amongst women compared to men. Globally, significant differences in all signs and symptoms of improvement were observed for all patient groups and all analyzed outcome measures.

When interpreting the results from the present study some limitations should be considered. One important issue is that results come from a retrospective study. This type of study has less validity than randomized prospective clinical trials due to issues of selection bias and confounding factors. Future prospective randomized clinical studies will be necessary to properly confirm these initial results. Also, it would be interesting to include objective biomarkers to assess the benefits of therapy with standardized PRGF in OSDs.

However, the data presented herein suggest that PRGF is safe and effective in reducing the signs and symptoms of a variety of OSDs in patients previously treated with AS or cyclosporine. Therefore, although further studies are needed, PRGF could be considered as an effective therapeutic alternative for these cases.

Disclosure Statement

The authors declare the following competing financial interest(s): E.A. is the Scientific Director of, and G.O. and L.B. are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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Plasma rich in growth factors (PRGF) eye drops stimulates scarless regeneration compared to autologous serum in the ocular surface stromal fibroblasts

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ABSTRACT

Autologous serum (AS) eye drops was the first blood-derived product used for the treatment of corneal pathologies but nowadays PRGF arises as a novel interesting alternative to this type of diseases. The purpose of this study was to evaluate and compare the biological outcomes of autologous serum eye drops or Plasma rich in growth factors (PRGF) eye drops on corneal stromal keratocytes (HK) and conjunctival fibroblasts (HConF). To address this, blood from healthy donors was collected and processed to obtain autologous serum (AS) eye drops and plasma rich in growth factors (PRGF) eye drops. Blood-derivates were aliquoted and stored at −80 °C until use. PDGF-AB, VEGF, EGF, FGFb and TGF-β1 were quantified. The potential of PRGF and AS in promoting wound healing was evaluated by means of proliferation and migration assays in HK and HConF. Fibroblast cells were induced to myofibroblast differentiation after treatment with 2.5 ng/mL of TGF-β1. The capability of PRGF and AS to prevent and inhibit TGF-β1-induced differentiation was evaluated. Results showed significant higher levels of all growth factors analyzed in PRGF eye drops compared to AS. Moreover, PRGF eye drops enhanced significantly the biological outcomes of both HK and HConF, and reduced TGF-β1-induced myofibroblast differentiation in contrast to autologous serum eye drops (AS). In summary, these results suggest that PRGF exerts enhanced biological outcomes than AS. PRGF may improve the treatment of ocular surface wound healing minimizing the scar formation compared to AS. Results obtained herein suggest that PRGF protects and reverses the myofibroblast phenotype while promotes cell proliferation and migration.

1. Introduction

Tear film plays a critical role in maintaining the homeostasis of ocular surface tissues. It provides moisture to the ocular surface and supplies a wide range of proteins that give nourishing, antimicrobial effects as well as epitheliotrophic and neurotrophic capacity (Klenkler et al., 2007). A decrease or lack of tear film production can cause damage to the ocular surface tissues and can lead to serious eye disorders including dry eye, persistent epithelial defects or corneal ulcers (Barton et al., 1998; Muller et al., 2003; Pflugfelder et al., 2002). Ocular surface pathologies can also lead to scarring, characterized by the development of stromal myofibroblasts which express several cytoskeletal proteins like smooth muscle actin (SMA), desmin or vimentin (Chaurasia et al., 2009; Wilson, 2012). Although myofibroblasts are associated with ocular injury repair and involved in wound contraction and extracellular matrix (ECM) deposition and organization, the persistence of these cells after wound healing is responsible for the development of corneal opacity and haze formation (Mohan et al., 2008; Sakimoto et al., 2006).

Conventional therapeutic options to treat ocular surface disorders include artificial tears, punctual occlusion, therapeutic contact

Abbreviations: PRGF, Plasma rich in growth factors; AS, Autologous serum; HK, Human corneal stromal keratocytes; HConF, Human conjunctival fibroblasts; PDGF-AB, Platelet-derived growth factor − AB (heterodimeric); VEGF, Vascular endothelial growth factor; EGF, Epidermal growth factor; FGFb, Basic Fibroblast growth factor; TGF-β1, Transforming growth factor-beta 1; SMA, Smooth muscle actin; ECM, Extracellular matrix; FBS, Fetal bovine serum; ELISA, Enzyme-linked immunosorbent assay; FM, Fibroblast medium; NS, non-stimulation; PBS, Phosphate buffered saline; TBS, Tris(hydroxymethyl) aminomethane-buffered saline; TBS-T, TBS buffer containing 0.1% Tween 20.

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lenses and topical anti-inflammatory agents. Artificial tears are the most widely used treatment to increase ocular surface humidity and to provide additional lubrication. The artificial tears try to emulate the natural tear in terms of its physicochemical characteristics, but are far to provide all the properties of the human tear. Also, most artificial tears is that they contain preservatives, which may induce ocular toxicity and cause irritation and epithelial damage (Geerling et al., 2001; Noecker, 2001).

Many recombinant growth factors and cytokines have been used as an alternative to natural tears to treat corneal injuries. The cornea tissue repair process is mediated by growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF) (Klenkler and Sheardown, 2004). Thus, recombinant growth factors and cytokines have been used as an alternative to natural tears to treat corneal injuries. The combination of these and other factors provides a better therapeutic response. However, the use of recombinant growth factors is expensive. A more cost-effective approach is the use of patient's own blood-derived products to induce tissue regeneration.

Autologous serum (AS) eye drops was the first blood-derived product used for the treatment of corneal pathologies (Fox et al., 1984; Tsubota et al., 1999a). Although AS may contain similar protein levels and physico-chemical properties compared with natural tears, they also have limiting drawbacks. First, the preparation of AS eye drops is still “home-made” and thus far from any regulatory acceptance. Second, AS dilution is also not standardized, ranging from 20% to 100% (Geerling et al., 2004). Third, AS contain pro-inflammatory agents such as metalloproteinases and acid hydrolases derived from leukocytes degranulation, which may induce negative effects in ocular tissue regeneration (Schnabel et al., 2007).

Plasma rich in growth factors (PRGF) is an autologous platelet rich plasma, that may represent a turning point in the use of blood-derivates in ophthalmology. By concentrating the platelets and avoiding the leukocytes, PRGF increases the amount of EGF, PDGF and FGF (Nurden et al., 2008) exerting multiple biological and antimicrobial properties (Anitua et al., 2012, 2008, 2009). Recent evidences suggest that PRGF is a potential approach for the treatment of several ocular surface diseases including dry eye, persistent corneal defects and ulcers, obtaining successful results even in those patients where autologous serum was ineffective (Lopez-Plandolit et al., 2010, 2011). However, until now, a complete characterization and biological comparison between AS and PRGF is lacking. In this study, we have evaluated the levels of different growth factors involved in tissue regeneration both in AS and PRGF. The effects of PRGF and AS on conjuctival fibroblast and keratocyte proliferation and migration has been determined as well as the protective and reverse effects of both blood-derived formulations against myofibroblast differentiation.

2. Methods

2.1. Cells

Cells involved in experiments were primary human cells including conjunctival fibroblasts (termed HConF) and corneal stromal keratocytes (termed HK) (ScienCell Research Laboratories, San Diego, CA) that were cultured according to manufacturer's instructions. Briefly, cells were maintained in culture until confluence in Fibroblast medium supplemented with Fibroblast Growth Supplement, 2% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) (Complete FM for routine culture) (ScienCell Research Laboratories, San Diego, CA, USA) and then were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion. Passage 4 cells were used in all experiments.

2.2. Characterization of cells

Immunolabelling of collagen type I (Chemicon – Millipore, Billerica, MA, USA), fibronectin and vimentin (Sigma–Aldrich, St Louis, MO, USA) were analyzed by phase-contrast microscopy in order to confirm the fibroblast-like morphology of cultured cells and the absence of dedifferentiation. Typical endothelial cells and hematopoietic progenitor cells markers CD105 (dilution 1:30) and CD34 (dilution 1:30) were also tested (BD Biosciences, San Jose, CA, USA); alpha-Smooth muscle actin (dilution 1:800) expression was analyzed (α-SMA, Sigma–Aldrich, St Louis, MO, USA) in order to check the spontaneous differentiation to myofibroblasts in culture. Corresponding secondary antibodies at 1:200 were used and finally, cells nuclei were counterstained with Hoechst 33342 (dilution 1:5000, Molecular Probes-Invitrogen, Grand Island, NY, USA) and visualized under a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany).

2.3. PRGF and autologous serum (AS) preparations

Blood from three healthy young donors was harvested after informed consent into 9-mL tubes with 3.8% (wt/v) sodium citrate or in serum collection tubes (Z Serum Clot activator, Vacuette, GmbH, Kremsmünster, Austria). The study was performed following the principles of the Declaration of Helsinki. Blood samples for PRGF were centrifuged at 580 g for 8 min at room temperature in an Endoret System centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain); the whole plasma column over the buffy coat was collected using Endoret ophthalmology kit (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) avoiding the layer containing leukocytes. Platelets and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpellier, France). Plasma preparations were incubated with Endoret activator (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at 37 °C for 1 h and PRGF supernatants were filtered, aliquoted and stored at −80 °C until use. Blood samples for autologous serum preparations were allowed to clot at room temperature for 20 min and subsequently centrifuged for 10 min at 2000 g; serum was isolated from the red series by the separation gel, filtered by PVDF filters and collected. Then, total serum was diluted to 20% with sterile serum saline, aliquoted and stored at −80 °C until use (termed AS). Growth factors (PDGF-AB, TGF-β1, VEGF, FGFb and EGF) were measured in both blood-derivates using commercially available Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) (Table 1).

2.4. Proliferation assay

Conjunctival fibroblasts and corneal stromal keratocytes were seeded at a density of 5000 cells per cm^2 on 96-well optical bottom black plates in serum-free medium supplemented with either: (i) the culture medium alone (FM) with 0.1% FBS as a control of non-stimulation (NS) (ii) 20% (v/v) PRGF or (iii) 20% (v/v) AS of the three donors for a study period of 72 h. DNA content corresponding with final number of cells in culture was estimated using the CyQUANT® Cell Proliferation Assay (Life Technologies, Invitrogen, Carlsbad, CA, USA). Cell treatments were removed and wells were washed carefully with phosphate buffered saline (PBS). Then microplate was freezed at −80 °C for efficient cell lysis for the CyQUANT® assay. Samples fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold

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Table 1
Characterization of PRGF and AS eye-drops. The concentration of platelets and leukocytes and levels of some representative growth factors are shown.

	Platelet count ($\times 10^6$/ml)	Leukocyte count ($\times 10^6$/ml)	Growth factor levels					
			Platelet enrichment	EGF (pg/ml)	PDGF-AB (ng/ml)	TGF-β1 (ng/ml)	VEGF (pg/ml)	FGFb (pg/ml)
Undiluted PRGF	386	0.1	1.7x	609.0 ± 104.1*	14.1 ± 4.9*	25.4 ± 7.2*	81.9 ± 4.2*	9.7 ± 4.7*
20% AS	227	6.4	1.0x	32.6 ± 15.4	3.3 ± 0.1	6.4 ± 1.7	34.7 ± 13.3	nd

*Statistically significant differences between PRGF and AS ($p < 0.05$).

