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

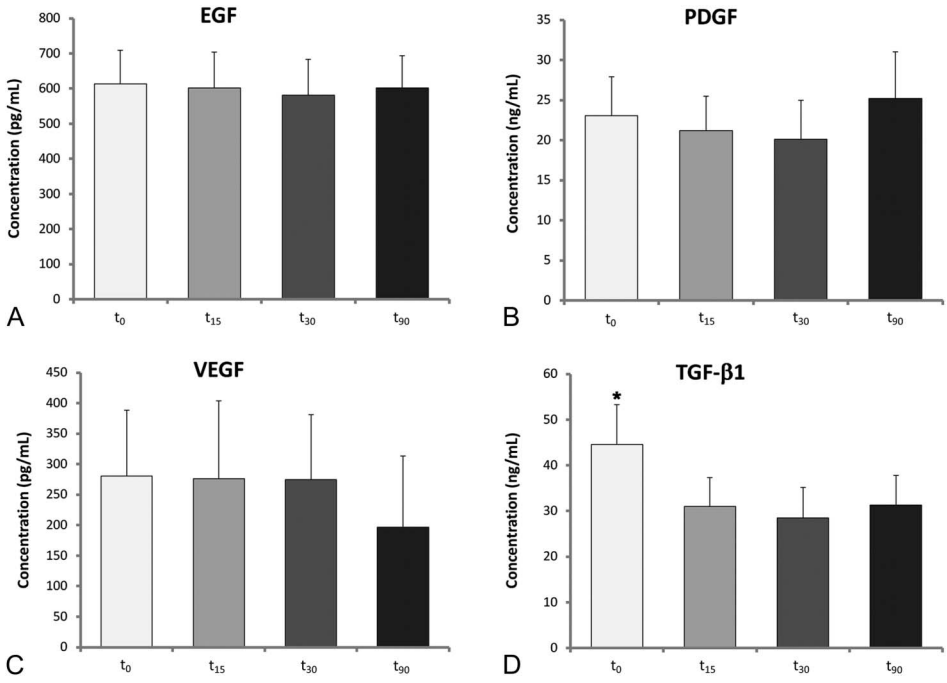

FIGURE 1. Growth factor concentration of fresh samples (t_0) of PRGF-Endoret eye drops and after storage at −20°C for 15, 30, and 90 days (t_{15}, t_{30}, and t_{90}, respectively; growth factor concentration values of storage at RT and at 4°C are not included). Levels of EGF (A), PDGF (B), VEGF (C), 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.

