



# Stability of freeze-dried plasma rich in growth factors eye drops stored for 3 months at different temperature conditions

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### Abstract

**Purpose:** The purpose of this study was to analyze the biological content and activity of freeze-dried plasma rich in growth factors eye drops after their storage at 4°C and at room temperature for 3 months with respect to fresh samples (time 0).

**Methods:** Plasma rich in growth factors was obtained after blood centrifugation from three healthy donors. After platelet activation, the obtained plasma rich in growth factors eye drops were lyophilized alone or in combination with lyoprotectant (trehalose), then they were stored for 3 months at room temperature or at 4°C. Several growth factors were analyzed at each storage time and condition. Furthermore, the proliferative and migratory potential of freeze-dried plasma rich in growth factors eye drops kept for 3 months at different temperature conditions was evaluated on primary human keratocytes.

**Results:** The different growth factors analyzed maintained their levels at each time and storage condition. Freeze-dried plasma rich in growth factors eye drops stored at room temperature or 4°C for 3 months showed no significant differences on the proliferative activity of keratocytes in comparison with fresh samples. However, the number of migratory human keratocytes increased significantly after treatment with lyophilized plasma rich in growth factors eye drops kept for 3 months compared to those obtained at time 0. No significant differences were observed between the freeze-dried plasma rich in growth factors eye drops whether mixed or not with lyoprotectant.

**Conclusion:** Freeze-dried plasma rich in growth factors eye drops preserve the main growth factors and their biological activity after storage at room temperature or 4°C for up to 3 months. Lyophilized plasma rich in growth factors eye drops conserve their biological features even without the use of lyoprotectants for at least 3 months.

### Keywords

Plasma rich in growth factors, stability, platelet growth factors, freeze-dried, platelet-rich plasma

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### Introduction

Ocular surface injuries include epithelial and stromal tissue disorders. The effective healing of these disorders is crucial to recover the main functions of the damaged ocular tissues. Healing is the net outcome of complex cellular and extracellular processes that are coordinated by different proteins including various growth factors such as epidermal growth factor (EGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), vascular endothelial growth factor

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(VEGF), and platelet-derived growth factor (PDGF), among others.<sup>1,2</sup>

In recent years, a type of platelet-rich plasma (PRP), denominated as plasma rich in growth factors (PRGF),<sup>3</sup> has been widely used in its eye drops formulations for the treatment of several ocular surface diseases such as dry eye, corneal ulcers, or persistent epithelial defects.<sup>4-10</sup> The beneficial effects obtained from the use of PRGF eye drops in the treatment of ocular pathologies are referred to their biological and biophysical properties, which are similar to the artificial tears, including pH, osmolarity, protein content, growth factors, as well as their anti-microbial and anti-inflammatory effects.<sup>11,12</sup>

Ocular surface disorders are chronic pathologies that require medium- or long-term treatment. Hence, it is essential that the stability and the biological activity of the treatments can be maintained for long periods of time in order to be used daily for months. In the case of eye drops derived from blood derivatives, several studies have demonstrated their safety and stability over several months, although their long-term storage and their maintenance during the period of application require the use and dependence of a cold chain (storage at  $-20^{\circ}\text{C}$  and  $+4^{\circ}\text{C}$  during their use).<sup>13</sup>

However, some patients are not suitable to be donors to obtain autologous hemoderivative products due to systemic inflammatory diseases, age, and other types of disorders or comorbidities. In these types of patients, an allogeneic blood-derived product could be an alternative to treat several ocular surface diseases, such as severe dry eye in patients with graft versus host diseases.<sup>14</sup> In these cases, the possibility of off-the-shelf storage would be advantageous for the use of blood-derived eye drops.<sup>15</sup>

Recently, our group demonstrated that PRGF eye drops can be lyophilized by maintaining their biological properties even without the use of lyoprotectants, such as trehalose (unpublished results), whose role in the protection of the ocular surface is well demonstrated.<sup>16</sup>

The purpose of this study was to analyze the optimal storage conditions of freeze-dried PRGF eye drops to preserve the composition and the biological activity after storage for 3 months at room temperature (RT) or  $+4^{\circ}\text{C}$  in comparison with the freshly prepared eye drops.