Technologies) and a DNA standard curve ranging from 7.8 to 500 ng/mL was included in all fluorescence quantifications in order to correlate fluorescence units with final amount of DNA.

2.5. Migratory capacity of corneal and conjunctival fibroblasts

To quantify their migratory potential conjunctival fibroblasts and keratocytes were seeded inside culture inserts (Ibidi, GmbH, Martinsried, Germany) at 20,000 cells/cm^2 and grown with complete FM until confluence. The migration device was carefully removed and two separated cell monolayers were created leaving a cell-free gap of approximately 500 μm thickness. The cells were washed with PBS and incubated with 20% PRGF or 20% AS from each donor in FM medium for 22 h; culture medium plus 0.1% FBS was used as control. After this period, the different culture mediums were removed and cells were incubated for 10 min with Hoechst 33342 in PBS. To quantify the number of migratory cells, phase contrast and fluorescence photographs of the central part of the created septum before and after treatment were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area and the migratory cells found in this septum after the time of treatment were measured using the Image J Software (NIH, Bethesda, Maryland, USA). The results were expressed as number of cell migrated per mm^2 of area.

2.6. Myofibroblast differentiation: protection and reversion assays

In order to analyze protective capacity of PRGF versus AS, HConF and HK cells were treated with either 2.5 ng/ml TGF-β1, 20% PRGF + 2.5 ng/ml TGF-β1 or 20% AS + 2.5 ng/ml TGF-β1 from each donor in serum-free FM medium for 72 h (TGF-β1, Chemicon-Millipore, Billerica, MA, USA). Another batch of experiments was performed to test the ability of myofibroblastic phenotype reversion of PRGF and AS. For this purpose both types of fibroblasts were pretreated for 72 h with 2.5 ng/ml TGF-β1 + 0.1% FBS; then, medium was removed, the wells were washed with PBS and finally cells were incubated with 20% PRGF + 2.5 ng/ml TGF-β1 or 20% AS + 2.5 ng/ml, using 2.5 ng/ml TGF-β1 + 0.1% FBS as a control. After 72 h, culture mediums from both sets of assays were discarded and the wells were rinsed with PBS. Depending on the experiment, cells were managed in different ways.

To analyze the influence of TGF-β1 over proliferation of conjunctival fibroblasts and corneal stromal cells were seeded in 96-well optical bottom black plates at 5000 cells per cm^2 and then the procedure was the same as explained in proliferation assay section, detecting final amounts of DNA.

In addition, cells were plated at a density of 5000 cells per cm^2 in 48-well tissue-culture plates to detect α-SMA and Ki-67 by immunolabelling. After performing protection and reversion assays, medium was removed and cells were fixed for 10 min in methanol. Cells then were blocked with 10% FBS in PBS for 30 min, and incubated for 1 h with mouse anti α-SMA antibody at 1:800 or rabbit anti-Ki67 antibody at 1:400 (Abcam, Cambridge, UK) followed by incubation with corresponding secondary antibodies for 1 h at room temperature. To finish cell nuclei were counterstained with Hoechst 33342 mounted using an anti-fade solution (Southern Biotech, Birmingham, AL, USA) and visualized under a fluorescence microscope (Leica DM IRB). Control isotype was performed by substituting the primary antibodies with 10% of FBS diluted in PBS.

Finally, another group of assays was designed to analyze expression of the fibrotic proteins α-SMA, desmin and vimentin by western blotting. For this purpose, HConF and HK cells were seeded in 6 well plates at 20,000 cells per cm^2 with complete FM. When cells reached confluence, complete medium was removed and wells were washed with PBS. Then, the protection and reversion of myodifferentiation assays were done. After 72 h of treatment culture mediums from both sets of assays were discarded, wells were rinsed with PBS and lysed with mammalian protein extraction reagent (M-PER, Piercenet, Rockford, IL USA) supplemented with protease and phosphatase inhibitors. Lysates were clarified by centrifugation at 14,000 g for 10 min, and the supernatants were then collected. In order to concentrate protein in the cell lysates, centrifugal filters (Merck Millipore, Darmstadt, Germany) were used following manufacturer's instructions. Protein concentration from the lysates was determined by BCA protein assay reagent (Piercenet Rockford, IL, USA). Equal amounts of protein were resolved through TGX precast gels (Bio Rad Lab, Hercules, CA, USA) electrophoresis and transferred to Trans Blot turbo PVDF membranes (Bio Rad Lab, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in tris(hydroxymethyl) aminomethane-buffered saline (TBS) containing 0.1% Tween 20 (termed TBS-T, Bio Rad Lab, Hercules, CA, USA and Sigma–Aldrich, St. Louis, MO, USA respectively) for 1 h at room temperature. After that, blots were incubated with corresponding primary antibodies: anti-α-SMA and anti-Vimentin (Sigma–Aldrich, St. Louis, MO, USA) and anti-Desmin (Abcam, Cambridge, UK) overnight at 4 °C. The day after, the blots were washed several times with TBS-T and were treated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Then, the blots were washed again with TBS-T and developed by chemiluminescence with substrate using a Chemi Doc™ XRS $^+$ image analyzer with Image Lab software (Bio Rad Lab, Hercules, CA, USA). The Stain-Free technology was used as loading control method (Colella et al., 2012).

2.7. Statistical analysis

Descriptive statistics were performed using absolute and relative frequency distributions for qualitative variables and mean values and standard deviations for quantitative variables. Different normality tests (Kolmogorov–Smirnov and Shapiro–Wilk) were performed on each sample.

The possible differences between the treatment groups were analyzed using ANOVA test of variance and a subsequent post-hoc analysis for multiple comparisons between groups, while in cases where no normality was detected, the nonparametric Kruskal–Wallis test with a subsequent Mann–Whitney analysis test for multiple comparisons between groups were used. Statistical significance level was set on $p > 0.05$. SPSS v15.0 for Windows statistical software package (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

3. Results

The human primary conjunctival fibroblasts (HConF) and corneal stromal keratocytes (HK) showed elongated and spindle-shaped aspect in routine culture. Both groups of cells represent heterogeneous populations with fibroblast characteristics and did not spontaneously differentiate into myofibroblasts as confirmed by the absence of α-SMA expression. Cells were positive for all the fibroblast markers (Collagen Type I, vimentin and fibronectin) and negative for markers of hematopoietic and endothelial cells (data not shown).

The number of platelets and leukocytes together with the level of some of the most important growth factors present in PRGF and autologous serum (AS) of the three donors are shown in Table 1. Mean platelet enrichment of the PRGF preparation was 1.7-fold over whole blood (386 10^6 platelets/mL). PRGF preparations contained no detectable levels of leucocytes. All the measured growth factor levels were significantly higher in PRGF than in AS.

3.1. PRGF augments fibroblasts proliferation

Proliferation of HConF and HK increased after treatment with PRGF and AS (20%PRGF or 20%AS) when compared with the control (data not shown). However, treatment of both types of fibroblasts with PRGF enhanced significantly their proliferation compared with AS ($p < 0.05$) (Fig 1).

3.2. Stimulation of migration by PRGF

Fig. 2 shows how 20% PRGF stimulated significantly the migratory capacity of both HConF and HK with respect to 20% AS ($p < 0.05$). The number of migrating cells was significantly higher with PRGF and AS preparations than cells cultured only with FM medium plus 0.1% FBS. Fig. 2A shows Hoechst images of HConF and HK cells after a 22-h period of migration and highlights the enhanced biological potential of PRGF versus AS.

3.3. Expression of fibrotic markers

In order to analyze the potential of PRGF and AS in preventing the TGF-β1-stimulated myofibroblastic differentiation cells were treated with either 20% PRGF or 20% AS in combination with TGF-β1.

As it shown in Fig. 3A and B, HConF and HK cells treated with 2.5 ng/mL TGF-β1 in combination with 20% AS showed a higher expression of the three fibrotic markers: α-SMA, desmin and vimentin compared to cells treated with 2.5 ng/mL TGF-β1 + 20% PRGF, becoming significant in the case of α-SMA and desmin. In addition, both fibroblastic cells treated with PRGF shown a significant reduction of the expression of α-SMA and desmin in comparison to cells treated with TGF-β1 alone, maintaining the expression of these proteins at baseline levels, similar to cells treated with 0.1% FBS (Fig. 3B). However, with the exception of the expression of α-SMA in HK cells and desmin on HConF cells, no differences were observed in the expression levels of fibrotic proteins in cells treated with SA regarding TGF-β1 alone treated cells, showing a lower capability of SA to protect fibroblastic cells against myofibroblast transformation in comparison to PRGF (Fig. 3B).

Then, the potential of both blood-derivates to reverse the myofibroblast phenotype was evaluated. Both fibroblastic cells pretreated with TGF-β1 for 3 days reduced significantly the expression levels of α-SMA and desmin after the treatment with both blood-derivates products (PRGF and SA). In addition, the expression of the three fibrotic markers in both types of fibroblasts was higher with AS compared to PRGF. In particular, the expression of α-SMA and desmin was significantly higher after culturing cells with 20% AS + TGF-β1 compared to cells cultured with 20% PRGF + TGF-β1 (Fig. 3C and D).

3.4. Effect of TGF-β1 over HConF and HK proliferation

TGF-β1 exerts an inhibitory effect on the proliferation of HConF and HK cells after 72-h-culture (Fig 4). After 3 days of stimulation with 2.5 ng/ml of TGF-β1, cells showed a limited growth compared to fibroblasts treated with PRGF + TGF-β1 and AS + TGF-β1 ($p < 0.05$). However, the proliferation of HConF and HK was significantly higher with PRGF compared with AS after three days of culture ($p < 0.05$) (Fig. 4A).

In a second set of experiments, cells were treated with TGF-β1 during 72 h and then with TGF-β1 alone, PRGF + TGF-β1 or AS + TGF-β1. Results showed once again that PRGF induced the highest cell proliferation profile rate (Fig 4B). Furthermore, in the case of HConF, the amount of DNA that correlates with the final number of cells was significantly higher after treatment with

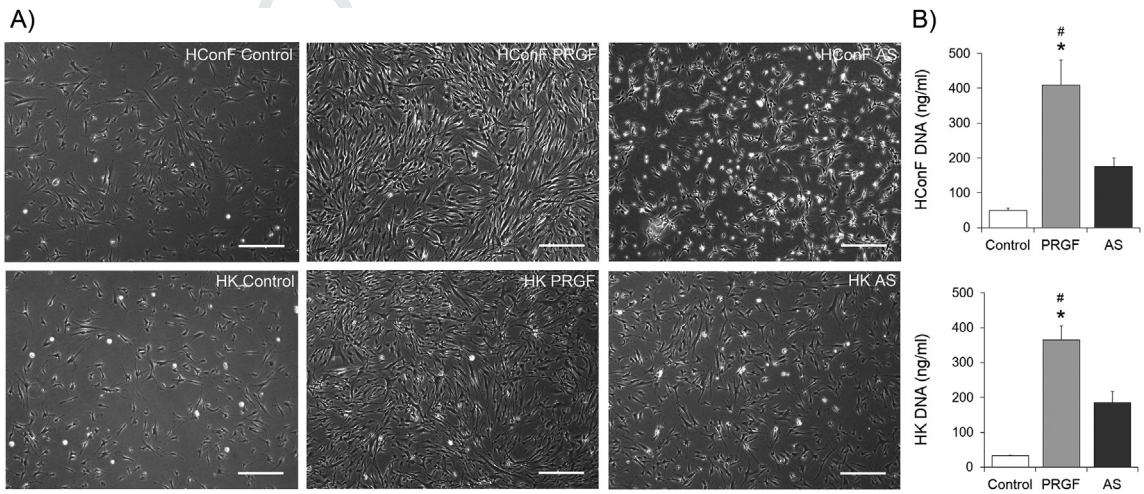

Fig. 1. (A) Phase contrast photomicrographs of HConF and HK cells cultured with PRGF or AS. Fibroblasts showed an elevated proliferation after treatment with PRGF and maintained spindle-shaped morphology in both cases (PRGF and AS). (B) 20% PRGF significantly increased proliferation of HConF and HK compared with 20% AS, measured as DNA content (ng/mL). * Statistically significances between PRGF and AS ($p < 0.05$). Scale bar 400 μm.

