BASIC INVESTIGATION

Short- and Long-Term Stability of Plasma Rich in Growth Factors Eye Drops

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Purpose: To analyze whether plasma rich in growth factors (PRGFs) eye drops preserve their activity and biological properties after storage for 9 and 12 months at −20°C, and at 4°C, and at room temperature (RT) for 3 and 7 days in comparison to fresh samples (t0).

Methods: PRGF eye drops were obtained from 6 healthy donors. Then, they were stored for 9 and 12 months at −20°C. At each time, different PRGF eye drops samples were thawed and maintained at RT or at 4°C for 3 and 7 days. Platelet-derived growth factor-AB, epidermal growth factor, transforming growth factor-β1, vascular endothelial growth factor, angiopoietin-1, and thrombospondin-1 were analyzed at each time and temperature of storage. In addition, the pH level, the microbial contamination, and the proliferative potential on primary human corneal stromal fibroblasts human keratocytes of each obtained PRGF eye drops were also evaluated.

Results: All growth factor levels were preserved at each time and storage condition. No differences were observed on the human keratocytes proliferation after treatment with PRGF eye drops at any studied time or temperature. No microbial contamination was observed in any of the PRGF eye drops. Finally, the pH levels increased significantly after 9 and 12 months of storage at −20°C compared with t0.

Conclusions: PRGF eye drops can be stored for up to 12 months without reduction of the main growth factors and proteins and without any microbial contamination. Furthermore, the biological activity of the PRGF eye drops is maintained after storing for 3 and 7 days at 4°C or at RT.

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Plasma rich in growth factors (PRGFs) eye drops has been successfully used for the treatment of several ocular surface disorders, such as dry eye, persistent epithelial defects, Sjögren syndrome, and neurotrophic keratitis, among others.[1–6] These fruitful results could be attributed to the high similarities found between the characteristics of PRGF eye drops and the tear film. Both contain a wide range of proteins and growth factors, such as platelet-derived growth factor-AB (PDGF-AB), epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), fibronectin, and vitamin A, among others, that are involved in the different biological processes of the ocular tissue regeneration.[7–9] Other important features of PRGF eye drops shared with the tear films are their antimicrobial, antifibrotic, and antiinflammatory properties.[10–12]

In addition, it is necessary to highlight that the PRGF eye drops therapy used for the treatment of eye disorders is produced under a standardized protocol in comparison to other blood-derived products.[13] Furthermore, PRGF eye drops manufacturing follows the regulatory framework published on May 23, 2013, by the Spanish Agency for Medicines and Health Products in which the classification of the nonsubstitute therapeutic use of autologous plasma and its fractions, components, or derivatives as drugs for human use to attend special needs was established.[14]

Most of the ocular surface disorders are chronic diseases that must to be treated for a long time to achieve successful outcomes. Therefore, therapies used for the management of these diseases should maintain their functionality and stability for several months to be used daily along this time. To ensure the preservation of the biological properties of PRGF eye drops in clinical practice, patients are instructed to store the PRGF eye drops containers in use at 4°C or at room temperature (RT), whereas the rest of the droppers should be stored at −20°C until required. Previous works have shown that PRGF eye drops can be stored for up to 6 months without reduction of the main proteins and growth factors implicated in ocular surface wound healing.[15,16] Furthermore, it was demonstrated that PRGF eye drops can preserve their composition and biological activity both at 4°C and at RT for 3 days.

However, some ocular disorders need a short treatment period, from days to a few weeks, to achieve a complete restoration of the ocular surface tissue. However, the symptoms

of the underlying chronic pathology may appear in a few months, thus requiring a new therapy application. On the other hand, in some countries such as Germany, competent authorities might require the quarantine of the eye drops preparations until the results of the bacterial contamination tests are available, which could take up to 1 to 2 weeks.[17] In this sense, the storage time of the blood derivative eye drops is already progressing. In addition, some ocular disorders are unilateral, affecting only one eye, in which case, the PRGF eye drops in use may last longer than the current 3 days treatment established for their short-term stability stored at RT or at 4°C. In this regard, it seems very interesting to evaluate whether long-term storage at −20°C could reduce the efficacy of the PRGF eye drops therapy and to investigate how long PRGF eye drops remain stable under different storage conditions.