## Materials and methods

### PRGF sample preparation

This study was conducted according to the principles of the Declaration of Helsinki following approval by the corresponding ethical committee. After collection of the informed consent, blood from three healthy donors was drawn-off into 9 mL tubes with 3.8% (wt/v) sodium citrate. Then, blood was centrifuged at 580 g for 8 min at RT

in an Endoret system centrifuge (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain). The whole plasma column was collected avoiding the layer containing leukocytes using Endoret ophthalmology kit (BTI Biotechnology Institute). Hematology analyzer (ABX Micros 60; Horiba, Montpellier, France) was used to analyze platelet and leukocyte concentration in each sample. Whole PRP volume was activated with calcium chloride and incubated for 1 h at  $37^{\circ}\text{C}$ . The supernatant enriched in growth factor was collected, filtered, and aliquoted in glass vials for lyophilization and divided into three groups: (1) PRGF: fresh PRGF supernatant (used as a control), (2) PRGF lyo: pure PRGF supernatant frozen at  $-80^{\circ}\text{C}$ , and (3) PRGF lyo + 2.5T: PRGF supernatant was mixed with 2.5% trehalose as lyoprotectant and frozen at  $-80^{\circ}\text{C}$ . Samples belonging to Groups 2 and 3 were introduced in the lyophilizer (LyoBeta; Telstar, Terrassa, Spain). The primary drying phase was carried out at  $-50^{\circ}\text{C}$  and 0.1 mBar for 24 h. Finally, secondary drying phase was performed at  $+20^{\circ}\text{C}$  and 0.1 mBar for 12 h. After lyophilization, one part of the samples was used immediately and the other part was divided into two halves: one half was stored for 3 months at  $+4^{\circ}\text{C}$  and the other half was stored at RT. The freeze-dried samples were reconstituted before their use with sterile water at the same volume used prior to the lyophilization process.

### Growth factor levels in PRGF eye drops

The concentrations of several growth factors, such as EGF, PDGF, TGF- $\beta$ 1, and VEGF, were analyzed to assay the stability of freeze-dried PRGF eye drops at each time and storage condition. These growth factors were analyzed using the commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

### Cell culture

The biological activity of the different eye drops stored at different times and conditions was studied on primary corneal keratocytes (called HK; ScienCell Research Laboratories, San Diego, CA, USA). HK were cultured following the manufacturer's instructions. Briefly, cells were cultured in fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories), antibiotics, and 2% fetal bovine serum (FBS; termed Complete FM) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  atmosphere until confluence.

Upon reaching confluence, the cells were detached using a commercial enzyme free of animal traces (TrypLE Select; Gibco-Invitrogen, Grand Island, NE, USA). Trypan blue dye was used to check the viability of the cells by exclusion method, based on the principle that non-viable cells take up the dye while viable cells do not.

### Cell proliferation

Human keratocytes (HK) were seeded in a 96-well dark-bottom plate at a density of 10,000 cells/cm<sup>2</sup> in serum-free medium supplemented with 20% (v/v) of the different PRGF samples kept at different storage times and conditions. At each time of the study, cells incubated with FBS diluted at 0.1% (v/v) were used as a control of cell growth and to normalize the results obtained at each study time. The study period was 72 h. CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA, USA) was used to analyze the cell density obtained with each PRGF sample. Briefly, the wells were washed carefully with phosphate-buffered saline (PBS) after removing the culture medium. Subsequently, to improve the cell lysis efficiency in the CyQUANT assay, the plate was frozen at -80°C. Then, the plate was thawed at RT and the samples were incubated with RNase A (1.35 kU/mL) diluted in cell lysis buffer for 1 h at RT. Before, the 2 × CyQUANT GR dye/cell lysis solution was added to each well of the plate, mixed gently, incubated for 5 min at RT, and protected from light. A fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany) was used to measure the fluorescence of the sample. After that, the fluorescence values obtained for each well were divided by the value obtained for the cells cultured with 0.1% FBS to normalize the results obtained at each time of the study.

