

Effects of heat-treatment on plasma rich in growth factors-derived autologous eye drop

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ABSTRACT

We have developed and characterized a new type of plasma rich in growth factors (PRGF) derived eye-drop therapy for patients suffering from autoimmune diseases. To determine the concentration of several growth factors, proteins, immunoglobulins and complement activity of the heat-inactivated eye-drop and to study its biological effects on cell proliferation and migration of different ocular surface cells, blood from healthy donors was collected, centrifuged and PRGF was prepared avoiding the buffy coat. The half volume of the obtained plasma supernatant from each donor was heat-inactivated at 56 °C for 1 h (heat-inactivated PRGF). The concentration of several proteins involved on corneal wound healing, immunoglobulins G, M and E and functional integrity of the complement system assayed by CH50 test were determined. The proliferative and migratory potential of inactivated and non-inactivated PRGF eye drops were assayed on corneal epithelial cells (HCE), keratocytes (HK) and conjunctival fibroblasts (HConF). Heat-inactivated PRGF preserves the content of most of the proteins and morphogens involved in its wound healing effects while reduces drastically the content of IgE and complement activity. Heat-inactivated PRGF eye drops increased proliferation and migration potential of ocular surface cells with regard to PRGF showing significant differences on proliferation and migration rate of HCE and HConF respectively. In summary, heat-inactivation of PRGF eye drops completely reduced complement activity and decreased significantly the presence of IgE, maintaining the biological activity of PRGF on ocular surface cells.

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1. Introduction

It is widely known that different ocular surface structures including cornea, conjunctiva, meibomian glands, goblet cells and lacrimal glands and mucosal immunity system contribute to the tear film composition and to the maintenance of normal cornea integrity. These components are interrelated to each other by

neural, hormonal and chemical feedback mechanisms (Mathers, 2000; Pflugfelder et al., 1986; Stern et al., 1998). Alteration of any of these components may modify this complex system, affecting the composition of the tear film and the homeostasis of the corneal tissues, increasing the risk to suffer a corneal injury (Muller et al., 2003; Nasu et al., 1984; Pflugfelder et al., 2002).

Several systemic immune-mediated disorders affecting the eye such as allergies, vasculitis, Primary or secondary Sjögren's syndrome, or graft versus host disease are characterized by a deregulated tear film composition and dry eye syndrome (Fox and Kang, 1992; Read, 2004; Tabbara et al., 2009). The soluble components of immune system, including immunoglobulins and the complement system have a direct role with the pathogenesis of these ocular diseases (Daha et al., 2011; Patriarca et al., 2006; Read, 2004; Stern et al., 2013). In addition, inflammation and ocular tissue injury are also common signs of these types of ocular diseases.

Local medications with preservatives, commonly used to treat ocular inflammation and to reduce corneal injuries, are not well tolerated by patients suffering from systemic immune disorders. Combination of lubricants and steroids are also usually required, although they show poor tolerance and unsatisfactory

Abbreviations: PRGF, plasma rich in growth factors; Inact-PRGF, inactivated PRGF; PRP, platelet-rich plasma; EGF, epithelial growth factor; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-beta 1; VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor I; IgG, immunoglobulin G; IgM, immunoglobulin M; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; PETIA, particle-enhanced turbidimetric immunoassay; HCE, human corneal epithelial cells; HK, human keratocytes; HConF, human conjunctival fibroblasts; FBS, fetal bovine serum; PBS, phosphate buffered saline; Fab fragment, variable fragment of immunoglobulins; Fc fragment, constant fragment of immunoglobulins.

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response (Abelson et al., 2003; Balaram et al., 2005; Stern et al., 2013).

Recently, the use of an autologous eye-drop based on platelet-rich plasma (PRP) has been proposed for treatment of several acute and chronic eye surface disorders including corneal epithelial defects and dry eye syndrome (Alío et al., 2007a, 2007b, 2007c, 2012; Lopez-Plandolit et al., 2010, 2011; Pezzotta et al., 2012). Plasma rich in growth factors (PRGF) is a type of platelet enriched plasma obtained from patient's own blood, which avoid the leukocytes collection, and which after activation with calcium chloride allows the release of a pool of biologically active proteins that influence and promote a range of biological process including proliferation, migration and differentiation, and protect against microbial contamination (Anitua et al., 2011, 2012; Freire et al., 2012).

Clinical results from patients with dry-eye syndrome from different etiologies treated with PRP eye-drops show that around 80% of the patients have a very significant or moderate improvement, while around 20% show minimal or no changes in the disease status (Alío et al., 2007b; Lopez-Plandolit et al., 2011; Pezzotta et al., 2012). However, in patients with systemic autoimmune syndromes results are worse. This may due to the concentration of immunologic components in the eye drops obtained from the own patient's blood. These immunologic components including immunoglobulins and complement (Jensen et al., 1999; Nguyen et al., 2007; Patriarca et al., 2006) may hamper correct wound healing of the cornea.

In this study we propose a new type of plasma rich in growth factors (PRGF)-derived eye-drop therapy for patients suffering from autoimmune diseases. The new formulation has been obtained after heating the eye-drops at 56 °C. The hypothesis is that the heat-inactivated PRGF eye-drops will have a reduced amount of immunoglobulins or complement. It has been reported that heating serum or plasma at 56 °C reduces the complement system activity and destroy microorganism and viruses (Triglia and Linscott, 1980; Ward, 1979). However, it is also known that heat inactivation has a wide spectrum of denaturing effects on serum and plasma protein and factors (Ayache et al., 2006). Assuming this, a new heat-inactivated PRGF eye-drop formulation has been obtained and characterized. The concentration of several growth factors, proteins, immunoglobulins and complement activity of the eye-drop has been determined. In addition, the effects of the heat-inactivated eye-drop on cell proliferation and migration of different ocular surface cells have been evaluated.