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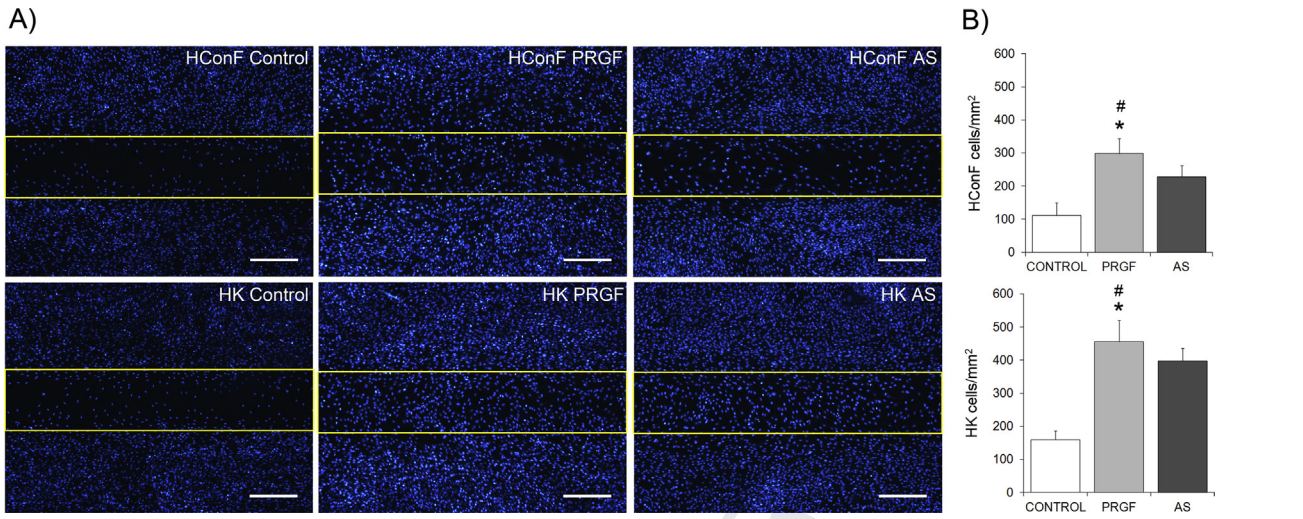

Fig. 2. (A) Immunofluorescence Hoechst images of HConF and HK fibroblasts after 22 h-migration with 20% PRGF and 20% AS. Yellow rectangle included in each image identifies the migration gap area (B) Cell migration was significantly higher when cells were cultured with PRGF. *Statistically significant differences between PRGF and AS ($p < 0.05$). Scale bar 400 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PRGF + TGF-β1 compared to AS + TGF-β1 ($p < 0.05$).

3.5. Expression of α-SMA and Ki-67

An immunofluorescence for α-SMA was performed after culturing the cells 3 days either with 20% of PRGF + 2.5 ng/mL TGF-β1 or with 20% of AS + 2.5 ng/mL TGF-β1. Results showed that α-SMA positive HConF and HK cells was lower in cells treated with PRGF than with AS. In parallel, the proliferation of both types of cells measured by Ki-67 expression was higher in fibroblasts treated with 20% PRGF + TGF-β1 than with 20% AS + TGF-β1 (data not shown).

A similar trend was observed in the myofibroblast phenotype reversion experiments (Fig 4C). After 72 h of pre-treatment with 2.5 ng/mL TGF-β1, all the cells were positive for α-SMA. When cells were treated for another 3 days with TGF-β1, PRGF + TGF-β1 or AS + TGF-β1, the expression was α-SMA was reduced with the use of blood-derivates and especially with PRGF, which provided the lowest expression of α-SMA. On the other hand, cell proliferation measured by the number of Ki-67 positive cells was higher with PRGF and AS compared to the TGF-β1-treated cell group.

4. Discussion

Ocular surface wound healing is mediated by a wide range of growth factors and cytokines that are produced by ocular surface cells and lachrymal glands (Imanishi et al., 2000; Wilson et al., 2001). These proteins are involved in cell proliferation and recruitment, cell differentiation and extracellular matrix synthesis (Klenkler and Sheardown, 2004).

Autologous serum (AS) eye drops are the first blood derivative product used for the treatment of ocular surface disorders (Geerling et al., 2004; Tsubota et al., 1999b). The preliminary results obtained with AS is due to the presence of growth factors (Bradley et al., 2009; Matsumoto et al., 2004). However, there are several limitations in the use of AS including its "home-made" preparation process, the large number of protocols available which make it difficult to standardize the approach and the presence of cytokines in its composition which may induce negative effects in ocular tissue regeneration.

Plasma rich in growth factors (PRGF) eye drops have been shown to be an effective treatment for patients with ocular surface injuries, and especially for those cases in which previous treatment with AS eye drops was ineffective. It has been suggested that this improvement in clinical outcomes may be due to the higher content of growth factors derived from platelets in PRGF eye drops (Lopez-Plandolit et al., 2010, 2011).

In this experimental study, we decided to compare for the first time the biological outcomes of PRGF and AS in two different ocular surface cell types. We first prepared both blood-derivates from the same healthy donors. The levels of some growth factors involved in ocular surface regeneration were determined in both samples. In addition, two primary culture cells from ocular surface tissue (corneal stromal keratocytes and conjunctival fibroblasts) were used as a cell culture model to investigate the influence of these blood products on cell proliferation, migration and myofibroblast differentiation.

The level of growth factors was significantly higher in PRGF eye drops compared with AS. It has been demonstrated that epidermal growth factor (EGF) promotes proliferation and migration on corneal epithelial cells (Kitazawa et al., 1990; Maldonado and Furcht, 1995). The higher EGF levels found in PRGF may be responsible of the increased proliferation and migration activity of this autologous approach, which may accelerate the wound healing time in corneal injuries (Freire et al., 2012, 2014). In addition, FGF, TGF-β and PDGF have critical roles in promoting proliferation and migration of stromal fibroblast in ocular surface tissues (Barton et al., 1998; Grant et al., 1992). Accordingly, the significant higher levels of FGFb, TGF-β1 and PDGF-AB detected in PRGF may explain the enhanced proliferation and migration activities of both fibroblastic cells when compared with AS.

Vascular endothelial growth factor (VEGF) promotes the proliferation of vascular endothelial cells and stimulates angiogenesis (van Setten, 1997). Although VEGF levels in PRGF are higher than in AS, no neovascularization has been detected in any of the patients treated with PRGF eye drops (Lopez-Plandolit et al., 2010, 2011). The latter may be explained by the pool of anti-angiogenic proteins including thombospondin, angiostatin and endostatin present also in PRGF, which may regulate the balance between pro and anti-angiogenic factors (Anitua et al., 2013a, 2013b).

One important concern in ocular surface wound healing is the persistence of myofibroblasts cells after tissue repairing, being identified as the primary biological step responsible for the development of scarring tissue (Garana et al., 1992; Jester et al.,

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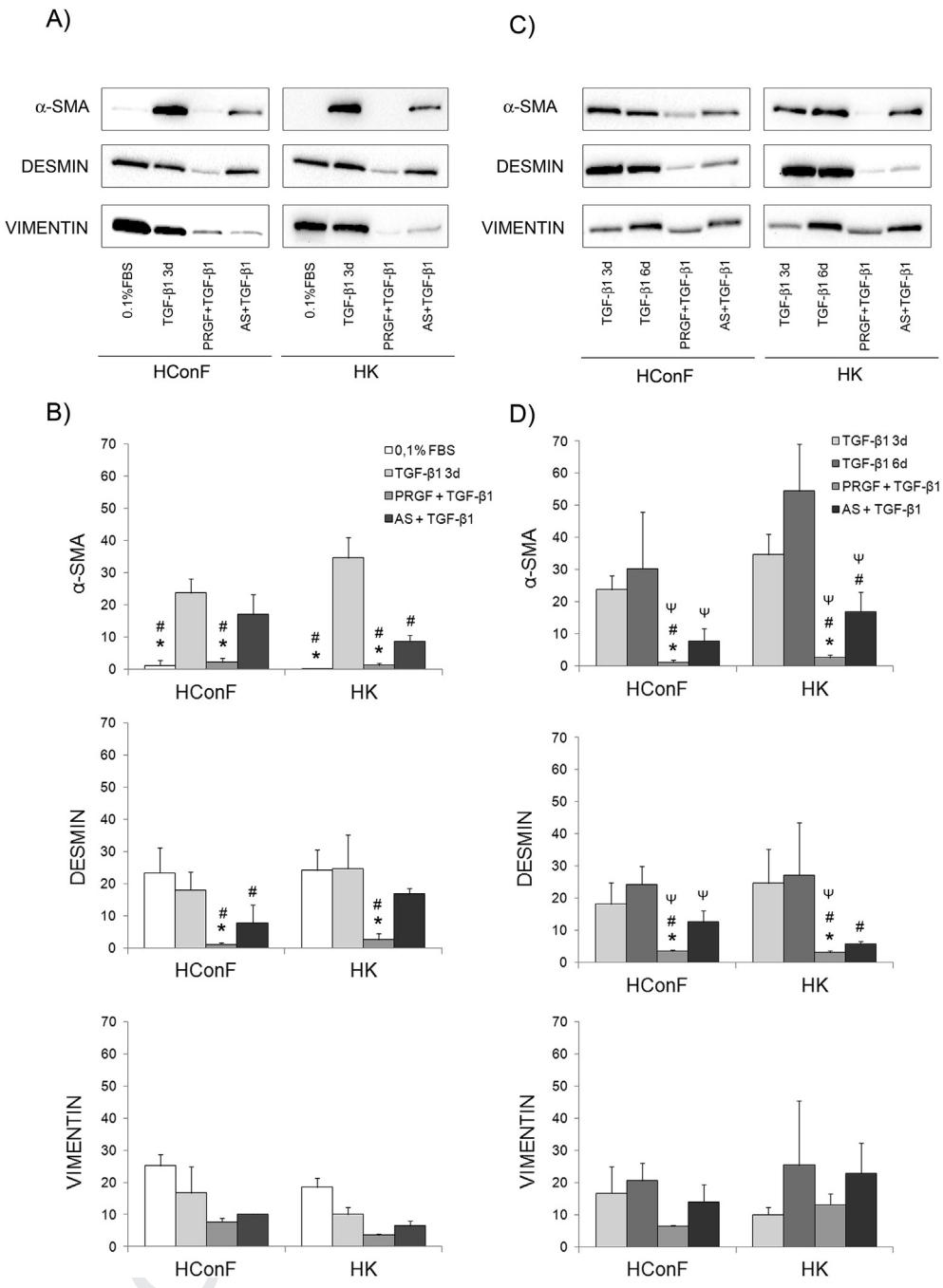

