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 reepithelialization. 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.

Key Words: wound healing, reepithelization, blood derivatives, cell migration

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 biochemical 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.7-10 Our group originally adapted a plasma preparation, which was first used in maxillofacial surgery and trauma,12 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 defects13 and dry eye syndrome.14 More recently, we have adapted the methodology to obtain s-PRGF to be used for the first time in a rabbit animal model18 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 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% CO2 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 epidermal 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% CO2 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 χ2 tests, as well as a time-dependently multiple comparison with the generalize linear model method.
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 mm², 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

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

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

The results are expressed as mean remaining denuded area ± SD in square millimeter.

*Statistically significant differences with respect to control (P ≤ 0.01) (time-dependently multiple comparison, generalized linear model method).

†Statistically significant differences with respect to control (P ≤ 0.05) (Mann-Whitney U test).

**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 ≤ 0.05) (χ² test).

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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).

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. An important determinant of the healing potential of tissues is their ability to increase blood supply through physiological angiogenesis. 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. Furthermore, these molecules may suppress inflammation in the case of impairment of epithelialization and also have antimicrobial effects.

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 amounts of growth factors, cytokines, and other signaling molecules that promote corneal epithelial healing. The results of this study indicate that s-PRGF is the most effective treatment for promoting corneal epithelial healing, followed by AS and PRP, with the control treatment being the least effective.

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

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

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

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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,⁴⁻³⁴ with some authors asserting that the presence of leukocytes can be highly beneficial because of their antimicrobial properties,⁴⁻²⁶ 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.²⁵

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,³⁶ 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.³² 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.³³ 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,³⁷ 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 cells³⁹ 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 deepithelialization 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 wounds²⁻¹⁸,⁴⁰ and corneal alkali burns,³¹ 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 previous20 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.

ACKNOWLEDGMENTS
The authors thank SGIker (UPV/EHU, MICINN, GV/EJ, and ESF) for providing technical support.

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