### Cell migration

In order to quantify the migratory/chemotactic potential of the different PRGF eye drop samples on HK, the cells were seeded at high density inside the culture inserts (ibidi GmbH, Martinsried, Germany) placed in a 24-well plate and were grown with Complete FM until confluence. The inserts were then carefully removed obtaining two separate cell monolayers leaving a cell-free gap of approximately 500 μm thickness. The cells were washed with PBS and incubated in quintuplicate with the different PRGF eye drops obtained in the study for 24 h. Like cell proliferation assay, some wells were also incubated with 0.1% FBS as a control of cell migration and to normalize the results obtained at each time of the study. After this period, the different culture media were removed and the cells were incubated with Hoechst 33342 (Invitrogen, Carlsbad, CA) for 10 min. To quantify the number of migrated cells, phase-contrast images were taken from the central part of the gap before treatment, and phase contrast and fluorescence after 24 h of treatment using a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB; Leica Microsystems, Barcelona, Spain). ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) was used to measure the gap area taken in each

image and the number of migrated cells after 24 h. After that, the number of migratory cells obtained for each well treated with PRGF samples was divided by the value obtained for cells cultured with 0.1% FBS to normalize the results obtained at each time of the study.

### Statistical analysis

Data are expressed as mean ± SD (see Supplementary Table). To evaluate the differences between the variables analyzed at the different storage temperatures (RT and 4°C) and times points (t0 and t3), non-parametric Friedman procedure was carried out followed by Wilcoxon test to discriminate among the statistical means. The significance level was established at  $p=0.05$ . SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses.

### Results

Endoret preparations had a mean platelet enrichment of 2.14-fold over the platelet concentration in peripheral blood. No detectable levels of leucocytes were observed in any of the PRGF preparations.

#### Protein levels in PRGF eye drop storage at different time and conditions

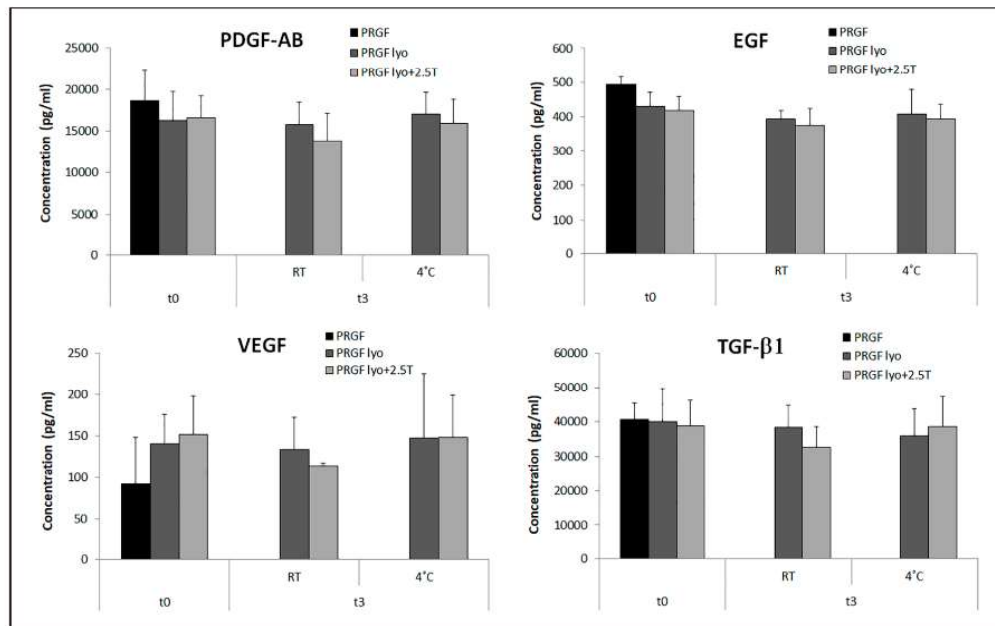
The characteristics of the different samples of PRGF eye drops used for this study were analyzed at the day of sample collection (time 0, t0), and after storage in freeze-dried form (PRGF lyo and PRGF lyo + 2.5T) at +4°C and at RT for 3 months (t3).

The results revealed that all concentration levels of the different growth factors analyzed in the study showed no significant changes after 3 months of storage at +4°C or RT in a freeze-dried state in comparison with the fresh samples (samples obtained at t0; Figure 1). Furthermore, no significant differences ( $p > 0.05$ ) were observed between the freeze-dried PRGF eye drops kept at 4°C or RT for 3 months.

#### PRGF eye drop effect over cell proliferation

Figure 2 shows the representative images of cells treated with freshly prepared PRGF eye drops (t0) and with freeze-dried PRGF eye drops stored at RT and 4°C for 3 months (t3). The HK proliferation index was not modified after treatment with PRGF eye drops obtained at t0 (fresh or freeze-dried samples evaluated at the obtaining time) or after storage at +4°C or RT for 3 months in lyophilized form (see Figure 2). Similarly, no significant differences were observed in the proliferative potential between freeze-dried PRGF eye drops stored at 4°C or RT for 3 months.