Fig. 3. (A) Western blot analysis of α-SMA, desmin and vimentin after three days of culture with 0.1% FBS, 2.5 ng/ml TGF-β1 alone or in combination with 20% PRGF or 20% AS. Results show the protective effect of PRGF over the myodifferentiation of fibroblasts. (B) Quantification of α-SMA, desmin and vimentin proteins in protection assay of PRGF and AS shows significant differences found between several treatments applied to HConF and HK. (C) Western blot analysis of α-SMA, desmin and vimentin after pretreatment with 2.5 ng/mL TGF-β1 and three additional culture days with 2.5 ng/ml TGF-β1 (TGF-β1 6d), TGF-β1 + 20% PRGF or 2.5 ng/ml TGF-β1 + 20% AS. Results show the capability of PRGF and AS to revert the myofibroblastic phenotype. (D) The expression of α-SMA and desmin were significantly reduced after culturing the cells with PRGF in comparison with AS. * Statistically significant differences with respect to AS + TGF-β1 ($p < 0.05$); # statistically significant differences respecting to 2.5 ng/ml TGF-β1 3d ($p < 0.05$); Ψ statistically significant differences with regard to 2.5 ng/ml TGF-β1 6d.

1992). Myofibroblast differentiation involves the expression of several cytoskeletal proteins like smooth muscle actin (SMA), desmin or vimentin (Kaur et al., 2009). Recently, we have reported the protective and inhibitory effect of PRGF eye drops on TGF-β1-induced myofibroblast differentiation (Anitua et al., 2011). In the present study we have compared the protective and inhibitory effect of PRGF and AS on TGF-β1-induced myofibroblast transformation. Our current findings confirm that PRGF technology protects and inhibits TGF-β1-induced myofibroblast on conjunctival fibroblasts and corneal stromal keratocytes unlike SA that shows a lower capability to protect fibroblastic cells against myofibroblast transformation. Our results showed that PRGF eye drops significantly protect and inhibit the myofibroblast transformation of both types of fibroblast cells in comparison to AS treatment, reducing the expression of all cytoskeletal proteins related to myofibroblast differentiation.

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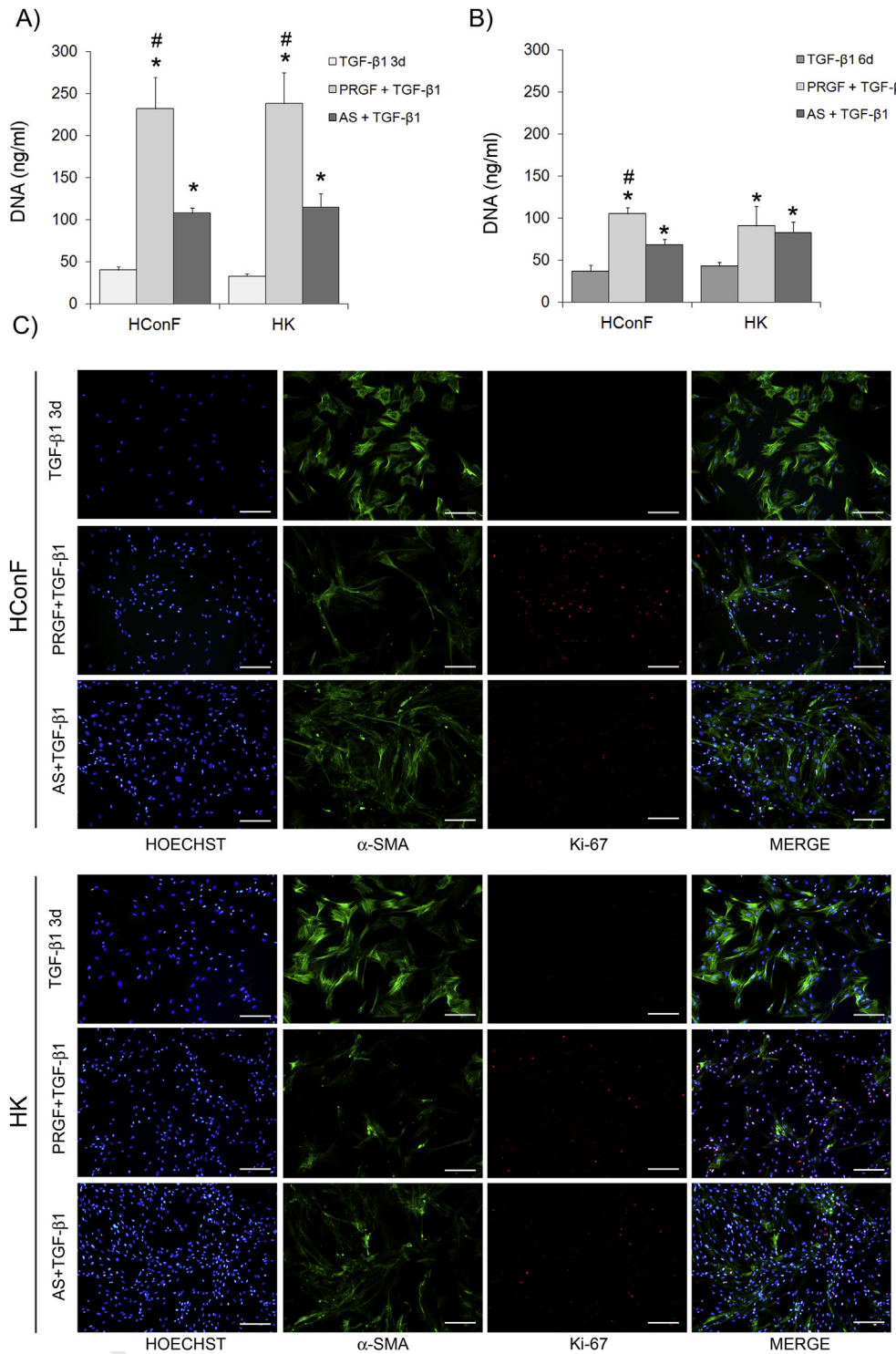

Fig. 4. (A) Effect of PRGF versus AS over HConF and HK proliferation treated simultaneously with TGF-β1 and PRGF or AS (protection assay). Cell treatment with PRGF + TGF-β1 increased significantly the proliferation of HConF and HK compared to AS + TGF-β1. (B) Evaluation of the pretreatment with TGF-β1 for 3 days and then treatment with TGF-β1 alone, PRGF + TGF-β1 or AS + TGF-β1 on the proliferation of HConF and HK (reversion assay). Results show that PRGF and AS increased cell proliferation. In HConF cells, proliferation was significantly higher with PRGF than with AS. * Statistically significant differences between TGF-β1 and hemoderivative products ($p < 0.05$); # Statistically significant differences between PRGF + TGF-β1 and AS + TGF-β1 ($p < 0.05$). (C) Immunofluorescence photomicrographs of α-SMA and Ki-67 staining. Hoechst expression shows total number of cells while α-SMA represents myofibroblast cells present in culture. Ki-67 expression indicates the proliferating cells. Merge illustrates proliferation of both fibroblasts and myofibroblasts after treatment with PRGF + TGF-β1 or AS + TGF-β1. Scale bar 200 μm.

Vimentin is the first cytoskeletal protein related with the myofibroblast transformation that is expressed in the first stage of fibrosis. Then, other proteins such as SMA and desmin are expressed to complete myofibroblast differentiation (Chaurasia et al., 2009). Although more studies are necessary, it is possible that vimentin could be the last protein to be expressed in the myofibroblast dedifferentiation. As a consequence, we speculate that longer culture times may be necessary to assess the real role of

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PRGF compared with AS.

Controversial studies are published about how myofibroblastic phenotype disappears after corneal wound healing. Some of them suggest a dedifferentiation myofibroblast process to fibroblast phenotype promoted by anti-fibrotic mediators such us MyoD, prostaglandin E2 or FGF (Garrison et al., 2013; Hecker et al., 2011; Maltseva et al., 2001). Other studies suggest that myofibroblast are terminally differentiated cells that have lost their ability to proliferate and then undergo an apoptotic process to finally disappear from the repaired tissue (Evans et al., 2003; Wilson et al., 2007). In this study, we have demonstrated that blood derivative products induce biological activity of fibroblasts and myofibroblasts even in the presence of TGF-β1 without induction to apoptosis. The results obtained in this study also showed that in general PRGF eye drops increased significantly the proliferative activity of both fibroblast cell types compared with AS treatment even in the presence of TGF-β1. Furthermore, the results of immunofluorescence showed that PRGF eye drops not only protected and reverted α-SMA expression induced by TGF-β1 but also promoted the proliferation of fibroblast and myofibroblast, showing a ki-67 nuclear positive staining. As it was reported by Hecker et al., these data suggest that myofibroblasts are not terminally differentiated cells and, although further studies are needed, they could be proliferate and undergo a dedifferentiation process induced by some factors present in the blood derived products, such as FGF (Hecker et al., 2011; Maltseva et al., 2001).

5. Conclusions

In summary, the results obtained in this study suggest that PRGF may improve the treatment of ocular surface wound healing minimizing the scar formation compared to AS. Although further studies are needed to determine the mechanisms underlying the effects of PRGF eye drops on myofibroblast dedifferentiation process, results from this study suggest that PRGF protects and reverses the myofibroblast phenotype promoting their biological activity.

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Autologous method for *ex vivo* expansion of human limbal epithelial progenitor cells based on plasma rich in growth factors technology

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Short title: PRGF in limbal culture/ Riestra et al

FOOTNOTES

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Abbreviations. AM: Amniotic membrane. BM: basal medium. CFE: colony forming efficiency. CFU: colony forming units. CK: cytokeratin. EGF: Epithelial growth factor. FBS: fetal bovine serum. LEPCs: limbal epithelial progenitor cells. PDGF: Platelet derived growth factor. PRGF: Plasma Rich in Growth Factors. SM: supplemented medium.

ABSTRACT

Purpose: Develop an autologous culture method for *ex vivo* expansion of human limbal epithelial progenitor cells (LEPCs) using Plasma Rich in Growth Factors (PRGF) as a growth supplement and as a scaffold for the culture of LEPCs. **Methods:** LEPCs were cultivated in different media supplemented with 10% fetal bovine serum (FBS) or 10% PRGF. The outgrowths, total number of cells, colony forming efficiency (CFE), morphology and immunocytochemistry against p63- α and cytokeratins 3 and 12 (CK3-CK12) were analyzed. PRGF was also used to elaborate a fibrin membrane. The effects of the scaffold on the preservation of stemness and the phenotypic characterization of LEPCs were investigated through analysis of CK3-CK12, ABCG-2 and p63. **Results:** LEPCs cultivated with PRGF showed a significantly higher growth area than FBS cultures. Moreover, the number of cells were also higher in PRGF than FBS, while displaying a better morphology overall. CFE was found to be also higher in PRGF groups compared to FBS, and the p63-α expression also differed between groups. LEPCs cultivated on PRGF membranes appeared as a confluent monolayer of cells and still retained p63 and ABCG-2 expression, being negative for CK3-CK12. **Conclusions:** PRGF can be used in corneal tissue engineering, supplementing the culture media, even in a basal media without any other additives, as well as providing a scaffold for the culture.