The purpose of this study was to analyze whether PRGF eye drops maintain their content in proteins and growth factors as well as their biological potential after preservation at −20°C for 9 and 12 months compared with the freshly prepared eye drops. The conservation of PRGF eye drops after 3 and 7 days both at 4°C and at RT was also determined. For these purposes, the concentration of different growth factors, the pH level, the proliferative potential, and the microbial contamination against the freshly obtained eye drops were evaluated at each time and storage condition.

MATERIAL AND METHODS

PRGF Preparation

The study was approved by the local clinical research ethics committee (number of protocol BTI-04-IV/18/2018-7). The study was performed following all the declaration of Helsinki principles. Blood from 6 healthy donors was collected after they signed the informed consent. PRGF eye drops from each donor were performed using the Endoret Ophthalmology kit (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). After blood centrifugation, the whole plasma column was drawn off, avoiding buffy coat collection containing the leukocytes. Platelet concentration was measured with a hematology analyzer (Micros 60; Horiba ABX, Montpelier, France). The harvested platelet-rich plasma was activated with calcium chloride and incubated until complete clot retraction. Finally, the obtained supernatant was filtered with a filter pore size of 0.2 μm (Merk Millipore, Carrigtwohill, Ireland) and aliquoted into the BTI eye dropper to be used as fresh samples (t0) or to be stored at −20°C for 9 (t9) months and 12 (t12) months. After this time, 5 aliquots from each donor and each storage time (t9 and t12) were removed from the freezer and thawed, and then, one of them was immediately used and the rest were stored for 3 (3 d) and 7 (7 d) days at 4°C or at RT until their use.

Sterility Analysis

Sterility analysis were carried out after the European Pharmacopoeia principles for the sterility test. One milliliter from each PRGF samples stored at different times and temperatures was collected to check the sterility. Briefly, thioglycollate broth for the culture of anaerobic bacteria and tryptic soy broth for the culture of both fungi and aerobic bacteria were used for qualitative determination of microbial noncontamination of the different PRGF 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. Increasing of broth turbidity was considered as a positive indicator of microbial contamination. Sterility was considered when no contamination of microorganisms occurred.

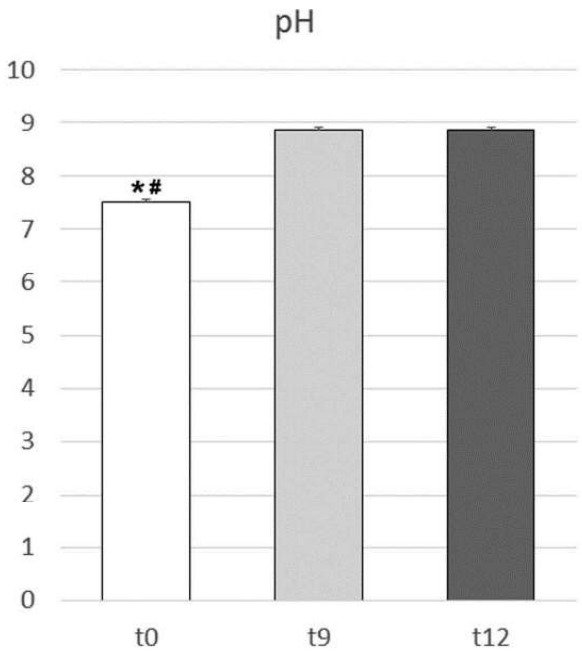

FIGURE 1. Levels of pH measured in fresh PRGF-Endoret samples (time 0) and in PRGF-Endoret eye drops after storage at −20°C for 9 (t9) and 12 (t12) months. Significant differences were observed among pH values obtained in t0 samples in comparison to t9 (*$P < 0.05$) and t12 (#$P < 0.05$).