**Figure 1.** Several growth factors involved in the ocular surface tissue regeneration were analyzed in the different study samples. No significant differences ( $p > 0.05$ ) were observed among the freeze-dried PRGF eye drop samples stored for 3 months at 4°C or RT and the PRGF eye drop samples obtained at time 0 (fresh samples) in any of the analyzed factors.

### Migratory potential of freeze-dried PRGF eye drops

Figure 3 shows the representative images of HK treated with PRGF eye drop samples obtained at t0 and after storage for 3 months at RT and 4°C in a freeze-dried manner. Migratory activity of HK increased significantly ( $p < 0.05$ ) after treatment with freeze-dried PRGF eye drop samples stored at RT or 4°C for 3 months with respect to t0 (Figure 3). These differences were observed among the different freeze-dried samples (PRGF lyo and PRGF lyo + 2.5T) stored for 3 months at RT and 4°C with respect to fresh PRGF samples. Significant differences were also detected among pure lyophilized PRGF eye drop samples (PRGF lyo) or mixed with 2.5% trehalose (PRGF lyo + 2.5T) stored for 3 months at +4°C or RT with respect to their freeze-dried sample obtained at t0 (see Figure 3). No differences ( $p > 0.05$ ) were observed in the HK migratory potential between the freeze-dried PRGF samples mixed with or without trehalose (PRGF lyo + 2.5T and PRGF lyo, respectively).

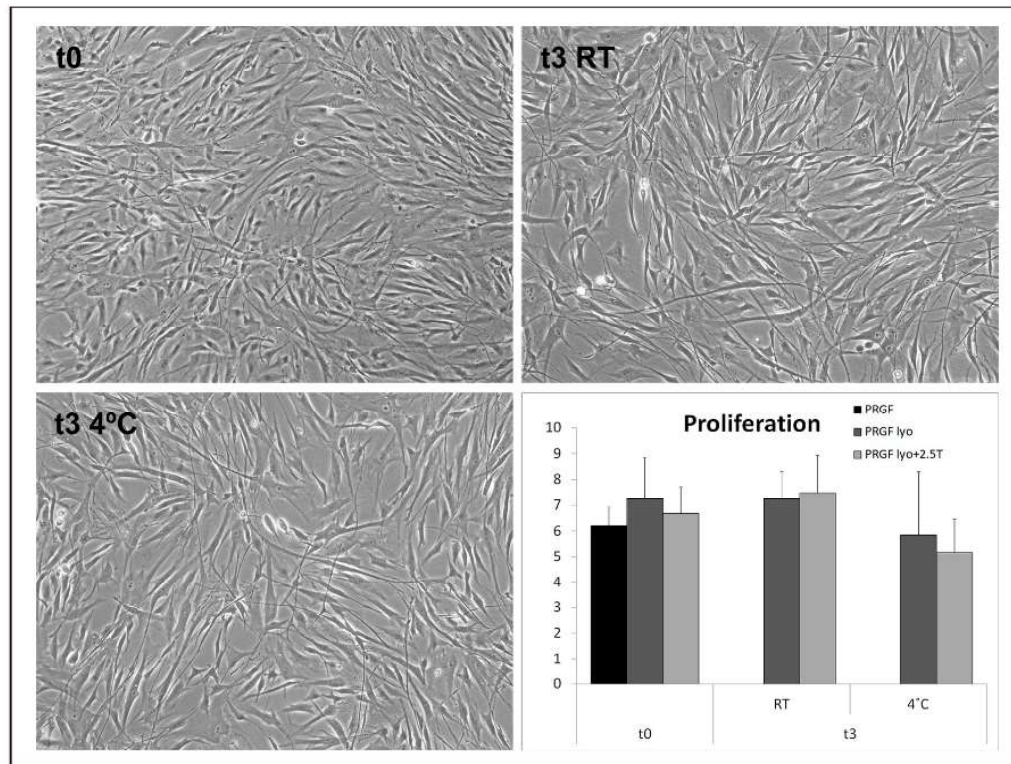
### Discussion

In recent years, hemoderivative products have been widely used for the treatment of different ocular surface diseases such as dry eye, persistent epithelial defects, and ocular ulcers.<sup>17-19</sup> The benefits of these type of products are mainly attributed to their content in growth factors that are