KEY WORDS: Cell culture techniques, Cell proliferation, Cell transplantation, Corneal epithelium, Culture media, Limbal deficiency, Limbus corneae, Plasma Rich in Growth Factors, PRGF**.**

I. Introduction

Transplantation of *ex vivo* expanded human limbal epithelial progenitor cells (LEPCs) can restore the integrity of the corneal surface. However, cultivation and transplantation methods of LEPCs differ significantly between groups performing transplantation, and in most protocols culture media include xenogeneic growth additives, such as fetal bovine serum (FBS), exogenous growth factors, hormones, cholera toxins, or 3T3 murine feeder layers [1].

Although FBS has often been the standard media supplement, it has several disadvantages when it is applied in clinical practice. FBS could cause a humoral or cellular response to bovine proteins, which can lead to the rejection of the implanted cells, and the transmission of pathogens from the animals, such as prions, is also possible [2]. Last but not least, the use of animal sources at the clinical level is strongly undesirable (sometimes prohibited), and most of these products do not meet the Good Manufacture Practice (GMP) exigencies [3]. As a consequence, regulatory guidelines for clinical usage of cellular products aim to replace FBS by human cell culture supplements to minimize the risk of transmitting infectious diseases from other animal species [4].

In the same way, different materials have been used as scaffold for LEPC culture and transplantation, with amniotic membrane (AM) being the most widely used [5]. However, the use of AM has some remarkable disadvantages due to the allogenic origin, the potential pathogen transmission, and the high costs of obtaining.

Plasma Rich in Growth Factors (PRGF) is an autologous platelet rich plasma which contains numerous growth factors involved in the maintenance of the ocular surface, including epithelial growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) among others, and the efficacy of PRGF stimulating corneal epithelium cell proliferation has been already investigated in a cell line and in an animal model [6-8].

One of the pivotal and distinguishing properties of PRGF is its great versatility, allowing the preparation of different therapeutic formulations [9]. These formulations include the eye drop, liquid formulation, the clot, and the autologous fibrin membrane. The clot and the fibrin membrane constitute a perfect biological scaffold embedded with growth factors [9, 10].

In the current study, we have evaluated the use of PRGF as a human-based serum additive of a supplemented and a basal culture medium and as a scaffold for the culture and expansion of LEPCs. The use of human PRGF for culturing human corneal cells may lead to a future customization of some therapies that require the use of cellular cultures while avoiding the risk of using xenogeneic products for the treatment of human diseases.

II. Materials and Methods

A. PRGF as a Substitute for FBS

1. Preparation of Liquid PRGF

Blood from healthy volunteers (age range, 30–40 years) was collected by venepuncture after informed consent was obtained from the subjects, following the explanation of the nature and possible consequences of the study and complying with the principles of the Declaration of Helsinki. The blood sample from each volunteer was processed according to the method described by Anitua et al. [11].

Briefly, 81 mL of human blood was collected into 9 mL tubes with 3.8% (wt/v) sodium citrate. Blood samples were centrifuged at 580g for 8 minutes at room temperature, the lower 2 mL of the plasma column (F2) was pipetted, avoiding the buffy coat, and stored at -20ºC for its posterior use for the preparation of PRGF fibrin membrane. The rest of the plasma column (F1) was drawn off avoiding the buffy coat and incubated with calcium chloride 10% at 37ºC for 1h, and finally, the released supernatants were collected by aspiration, filtered, aliquoted, and stored at -80ºC until use.

2. Cell Isolation and Culture of LEPCs

Human tissue was handled according to the Declaration of Helsinki. Thirty-two corneoscleral rings from different donors were obtained after penetrating keratoplasty surgeries from the Fernández-Vega Eye Institute (Instituto Oftalmológico Fernández-Vega, Oviedo, Asturias, Spain). The donors' ages ranged from 40 to73 years. All tissues were maintained at 4ºC in Eusol-C storage medium (Alchimia, Ponte S.Nicolò, Italy) for less than 10 days before the study.

The limbal region was carefully dissected under a dissecting stereomicroscope according to its anatomical position and then cut into small pieces of approximately 1-2 mm^2. The tissue pieces were placed as explants in a culture plate with culture medium. Of the 32 corneoscleral rings, 14 were used for the measurement of the extent of outgrowth from the explant and for the immunocytochemistry; 14 were used for the measurement of the number of LEPCs, the morphological analysis and the percentage of colony forming units (CFU) assessment; and 4 were used to cultivate onto the PRGF fibrin membranes. Explants that did not remain attached or that floated off the dish were removed from the dish. From each sample, 6 explants of the limbal region were cultured in the different culture media:

Supplemented medium (SM): DMEM:F12 2:1 mixture (Life technologies, CA, USA), supplemented with 5 µg/mL insulin, 8.33 ng/ml cholera toxin, 24 µg/mL adenine, 1.3 ng/mL

triiodothyronine (T3), 0.4 μg/mL hydrocortisone, 100U/mL penicillin and 0.1mg/mL streptomycin and 10% FBS (SM-FBS [Sigma-Aldrich, MO, USA])or 10% PRGF (SM-PRGF). These media were used in the first three days of culture; after that, cells were cultured in the same media with 10 ng/mL EGF (Austral Biologicals, CA, USA).

Basal medium (BM): Dulbecco's modified Eagle's medium, Ham's F12, (DMEM:F12) 2:1 mixture supplemented with 100U/mL penicillin and 0.1mg/mL streptomycin and 10% FBS (BM-FBS) or 10% PRGF (BM-PRGF).

The cultures were incubated in a 37°C, 5% CO_2-95% air, water-jacketed incubator for 7 days with the medium changed twice a week. Explants were left in the culture dish for the duration of the experiment.

3. Examination of Cell Cultures

a. Measurement of the Extent of Outgrowths of Cultures of LEPCs

Cultures were examined every day using a Leica DMIL LED phase contrast microscope (Leica SP2; Wetzlar, Germany).

After 7 days of culture, the epithelial outgrowths and explants of 14 corneal rings were fixed in ice-cold methanol for 10 minutes, followed by rinsing in PBS. Photographs of the outgrowths were taken using a Leica DMIL LED phase contrast microscope with an attached EC3 camera (Leica, Wetzlar, Germany), and the area of each outgrowth was measured (mm^2) using ImageJ software (NIH, Bethesda, MD, USA).

b. Measurement of the Number of LEPCs

After 9 days of culture, LEPCs from 14 corneal rings were trypsinized, and the total number of cells was obtained by counting the cells in a hemocytometer.

c. Colony Forming Efficiency

In order to evaluate the colony forming efficiency (CFE), $1x10^6$ lethally irradiated 3T3/J2 cells were seeded in a 6 well plate and maintained in SM-FBS for 24 hours. After that, plates were washed in PBS and LEPCs, previously cultured in the different culture media during 9 days, were

seeded as cell suspensions at 1,000cells/cm^2 and incubated with its corresponding media for 12 days.

To evaluate the number of colonies, plates were washed in PBS, stained with rodamine 2% diluted in 4% paraformaldehyde, and rinsed in running water. Number of colonies was counted and colony forming units (CFU) was calculated according to the following formula:

$$\%CFU = \frac{number\ of\ colonies}{number\ of\ seeded\ cells} x\ 100$$

d. Morphological Analysis

After 9 days of culture, cultures were classified based upon overall morphological characteristics by two independent blinded researchers, and representative photos were taken using a phase contrast microscope (Leica, Welztar, Germany). Those whose cells were small, rounded, and uniform were classified as undifferentiated; the ones that presented an increased cell size or enlarged morphology were classified as differentiated.

e. Immunocytochemistry and quantification of p63-α HLCEs

Immunocytochemistry for the transcription factor p63-α (Cell Signaling, MA, USA) was performed in order to identify undifferentiated LEPCs. CK3-CK12 (Abcam, Cambrigde, UK) was used to identify differentiated corneal epithelial cells. Briefly, fixed cells were rinsed with PBS solution twice for 10 minutes, and permeabilized in a PBS solution containing 0.3% Triton X-100 for another 5 minutes. Following this, the samples were incubated at 4ºC overnight with primary mouse and rabbit polyclonal antibodies (1:100) containing 10% normal goat serum (Life Technologies, CA, USA) as a blocking agent. Immunolabeled cells were visualized by indirect immunocytochemistry and stained with 4',6-diamidino-2-phenylindole (DAPI) to allow nuclei visualization. Five photos of random fields were captured using Leica DFC310FX camera at 20x magnification counting the number of positive cells for p63-α out of the total of DAPI positive cells (%) using ImageJ software (NIH, Bethesda, MD, USA).

4. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics v.22 software (IBM, NY, USA). Quantitative data is expressed as mean±SEM while qualitative data is expressed as absolute

frequencies and percentages. Significant differences among defined groups were tested using the non-parametric Wilcoxon test after applying the Shapiro–Wilk normality test. Qualitative data was analyzed by a Pearson's Chi squared test and by calculating an Odds Ratio (OR) estimate of chances. A difference level of $p<0.05$ was considered to be statistically significant and $p<0.01$ statistically very significant.

B. PRGF fibrin membrane as a scaffold for LEPCs Culture

1. Preparation of PRGF Fibrin Membrane

The lower 2 mL of the plasma column (F2) was pipetted, avoiding the buffy coat. For eachmembrane, 5 mL of recovered plasma is incubated in the presence of 250 μL of 10% $CaCl_2$ in a 35 mm diameter dish at 37ºC for 30 minutes. Once a gel is formed, PRGF fibrin membranes were obtained by flattening for 30 seconds using a 500 μm fibrin membrane shaper (BTI, Vitoria, Spain). The membranes obtained were placed onto a nitrocellulose disk to improve its manageability.

2. Culture of HLCEs onto PRGF Fibrin Membranes

Four corneoscleral rings were processed as described above, and cultured as explants in BM-PRGF in a 12-well culture plate. Once confluent, cells were trypsinized, seeded on a PRGF fibrin membrane and cultured for 24 h. After that, PRGF fibrin membranes were fixed in ice cold methanol and processed for histological analysis.

Methanol-fixed cultures were immunostained as described in the previous section, using antibodies against CK3-CK12, ABCG-2 and p63 (1:100) (Abcam, Cambridge, UK).

3. Scanning Electron Microscopy (SEM)

The surface morphology of PRGF fibrin membranes with or without cells was observed under SEM. The membranes, previously fixed in ice-cold methanol for 10 minutes, were dehydrated in a graded series of acetone (30%, 50%, 70%, 90% and 100%) for 10 minutes, respectively, and dried by the critical-point method. Afterwards, they were coated with gold under vacuum and observed with a JEOL 6610LV scanning microscope, (JEOL co., Tokyo, Japan) at 20kV accelerated voltage.