PRGF Eye Drops Characterization

The stability of PRGF eye drops at each time and storage temperature was evaluated by the quantification of several growth factors involved in ocular surface tissue regeneration such as EGF, PDGF-AB, TGF-β1, VEGF, angiopoietin-1 (ANG-1), and thrombospondin-1 (TSP-1). They were measured using commercially available Quantikine Colorimetric Sandwich enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The pH was analyzed with a pH meter (Thermo Scientific, Madrid, Spain) on the fresh samples (t0) and on those that have been stored at −20°C for 9 and 12 months obtained from each donor.

Cell Culture

Primary human corneal stromal fibroblasts [termed human keratocytes (HK)] (ScienCell Research Laboratories, San Diego,

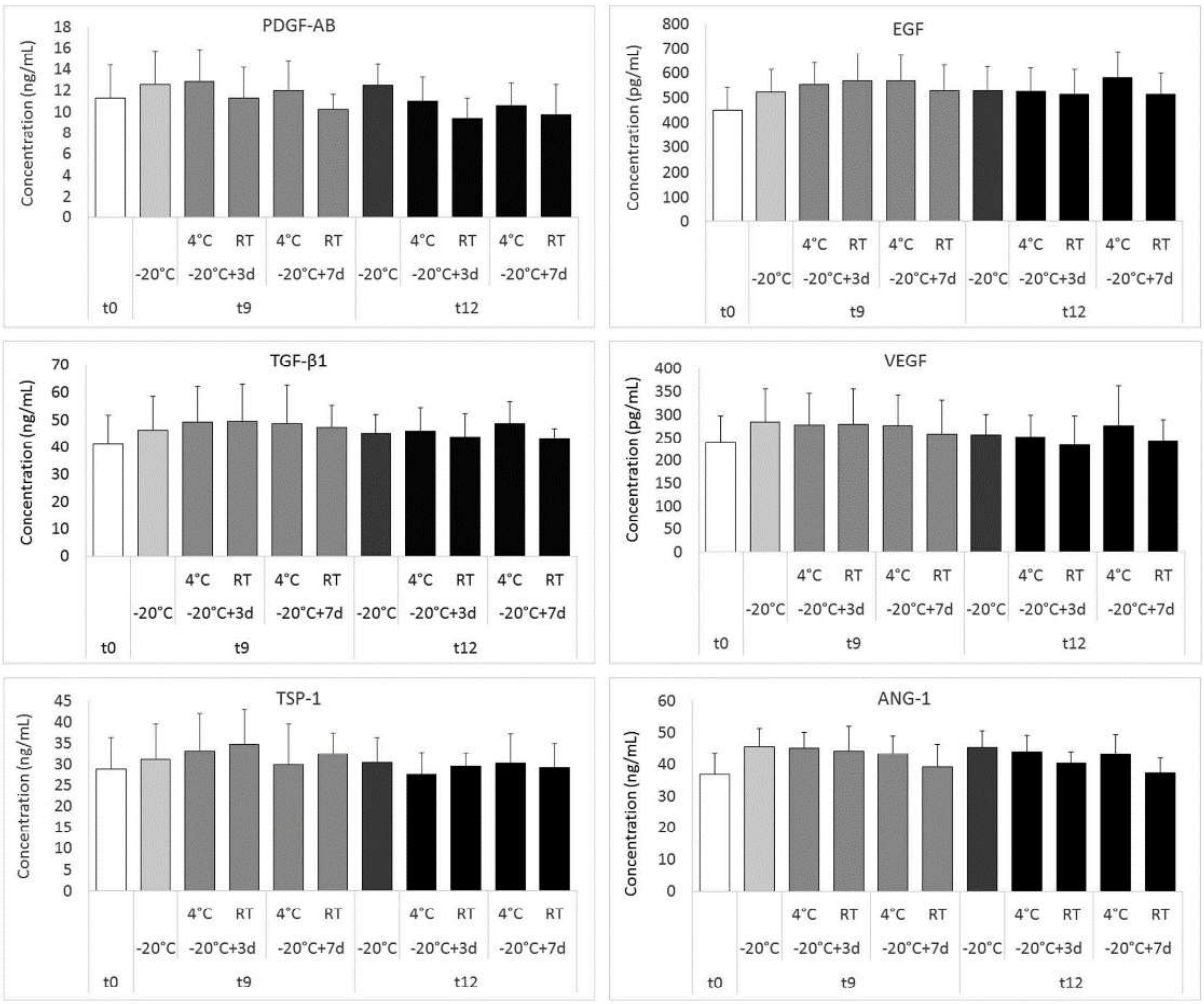

FIGURE 2. Concentration of the different growth factors analyzed in the study PDGF-AB, EGF, TGF-β1, VEGF, TSP-1, and ANG-1 in the PRGF eye drops samples stored at different times and temperature conditions. No significant differences ($P > 0.05$) were observed among the distinct time and storage conditions of the growth factors analyzed in the PRGF eye drops.

CA) were cultured following the manufacturer's instructions. Briefly, the cells were cultured at 37°C and 5% CO_2 atmosphere in a fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories), 2% fetal bovine serum, and antibiotics until confluence. Then, animal origin-free trypsin-like enzyme (TrypLE Select; Gibco-Invitrogen, Grand Island, NY) was used to detach the cells, and trypan blue dye exclusion method was carried out to assess the cell viability. Finally, these cells were used in proliferation assays.

Cell Proliferation

Cells were seeded at a density of 5000 cells/cm^2 on 96-Well Optical-Bottom black microplates in serum-free medium supplemented with 20% (vol/vol) of PRGF eye drops obtained from each donor and kept at each time and temperature condition mentioned above. Then, the cells were incubated with each PRGF sample for 96 hours. The cell density was analyzed using the CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA). Briefly, the culture medium was removed, and the wells were carefully washed with phosphate-buffered saline for not disrupting the cellular monolayer. Later, the plate was frozen at −80°C to improve the cell lysis efficiency. After that, the plate was thawed at RT by performing the CyQUANT assay after leaflet instructions and including a RNase A treatment. The fluorescence of the sample was measured using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany). A control group (control) was included on each plate as an internal control of basal cell growth and as a