involved in the regeneration of the ocular surface tissues such as EGF, TGF-β1, VEGF, or PDGF, levels of which are similar to those observed in the natural tears.<sup>1,2,20</sup> It is very common that the diseases mentioned above need long-term treatments, being necessary to store these blood derivative products at low temperatures in order to maintain their biological characteristics during this period of application.<sup>21</sup> However, the long-term storage of blood-derived products and their application during the period of use requires the dependence of a cold chain (storage at -20°C to keep it during a large period of time and +4°C during its use).<sup>13</sup> In this study, we have demonstrated that freeze-dried PRGF eye drops maintain the levels of different growth factors involved in ocular surface tissue regeneration as well as their biological activity after their storage at RT or at 4°C for at least 3 months.

The cause of a significant increase in the migratory activity of keratocytes after treatment with freeze-dried PRGF eye drops stored for 3 months in contrast to the PRGF eye drops obtained at t0 remains unknown. One possible explanation is that some proteins or growth factors that could be involved in the control/inhibition of cell migration could be partially or totally denaturalized during the storage period. Although in previous studies a slight increase in migratory capacity of HK was observed after treatment with PRGF eye drops stored at -20°C for 3 months,<sup>13</sup> these changes did not become significant, hence further investigations will be needed to evaluate the results obtained at this point in this study.





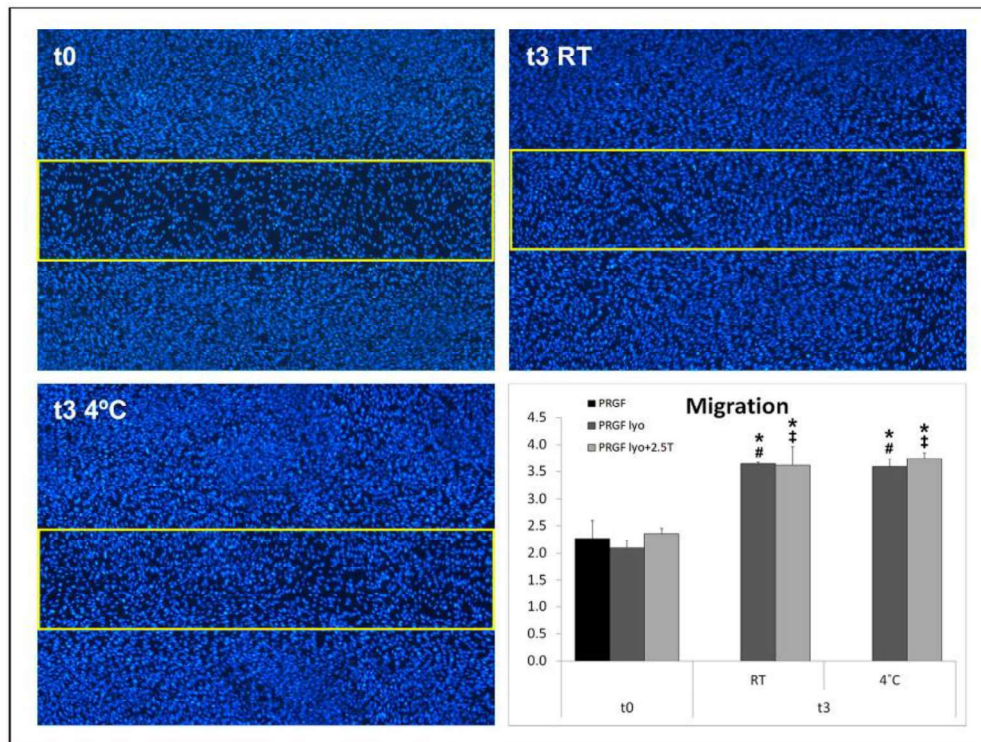
**Figure 2.** Proliferation of HK after treatment with freeze-dried PRGF eye drops. Representative phase-contrast photomicrographs showing human primary keratocytes cultured with fresh PRGF-Endoret samples (t0) and freeze-dried PRGF-Endoret eye drops after storage for 3 months at room temperature (t3 RT) and at 4°C (t3 4°C). No significant differences ( $p > 0.05$ ) were observed among the proliferation induced by the PRGF eye drops at any time and condition of storage (with or without the use of trehalose).