II. Results

A. PRGF as Substitute for FBS

In this study, we compared the outgrowth area of LEPCs explants cultured in different supplemented and basal media (Figure 1).

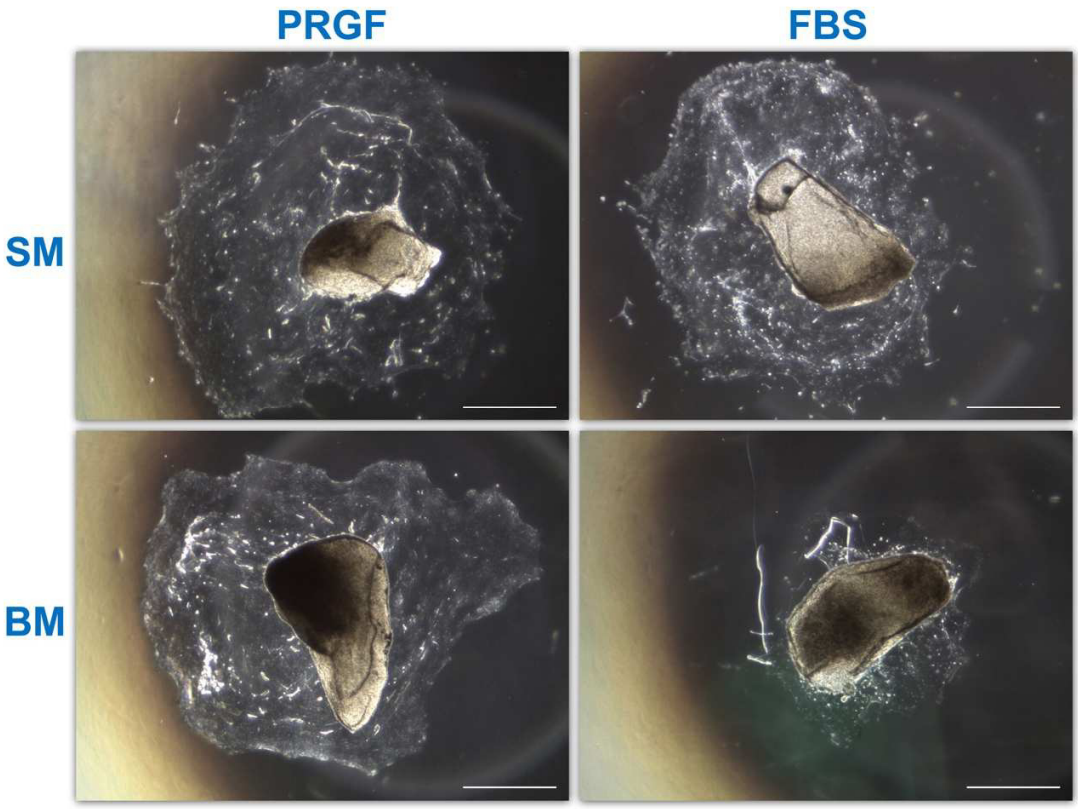

Figure 1: Phase-contrast microphotographs of the limbal explants after 7 days of culture in a supplemented (SM) or basal medium (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). Scale bar 1,000 μm.

Phase-contrast micrographs revealed significantly better results ($p<0.01$) in growth areas from the limbal explants in the SM compared to BM, in both PRGF (122.97±17.14 mm^2; 89.79±15.24 mm^2) and FBS (77.91±12.3 mm^2; 46.89±10.44 mm^2), as shown in Figure 2. Limbal cultures supplemented with PRGF showed an increase in the growth areas; moreover PRGF-supplemented media showed significant differences when compared to FBS-supplemented media, both in SM ($p<0.05$) and BM ($p<0.01$). Although the growth area of BM-PRGF is higher than the growth area of SM-FBS, no significant differences were found.

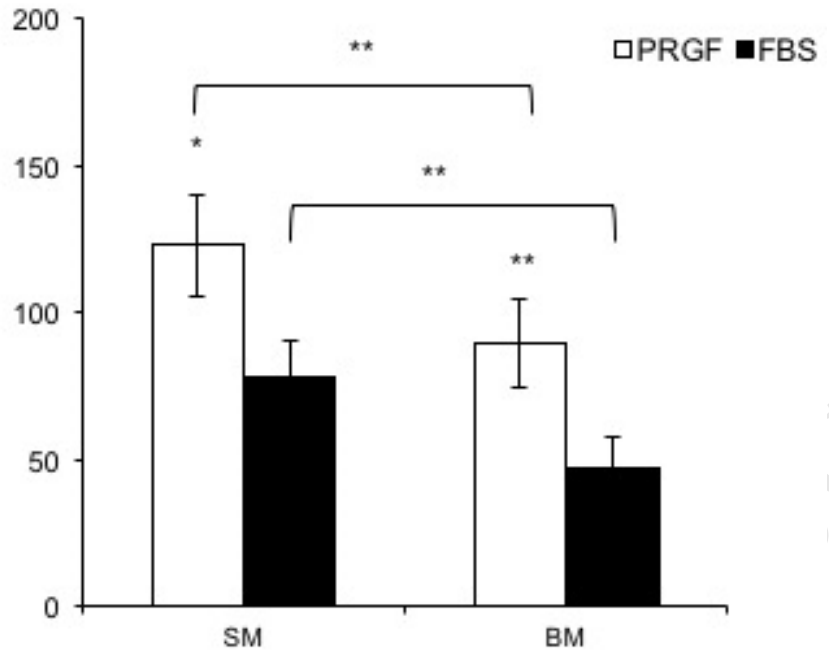

Figure 2: Growth area (mm^2) from the limbal explants (mean ± SEM) after 7 days of culture in a supplemented (SM) or basal medium (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). (*p<0.05 and **p<0.01).

On the other hand, after 9 days of culture, statistically significant differences (p<0.05) were found in the total number of cells when groups supplemented with PRGF or FBS were compared (Figure 3), both in SM (84,464±15,089; 46,116±6,895 cells) and BM (78,214±14,395; 38,839±7,418 cells).

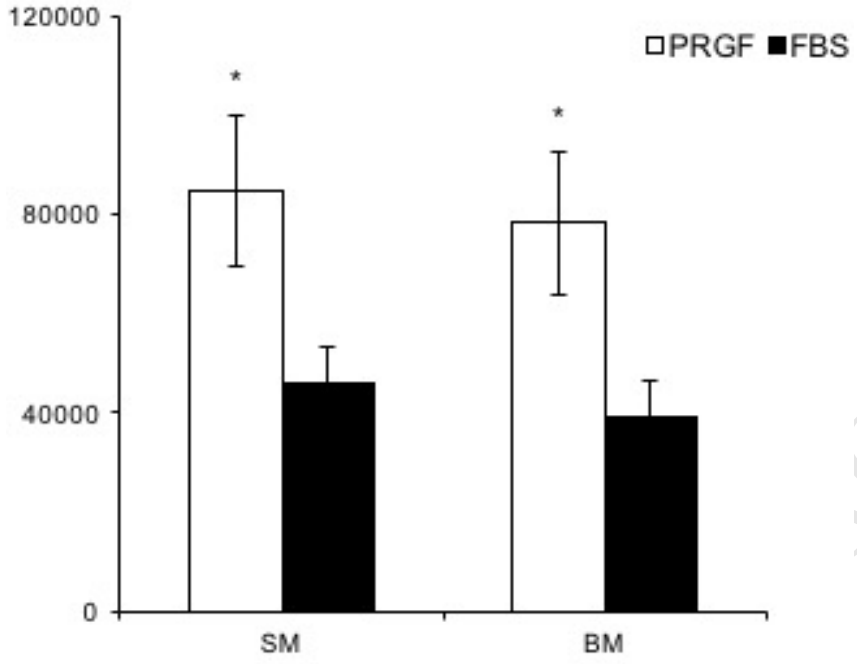

Figure 3: Total number of limbal epithelial cells (mean ± SEM) after 9 days of culture in a supplemented medium (SM) or basal (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). (*$p<0.05$).

When cellular morphology was compared under phase contrast microscopy, limbal epithelial cells cultured in media supplemented with PRGF appeared as more homogeneous, ovoid-to-round in shape, with a cobblestone pattern, while cultures supplemented with FBS showed a heterogeneous cell population with bigger cells. When classifying the cultures in accordance with its morphological characteristics (Figure 4), statistically significant differences were found in the percentage of cultures that presented an undifferentiated morphology when cultured in the presence of PRGF (56.8%) or FBS (28.9%), in both SM and BM, displaying an odds ratio of 4.62 and 2.25, respectively. Moreover, cultures in BM-PRGF display an odds ratio of 3.08 compared to cultures in SM-FBS. Overall, cultures in media supplemented with PRGF were 3.22 times more likely to display an undifferentiated morphology than cultures in media supplemented with FBS (Table 1).

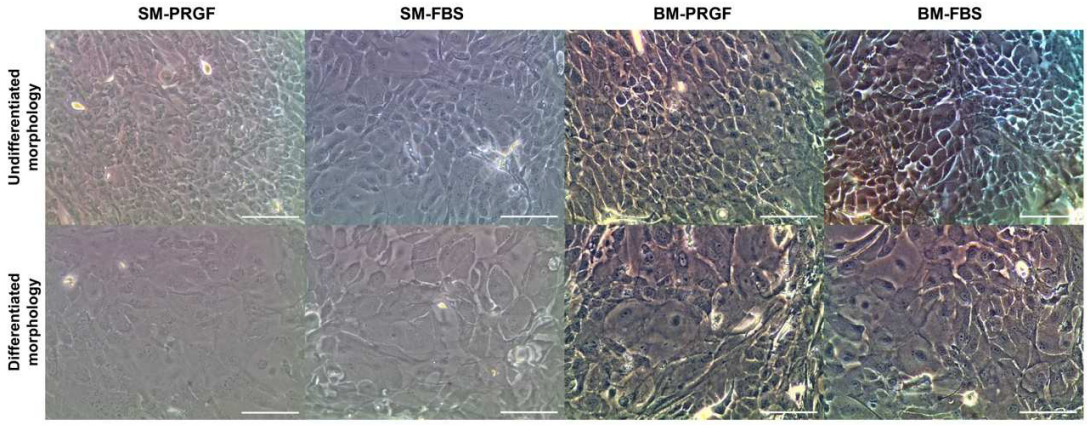

Figure 4: Representative micrograph of the overall morphology of the limbal epithelial cells after 9 days of culture in a supplemented (SM) or basal medium (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). The cultures were classified in accordance to its undifferentiated morphology by two blinded, independent observers. Scale bar 200 μm.

	SM-PRGF (n=60)	SM-FBS (n=62)	p value	OR
Undifferentiated	37	16	<0.001	4.62
Differentiated	23	46		0.21
	BM-PRGF (n=58)	**BM-FBS (n=59)**	**p value**	**OR**
Undifferentiated	30	19	<0.05	2.25
Differentiated	28	40		0.44
	PRGF (n=118)	**FBS (n=121)**	**p value**	**OR**
Undifferentiated	67	35	<0.001	3.22
Differentiated	51	86		0.31
	BM-PRGF (n=58)	**SM-FBS (n=62)**	**p value**	**OR**
Undifferentiated	30	16	<0.01	3.08
Differentiated	28	46		0.32

Table 1: Contingency table of the classification of the cultures according its morphology after 9 days of culture in a supplemented (SM) or basal medium (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS).