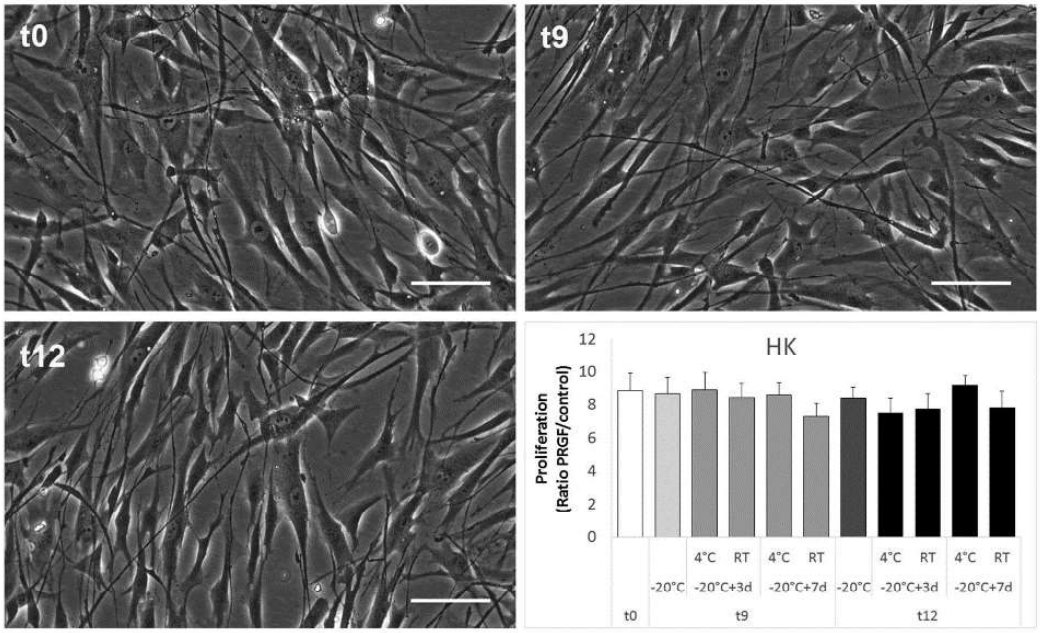

FIGURE 3. Representative phase contrast photomicrographs showing the proliferation of HK cells treated with fresh PRGF samples (t0) and with samples stored at −20°C for 9 (t9) and 12 months (t12). There were no significant differences ($P > 0.05$) among the proliferation rate induced by PRGF eye drops stored at any time and temperature condition.

reference control of cell growth between the different plates used in the study. For this control, the cells were incubated with basal fibroblast medium and 0.1% fetal bovine serum and antibiotics. To normalize the cell growth of the samples incubated in different plates, the fluorescence value obtained in each PRGF sample well was divided by the mean value of the control obtained on the corresponding plate (ratio = PRGF sample/control).

Statistical Analysis

Data are expressed as mean ± SD. After the analysis of the normal distribution and homoscedasticity of the results, one-way analysis of variance was used to assess the differences among the variables at the 3 different times points (t0, t9, and t12) and at distinct temperatures of storage (−20°C, RT, and 4°C), whereas the nonparametric Kruskal–Wallis test with a subsequent Mann–Whitney analysis test for multiple comparisons between groups were used in cases where no normality was detected. 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 PRGFs Samples

The different samples of PRGF eye drops were obtained from 6 healthy donors (3 women and 3 men) with a mean age of 46 years (SD = 8), ranging from 31 to 52 years. PRGF samples showed a 1.95-fold increase on platelet concentration over peripheral blood. PRGF contained no detectable levels of leukocytes.

Sterility Analysis

No microbiological contamination was detected in any of the PRGF eye drops stored at different times and temperature conditions. Furthermore, no sign of microorganism growth was observed in any of the cell cultures assayed for proliferation with the different PRGF eye drops.

pH Levels

The pH levels of PRGF eye drops obtained on the same day of the assay (fresh samples, t0) or those samples stored at low temperatures (−20°C) for 9 (t9) or 12 (t12) months are represented in Figure 1. The pH levels (mean ± SD) increased significantly from the t0 (7.51 ± 0.04) to t9 (8.85 ± 0.06) and t12 (8.87 ± 0.04).

Levels of Growth Factors at Different Time and Temperature of Storage

The content of different growth factors related to the ocular tissue regeneration were measured in different PRGF eye drops samples on the day of collection (fresh samples, t0) and after storage at −20°C for 9 (t9) and 12 (t12) months. The different growth factor concentrations measured at each storage time is represented in Figure 2. None of the growth factors analyzed in the study showed a significant change ($P > 0.05$) after PRGF eye drops storage for 9 and 12 months

in comparison to the fresh samples (t0). In fact, the concentration of all growth factors analyzed in this study remained unchanged at each studied time.

At each study time (9 and 12 mo), 4 samples from each donor were thawed from the freezer and 2 of them were stored for 3 days at 4°C and RT and the other 2 were kept for 7 days at 4°C and RT. The levels of several growth factors (PDGF-AB, TGF-β1, VEGF, EGF, ANG-1, and TSP-1) analyzed in the eye drops stored during 3 and 7 days at 4°C or RT are presented in the Figure 2. No significant differences ($P > 0.05$) were observed in the content of the different growth factors among the different times and storage conditions evaluated in this study.

Proliferation Assay

Representative images of HK cells incubated for 96 hours with fresh PRGF eye drops (t0) and with PRGF eye drops stored at −20°C for 9 and 12 months are shown in Figure 3. The proliferation rate of HK cells showed no significant differences ($P > 0.05$) after treatment with the PRGF eye drops maintained at different time and temperature conditions.