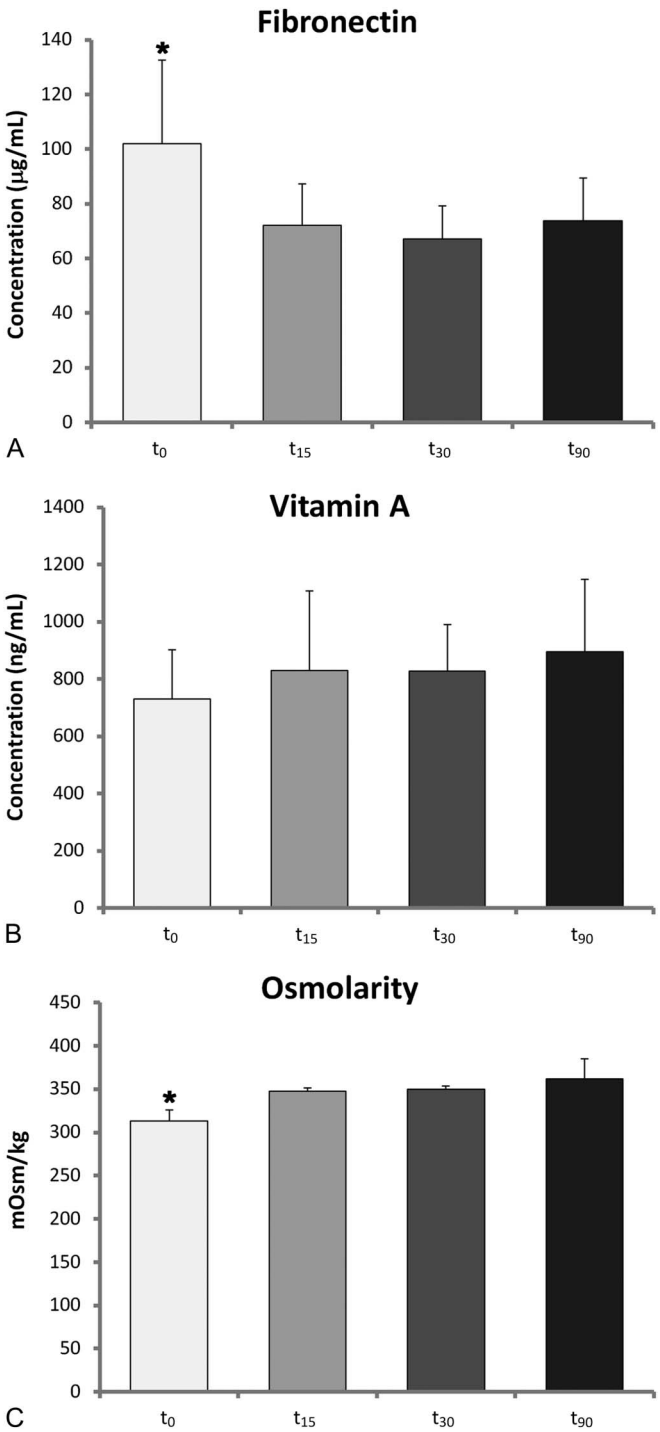

FIGURE 2. Characteristics of PRGF-Endoret eye drops for each time of the study [fresh samples (t_0) and after storage solely at −20°C for 15, 30, and 90 days]. Fibronectin concentration (A), vitamin A levels (B), and osmolarity (C). *Significant differences ($P < 0.05$) in the composition between PRGF-Endoret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

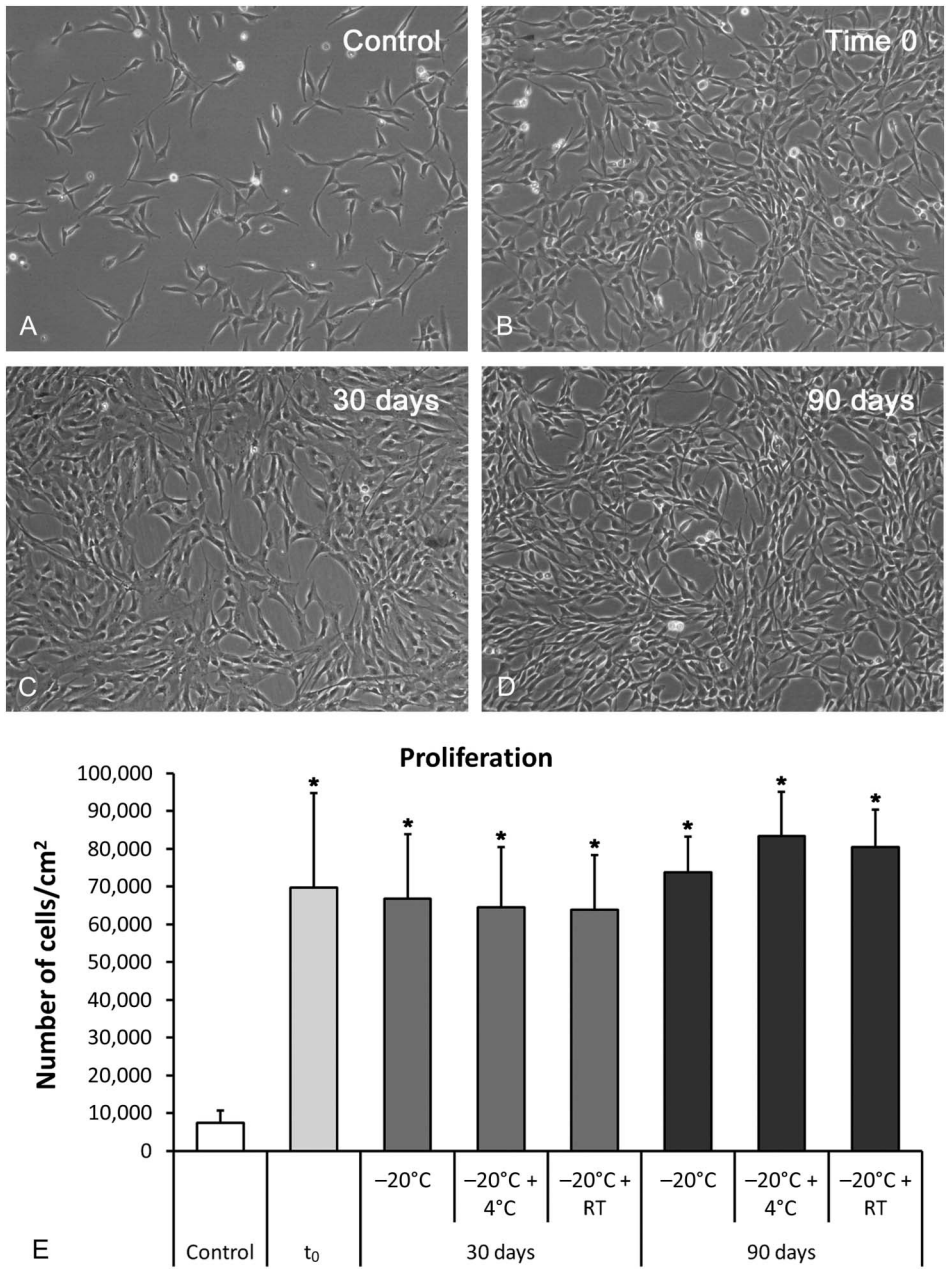

FIGURE 3. A–D, Phase contrast photomicrographs illustrating the proliferation rate of HK cells cultured with a control medium (A), with fresh PRGF-Endoret (B), or with PRGF-Endoret eye drops stored at −20°C for 30 (C) and 90 days (D). Proliferation of the HK cells after treatment with fresh or frozen 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