In recent years, several studies have been carried out to evaluate the stability of freeze-dried hemoderivative products.<sup>22,23</sup> Although remarkable results were observed in these studies, the lyophilized products were stored at temperatures under 4°C for their maintenance. In this study, it has been shown that lyophilized PRGF eye drops can be stored at RT for up to 3 months preserving their biological features like those of fresh PRGF eye drops. Hence, it is necessary to highlight that, one important benefit of the freeze-dried PRGF eye drops is their easy storage, allowing to keep this product at RT for at least 3 months avoiding the dependence on cold chain.

The freeze-drying process could alter protein structures due to the decrease in temperature and as a consequence of solute concentration increase during the freezing procedure.<sup>24</sup> The low temperatures encourage protein denaturation by disrupting the interactions between proteins in a similar way as thermal denaturation.<sup>25,26</sup> Multiple lyophilized products contain cryoprotectants or lyoprotectants to avoid protein denaturation during the freeze-drying process. The most common protectants used in freeze-dried protein formulations are disaccharides, such as sucrose or trehalose, due to their capability to substitute water molecules undergoing protein-stabilizing effects.<sup>27</sup> However, the use of

trehalose could cause detrimental results in ocular surface fibroblast proliferation, thus reducing their regenerative capability.<sup>28</sup> In a recent work sent for publication, we have demonstrated that the developed freeze-dried PRGF eye drops without lyoprotectants maintain the growth factor levels and the biological activity in a similar way to the lyophilized PRGF eye drops mixed with 2.5% or 5% trehalose (unpublished results). In the present work, we have used the PRGF combined with 2.5% trehalose to evaluate whether freeze-dried PRGF eye drops alone preserve their biological potential in a similar manner as the first ones for 3 months of storage at 4°C or RT. The results obtained showed that freeze-dried PRGF eye drops without trehalose kept the concentrations of different growth factors and their biological potential in similar levels to those lyophilized PRGF samples mixed with trehalose. Thus, PRGF eye drops maintain their endogenous origin avoiding exogenous preservatives which can increase the risk of chemical toxicity.<sup>29</sup> Furthermore, the lyophilization process would permit off-the-shelf storage of the PRGF eye drops, facilitating the accessibility to this therapy to those patients who need several applications for a long period of time or for those who require an allogeneic application due to the unsuitability to obtain autologous hemoderivative products.<sup>30</sup>





**Figure 3.** Migratory potential of lyophilized PRGF eye drops on HK. Representative fluorescence Hoechst images of HK after treatment with fresh PRGF-Endoret samples (t0) and with freeze-dried PRGF-Endoret eye drops stored for 3 months at room temperature (t3 RT) and at 4°C (t3 4°C). Yellow rectangle included in each image identifies the migration gap area. The graph shows that cell migration was significantly higher when cells were cultured with freeze-dried PRGF eye drop samples (with or without trehalose, PRGF lyo, and PRGF lyo + 2.5T, respectively) stored at RT or 4°C for 3 months in comparison with time 0. Furthermore, freeze-dried PRGF eye drops without (PRGF lyo) or with trehalose (PRGF lyo + 2.5T) stored for 3 months increased significantly the migratory potential of HK regarding their respective freeze-dried PRGF eye drops obtained at time 0. \*Statistically significant differences among the different freeze-dried PRGF samples stored for 3 months and the fresh PRGF eye drops ( $p < 0.05$ ). #Statistical significance of PRGF lyo eye drops stored for 3 months with respect to PRGF lyo obtained at time 0 ( $p < 0.05$ ). ‡Statistically significant differences of PRGF lyo + 2.5T samples stored for 3 months regarding PRGF lyo + 2.5T obtained at time 0 ( $p < 0.05$ ).

In summary, this study suggests that freeze-dried PRGF eye drops stored for up to 3 months at RT or 4°C preserve the principal growth factors and proteins involved in ocular surface tissue regeneration. Furthermore, lyophilized PRGF eye drops maintain their biological activity for 3 months stored at 4°C or at RT. Finally, it is important to highlight that freeze-dried PRGF eye drops conserve their biological characteristics even without the use of lyoprotectants for at least 3 months.

#### Declaration of conflicting interests


The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: F.A. is the Scientific Director and M.d.l.F. and F.M. are scientists at BII Biotechnology Institute, a company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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#### Supplemental material

Supplemental material for this article is available online.

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