DISCUSSION

In this study, the stability of PRGF eye drops stored at −20°C for 9 and 12 months and for 3 and 7 days at RT or at 4°C was evaluated. Recent studies have demonstrated the efficacy of PRGF eye drops for the treatment of several ocular surface disorders.[2,18,19] However, the storage of the PRGF eye drops for long- and short-term use could increase, assuming that some clinical doses are based on a daily use of the eye drops from 2 to 4 instillations and that the treatment time could last more than 9 months.[2,20] In this sense, we decided to evaluate the composition and biological activity of PRGF eye drops after different storage conditions.

Our results show that no significant differences ($P > 0.05$) were found in the levels of different growth factors and cytokines involved in ocular surface tissue regeneration such as TGF-β1, EGF, VEGF, PDGF-AB, TSP-1, and ANG-1 in the PRGF eye drops samples after their storage both at 9 and 12 months at −20°C. In addition, the levels of these proteins remain similar in comparison to the PRGF eye drops obtained at time 0 from the different donors (fresh samples). Current results could also simplify the logistic for patients depending on the procedure for chronic disorders treatment over long-term periods. For instance, this could allow patients to initiate the treatment again when symptoms reappeared without further blood collection and eye drop manufacturing.

Some stability studies of blood derivative products have shown that several growth factors and proteins analyzed at different storage temperatures for up to 24 hours were reduced in their concentration levels after storage at RT or 4°C.[21] In consequence, patients are specifically instructed to avoid keeping the eye droppers in their pocket or near to a warm place for more than a few moments and to discard those dispensers that have been left unrefrigerated for more than 2 to 3 hours. However, no differences in the concentration of the main protein levels were observed in our study among the PRGF eye drops obtained at time 0 (fresh samples) or stored at −20°C for 9 and 12 months and the eye drops stored at RT or 4°C for 3 and 7 days. In addition, no significant differences ($P > 0.05$) were observed in corneal stromal fibroblast proliferation after treatment either with the PRGF obtained at time 0 (fresh samples) or stored up to 9 and 12 months at −20°C and maintained at 4°C or RT for 3 and 7 days. The latter results need to be highlighted because they significantly improve the dosage of autologous eye drops, allowing the consumption of the PRGF eye drops in use for up to 7 days without dependence on a cold chain.

The pH of the tear films has similar values to the physiological levels (pH = 7.4). Likewise, eye drops obtained from blood-derived products also show comparable pH values as the tear film.[17,22] However, the eye is able to tolerate pH values ranging from 3.5 to 9, thanks to the buffering capacity of the tears.[23] In this work, the pH levels increased significantly in PRGF eye drops from mean values of 7.5 in fresh samples to values approximately 8.8 in PRGF eye drops stored at −20°C for 9 and 12 months. Despite that, our results show that pH levels remained in values below 9 during the entire study period; hence, PRGF eye drops stored at −20°C for up to 12 months should be perfectly tolerated by the ocular tissues.

To avoid the risk of chemical toxicity, blood-derived eye drops are commonly used without preservatives.[24] Recent studies have demonstrated the natural antimicrobial properties of the blood-derived products.[10,25] However, special care is taken to avoid the potential microbial contamination of the eye drop dispenser related to its long-term use.[26,27] As a consequence, it is recommended to keep the eye drops dispenser in use at 4°C for 5 to 7 days.[28] Despite this recommendation, microbial contamination was found in up to 25% of the eye drops dispensers analyzed.[24,29,30] In this study, no microbial contamination was detected in any of the eye droppers containing PRGF eye drops from each donors examined at each time point and at any of the evaluated temperature conditions. In addition, no microbial contamination was observed in the corneal stromal fibroblast cultures treated with PRGF eye drops stored at different times and temperatures.

In summary, this study shows that PRGF eye drops can be stored for up to 12 months without reduction of the main growth factors and proteins implicated in ocular surface tissue regeneration and without any microbial contamination. Furthermore, the biological activity of the PRGF eye drops is maintained when they are stored for 3 and 7 days at 4°C or at RT.

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