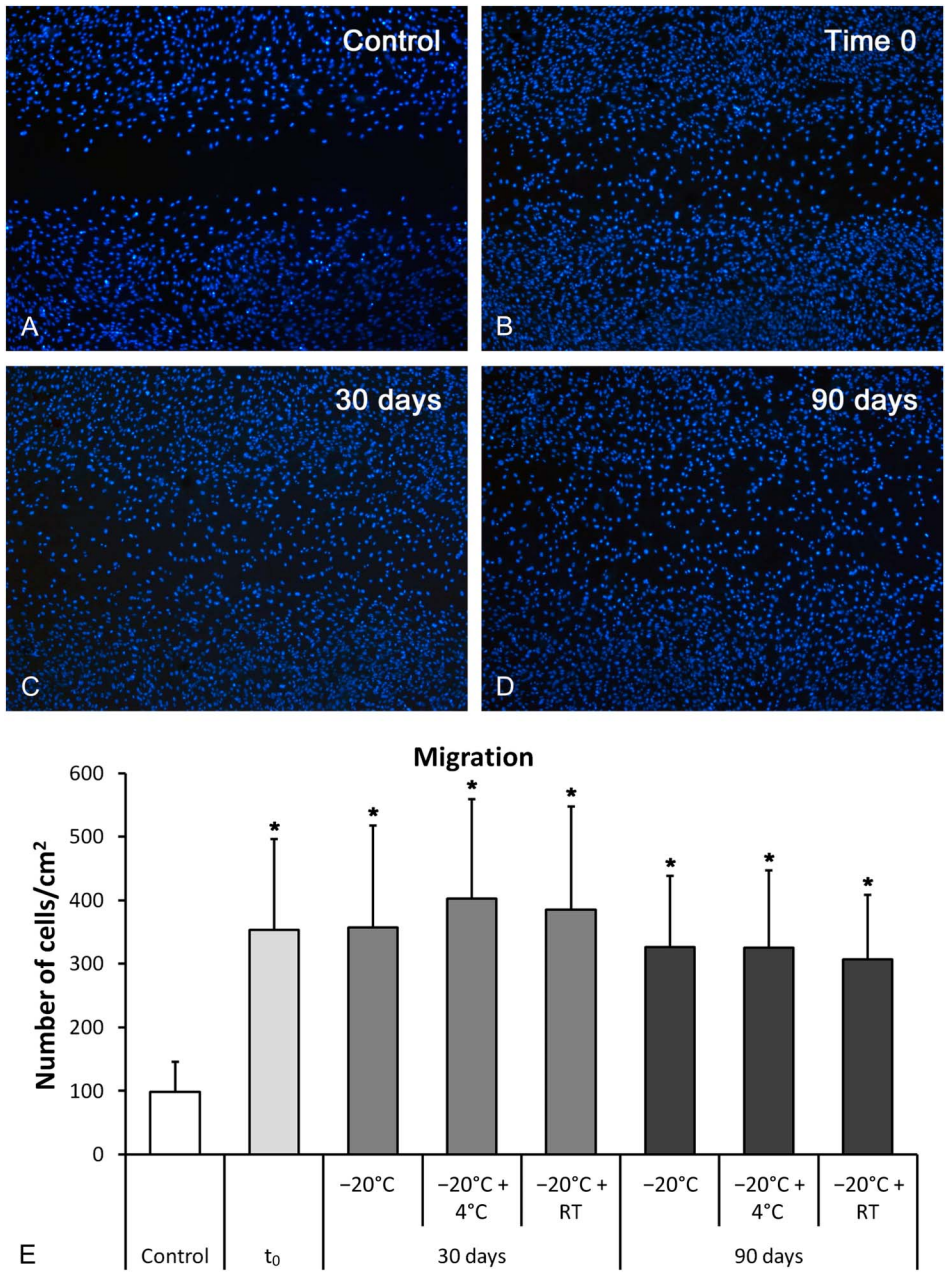

FIGURE 4. Migration assay in HK cells. (A–D) Images of Hoechst counterstaining of the migration assay in HK cells cultured with control medium (A), with fresh PRGF-Endoret (B), or with PRGF-Endoret eye drops stored at −20°C for 30 (C) and 90 days (D). Migration of the HK cells after treatment with fresh 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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