Finally, the expression of p63-α showed no significant differences between BM-PRGF and SM-PRGF (63.00±1.93%; 64.84±2.01%). However, significant differences ($p<0.01$) were found between BM-FBS and SM-FBS (54.97±3.28%; 66.75±2.44%) cell cultures (Figures 5 and 6). Moreover, significant differences ($p<0.05$) were also found between SM-PRGF and BM-FBS (64.84±2.01%; 54.97±3.28%) cultures.

It is worthy of mention that CK3-CK12, markers of differentiated cornea epithelial cells, were only clearly expressed in BM-FBS cultures (Figure 6).

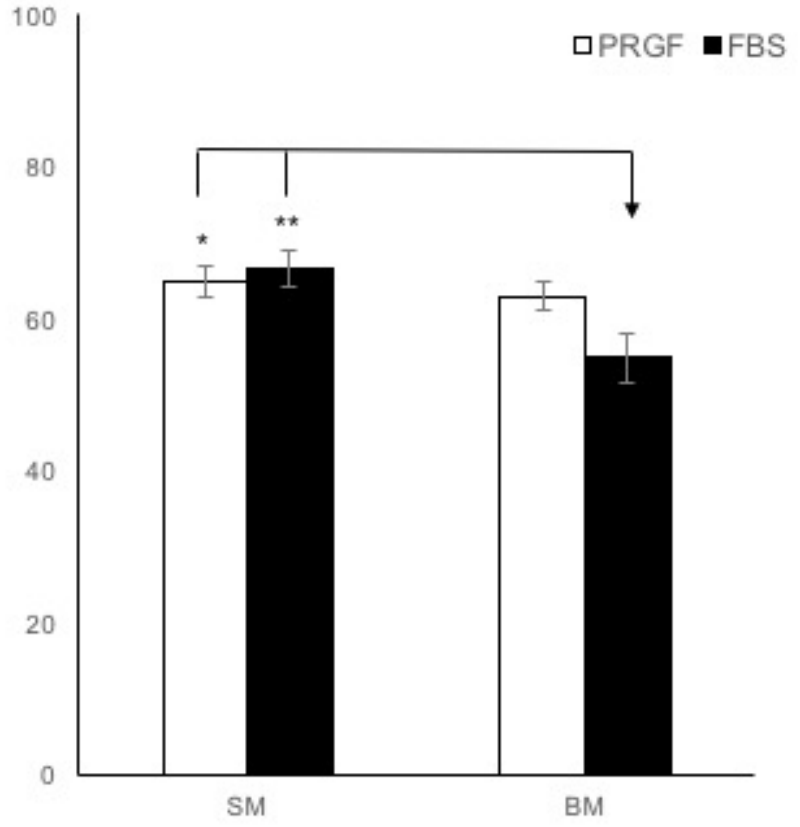

Figure 5: Percentage of p63-α expression (%) of the limbal epithelial cells at 7 days of incubation in a supplemented medium (SM) or basal (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). *p<0.05; **p<0.01.

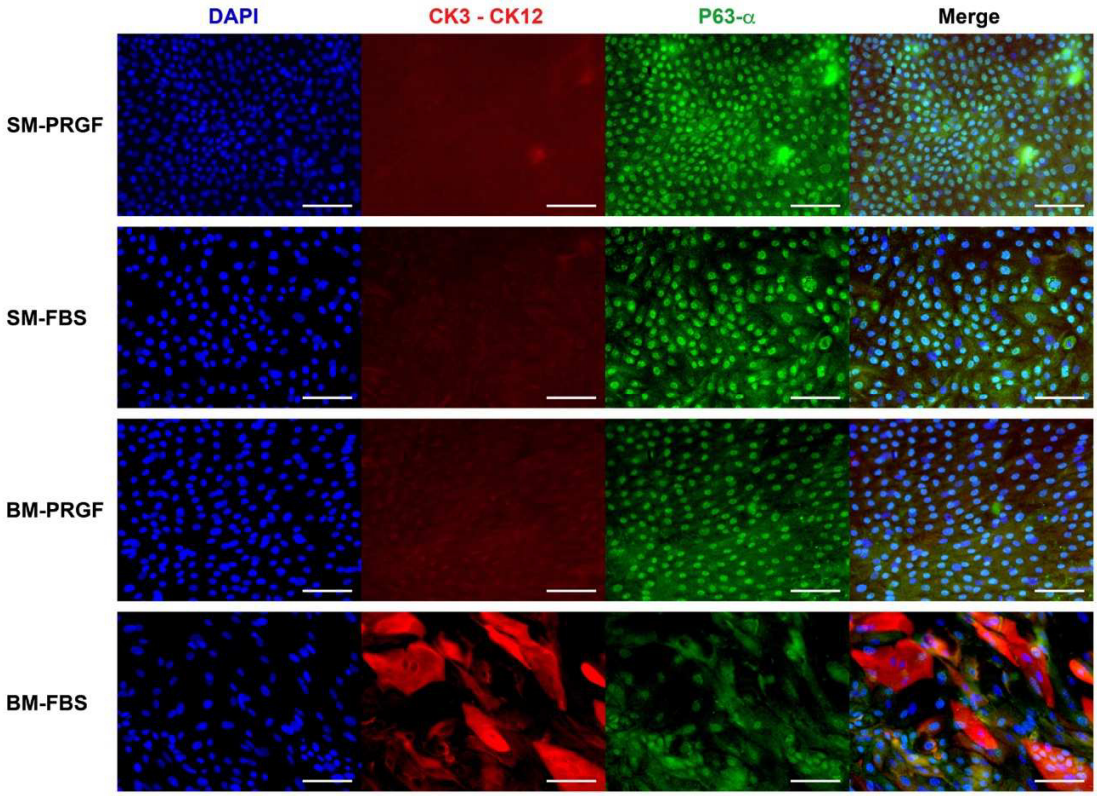

Figure 6: Immunofluorescence microphotographs for DAPI (blue), CK3-CK12 (red) and p63-α (green) after 7 days of culture in a supplemented (SM) or basal medium (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). Scale bar 100μm.

Colony-forming efficiency (Figures 7 and 8) revealed a significantly higher ($p<0.01$) percentage of colonies in the cultures supplemented with PRGF compared with the ones supplemented with FBS, both in SM (5.06±0.44 and 2.19±0.30) and BM (3.89±0.31 and 0.61±0.07). Moreover, statistically significant differences ($p<0.01$) were found between BM-PRGF (3.89±0.31) and SM-FBS (2.19±0.30).

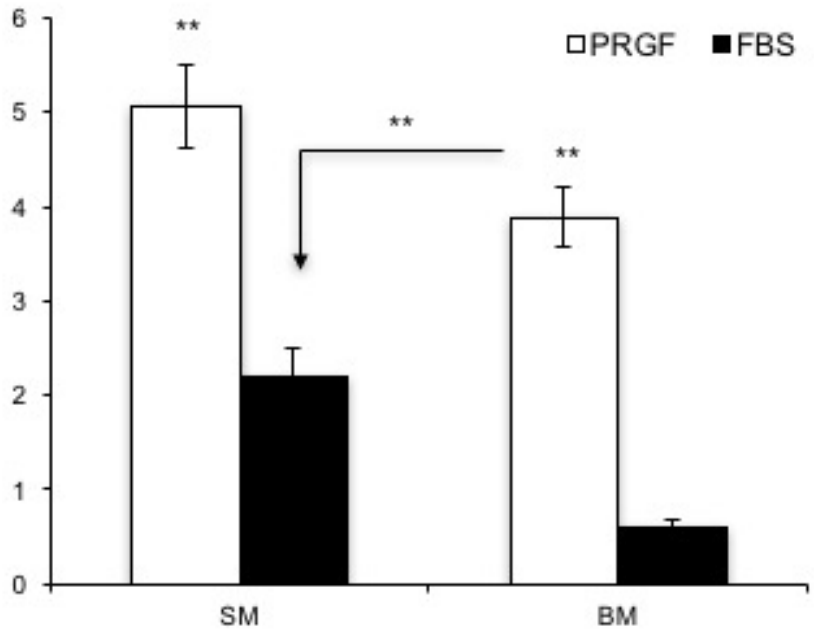

Figure 7: Colony forming unites percentage in incubation in a supplemented medium (SM) or basal (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS). **p<0.01.

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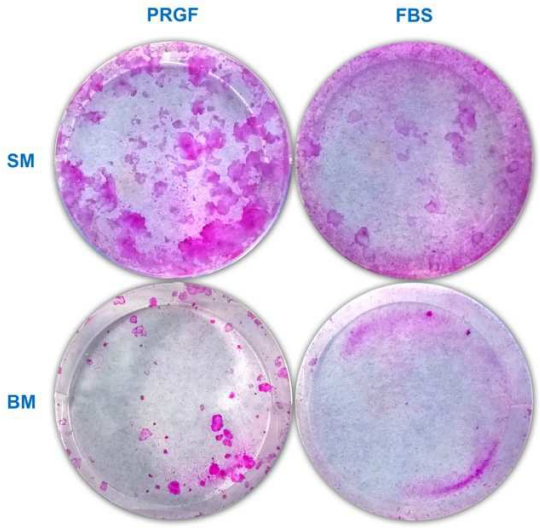

Figure 8: Representative macroscopic view of the colonies stained with rodamine after 12 days of culture in a supplemented medium (SM) or basal (BM) supplemented with 10% of plasma rich in growth factors (PRGF) or fetal bovine serum (FBS).

B. PRGF Fibrin Membrane as a Scaffold for LEPC Culture

PRGF fibrin membranes were effectively obtained by the protocol here described (Figure 9).

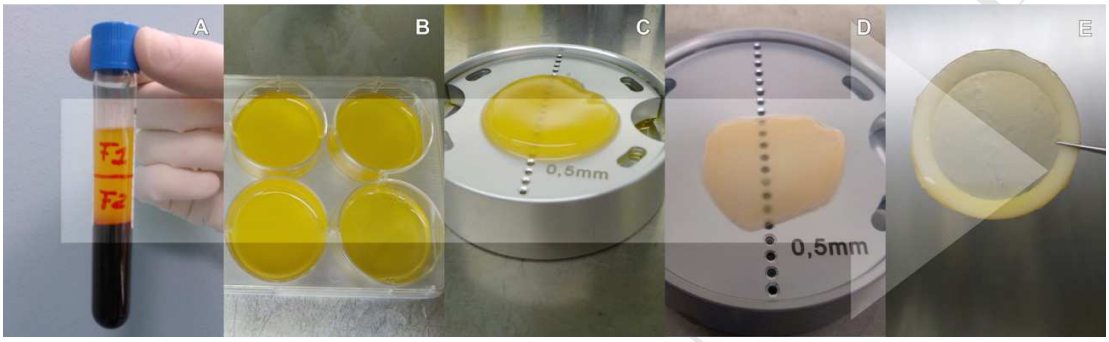

Figure 9: Sequence for obtaining a PRGF fibrin membrane. (A): Blood obtained in a citrated blood tube. (B): PRGF-fibrin gel after adding $CaCl_2$. (C): PRGF-fibrin gel placed in the fibrin membrane shaper. (D): PRGF-fibrin membrane after being flattered against the 0.5 mm thick fibrin membrane shaper. (E): Macroscopical appearance of the PRGF-fibrin membrane onto a nitrocellulose disk.

LEPCs expanded on PRGF fibrin membranes were evaluated by hematoxylin-eosin stain and immunofluorescence against phenotypical markers CK3-CK12, ABCG-2, and p63 (Figure 10).

LEPCs cultured on PRGF fibrin membrane appeared as a confluent monolayer expressing limbal progenitor cell markers, ABCG-2 in the cytoplasm and p63 in a nuclear localization. None of the cells cultured on PRGF fibrin membrane expressed the markers of differentiated epithelial cells CK3-CK12.

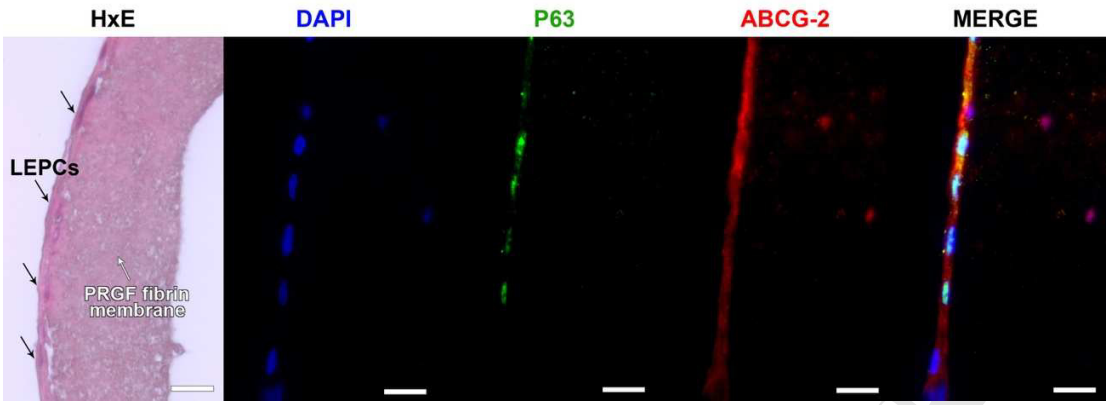

Figure 10: Hematoxylin-eosin and immunofluorescence against ABCG-2 (red) and p63 (green) of cultured limbal epithelial cells onto PRGF fibrin membrane. Nuclei are stained in blue. Scale bar 20 μm.

SEM images showed that the PRGF fibrin membrane has a complex three-dimensional fibrillar structure capable of allowing LEPCs adhesion and proliferation displaying their typical polygonal morphology (Figure 11).

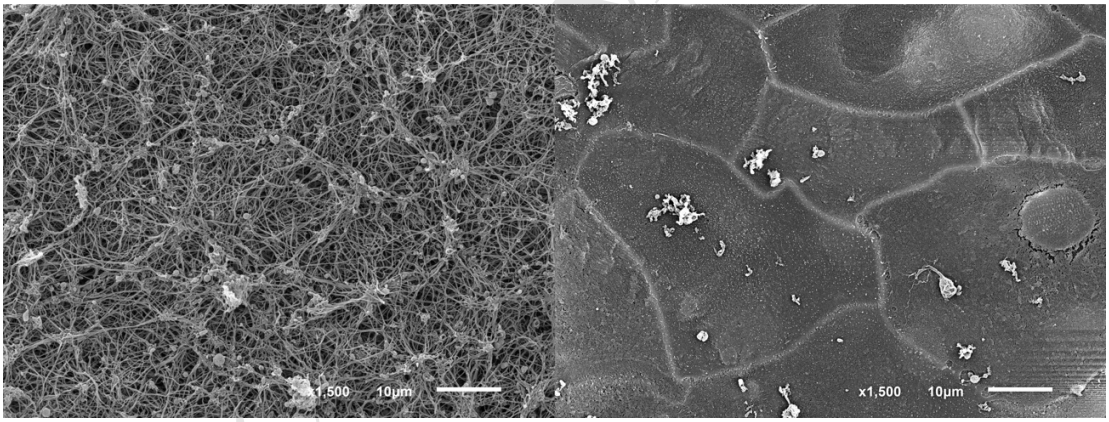

Figure 11: SEM micrographs of PRGF fibrin membrane without (left) or with cultured limbal epithelial cells (right).

IV. Discussion

Ex vivo expansion of limbal epithelial stem cells is a promising treatment for limbal stem cell deficiency, because it increases the performance of the conventional treatment, minimizing the required amount of donor limbus. Despite the good results published in the literature [12-14], the expansion of human limbal stem cells from the limbus for the regeneration of the ocular surface has a number of limitations, including the use of xenogeneic factors.

Classically, culture media include in their composition substances that promote the proliferation of cells, such as FBS, insulin, hydrocortisone, EGF, T3, adenine, or cholera toxin [15]. Serum is the most commonly used supplement in cell culture and tissue engineering because of its effect on the proliferation and maintenance of an undifferentiated state [16]. The majority of serums are of animal origin, mainly bovine, implying significant risks as the development of antibodies against animal proteins or pathogens transmission [17].

In the current study, an autologous method has been developed using PRGF as a single supplement of a basal culture medium and as a fibrin scaffold for culturing and delivering LEPCs.

PRGF as a supplement of the cell culture media has been shown to induce a greater cell proliferation against the FBS, measured both in growth area from the limbo explant as in number of cells, regardless of the medium used was SM or BM. Notably proliferation area and cell number was higher in the BM-PRGF than SM-FBS, showing statistically significant differences in the number of cells. We can say that a BM supplemented only with PRGF achieves greater proliferation of limbal cells than a SM supplemented with FBS, which includes in its composition substances unsuitable for clinical application. Comparing the concentration of growth factors described in PRGF and FBS, it is observed that the concentration of PDGF-AB is substantially higher in the PRGF to FBS, while the concentration of TGF-β, with inhibitory effects on the growth of epithelial cells, is lower [7,18]. These differences in the levels of growth factors may explain the greater effect on proliferation observed.

The use of human autologous or allogeneic serum has also been proposed as a substitute for the FBS in the culture of conjunctival, corneal and oral epithelia for ophthalmic application [19,20]. However, PRGF contains a higher concentration of EGF, among other growth factors, than the human serum, and even than other platelet-rich plasmas, that has resulted into a greater effect on the proliferation of epithelial corneal cells both *in vitro* and *in vivo* studies [7, 8, 21].

Regarding cellular morphology, in media supplemented with PRGF, cells were smaller and homogeneous with an oval shape, which corresponds to the typical morphology of undifferentiated

epithelial cells [22,23]. Moreover, when PRGF supplemented media are used the probability of obtaining cells with an undifferentiated morphology is multiplied by three.

We confirm that the PRGF helps to maintain the undifferentiated state of limbal cells by analyzing the expression of epithelial progenitor cell marker p63-α and differentiated epithelial marker CK3-CK12 [24]. The data shows that when using PRGF as media supplement, the expression of p-63α is maintained even in a BM, while the differentiated epithelial marker CK3-CK12 was only clearly expressed in BM-FBS cultures. However, Suri et al., found no differences in the expression of ABCG2 and p63-α between a complete medium supplemented with FBS or platelet lysate, which also included cholera toxin [25]. This could be due to a lower concentration of growth factors by lower efficiency in the lysate for the excretion of proteins from the granules-α.

Cell clonogenicity was assessed by determining the percentage of CFU present under each culture medium. A higher percentage of CFU was found in media supplemented with PRGF, and even highly statistically significant differences between the BM-PRGF and SM-FBS were observed. The CFU rates obtained are higher than those reported in the literature for explant culture methods without murine 3T3 fibroblasts, it could be attributed to the culture method described [26]. Several studies have shown that platelet lysate, both allogeneic and autologous, is superior to FBS in stimulating the proliferation of mesenchymal cells, reducing the time to confluence and increasing the proliferation and size of the CFU, these results support the findings in this work [27]. These results are consistent with the increased presence of cells expressing phenotype of undifferentiated cells as undifferentiated limbal cells have a greater capacity for proliferation and clonogenic cells than differentiated corneal epithelial cells [28]. The discrepancies between the p-63α expression and the percentage of CFU may be due to the fact that not only the p-63α expressing cells have clonogenic capacity , since it have been described that holoclones derived from the limbus are rich in p63-α, but meroclones contain little, and paraclones contain none [29].

Another factor involved in cell dedifferentiation is the interaction between fibroblasts and limbal epithelial cells [30]. This interaction can be achieved by growing on a layer of human or murine feeder cells, by the presence of native fibroblast included in the limbo explant or after cell isolation by collagenase A [31-34]. In the present study we opted for a completely autologous culture method, avoiding the use of xenogeneic products such as murine feeder cells, facilitating cultivation and preventing possible cell damage from the use of enzymes. The proposed cultivation method maintains native stromal cells in the limbal explant, enhancing the proliferation of the limbal cells.

The number of undifferentiated cells present in culture is a prognostic factor for the success of the transplantation of *ex vivo* expanded limbal cells [35], so it is expected that with cells grown in the presence of PRGF, better clinical results are obtained in the treatment of LSCD while patients safety is increased.

Several materials have been proposed as a scaffold for ocular surface reconstruction, the AM being the most widely used [36]. The growth factors present in the AM favor the proliferation and undifferentation of the limbal cells [22]. In addition, AM has shown to inhibit TGF-β-induced myofibroblast differentiation in cultured human corneal and limbal fibroblasts [37] and to reduce haze in an animal model of laser keratectomy [38]. In the present work, a PRGF fibrin membrane was developed with the patient's own blood. SEM studies showed that the PRGF membranes have a fibrillar ultrastructure, and it is already described to have embedded components such as fibrin, which favor cell adhesion [9]. PRGF membranes allowed the adhesion and proliferation of LEPCs, maintaining the morphology and marker expression of progenitor cells. This is an essential factor for the prognosis of the limbal transplant, since the LEPCs will maintain the epithelial cell turnover. As the AM, PRGF provides fundamental growth factors for tissue regeneration, it has anti-infectious and proliferative properties and inhibits fibrosis [7,38,39], having been shown to stimulate corneal wound healing and reduce haze formation in an animal model of photorefractive keratectomy [39]. Moreover, PRGF fibrin membrane presents several advantages over the amniotic membrane, like the autologous origin, the widely access, its versatility and lower costs.

III. Conclusions

PRGF could be used in corneal tissue engineering, supplementing the culture media, even in a basal media without any other additives, as well as scaffold for the culture of the LEPCs. The novelty of the present results resides in the culture of LEPCs on a PRGF fibrin membrane using PRGF as a human-derived supplement of the culture medium. Although additional studies would be necessary to assess its efficacy *in vivo* this approach represents a promising alternative to the actual xenogeneic supplementation in the cell therapy for corneal diseases, minimizing the receptor risks due to the use of receptor own blood.

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