2. Materials and methods

2.1. Preparation of plasma rich in growth factors (PRGF) eye drops

Blood from four healthy donors was collected after informed consent into 9-mL tubes with 3.8% (wt/v) sodium citrate. The study was performed following the principles of the Declaration of Helsinki. Samples were centrifuged at 580 g for 8 min at room temperature in an Endoret System centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Whole column of plasma rich in growth factors was collected after centrifugation avoiding the buffy coat that contains the leukocytes using Endoret ophthalmology kit (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Platelets and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpellier, France). Whole PRGF volume was activated with Endoret activator (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) and incubated at 37 °C for 1 h. The growth factor enriched supernatants were collected by aspiration after centrifugation at 1000 g for 10 min. Then, the half volume of the obtained plasma supernatant from each donor was

incubated at 56 °C for 60 min to plasma inactivation. Finally, both samples obtained from each donor, inactivated PRGF (Inact-PRGF) and non-inactivated PRGF (PRGF), were filtered with a filter pore size of 0.2 µm (Fisher Scientific, Madrid, Spain), aliquoted and stored at –80 °C until use.

2.2. Measurement of plasma peptide concentrations

To check the heat inactivation effect of PRGF eye drops, concentration of proteins related to ocular surface wound healing, such as, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I) and fibronectin; and immunoglobulins Ig G and Ig M, were measured using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. PDGF-AB, EGF, VEGF, IGF-I and TGF-β1 ELISA kits were purchased from R&D Systems (Minneapolis, MN). Fibronectin ELISA kit was acquired from Takara Bio (Shiga, Japan), and Ig G and Ig M ELISA kits were acquired from Cusabio Biotech (Wuhan, China).

The immunoglobulin E (IgE) was measured using commercially available particle-enhanced turbidimetric immunoassay (PETIA) obtained from Biokit (Quantex IgE kit, Barcelona, Spain). PETIA technique was performed according to the manufacturer's instructions.

2.3. Complement activity analysis

Total classical complement activity was measured using a commercial CH50 assay kit from Wako Chemicals (Autokit CH50, Richmond, VA) based on the technique of liposome immunoassay developed by Yamamoto et al. (1995). CH50 assay was performed according to the manufacturer's instructions.

2.4. Cells

Biological activity of PRGF samples (PRGF and Inact-PRGF) were assayed in different types of ocular surface cells including Human SV-40 immortalized corneal epithelial cells (termed HCE) (RCB1384: HCE-T, Riken Cell Bank, Ibaraki, Japan), and primary human keratocytes (termed HK) and conjunctival fibroblasts (termed HConF) (ScienCell Research Laboratories, San Diego, CA). HCE Cells were cultured at 5% CO₂ at 37 °C in DMEM/F12 medium (Invitrogen-Gibco, Grand Island, NY, USA), supplemented with 0.5 µg/mL insulin (Sigma–Aldrich, St Louis, MO, USA), 10 ng/mL EGF (Sigma–Aldrich, St Louis, MO, USA), 1% DMSO (Sigma–Aldrich, St Louis, MO, USA) and 7.5% fetal bovine serum (FBS) (Biobrom AG, Berlin, Germany). On the other hand, HK and HConF cells were cultured in Fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories, San Diego, CA).

After confluence, cells were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion.

2.5. Proliferation assay

Ocular surface cells were seeded on 96 well optical bottom black plates at a density of 20,000 cells per cm² for HCE cells and 10,000 cells per cm² for HK and HConF, and cultured with serum-free medium supplemented with either: (i) the culture medium alone with 0.1% FBS as a control of non-stimulation (Control) (ii) 20% (v/v) of PRGF, or (iii) 20% (v/v) of Inact-PRGF. The study period was 48 h. Proliferation assay were done in quintuplicate for each treatment

and cultured cell. Density of cells in culture was estimated using the CyQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA). Briefly, medium was removed and wells were washed carefully with phosphate buffered saline (PBS). Then microplate was frozen at -80°C for efficient cell lysis in the CyQUANT assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Ku/ml) diluted in cell lysis buffer for 1 h at room temperature. Then $2\times$ CyQUANT GR dye/cell lysis buffer was added to each sample well, mixed gently and incubated for 5 min at room temperature protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany). A DNA standard curve was included in all fluorescence quantifications. As an index of cell number, calibration curves ranging from 10,000 to 100,000 cells per cm^2 were established using the CyQUANT assay.

2.6. Wound healing and migration assay in response to PRGF and Inact-PRGF

In order to quantify the migratory potential of ocular surface cells after treatment with PRGF or inactivated PRGF, cells were plated in culture inserts (Ibidi, GmbH, Martinsried, Germany) placed on a 24-well plate at high density and were grown until confluence. After

carefully remove the inserts, two separated cell monolayers leaving a cell-free gap of approximately $500\ \mu\text{m}$ thickness were obtained. The cells were washed with PBS and incubated with the same treatments as in the proliferation assay (0.1% FBS, 20% PRGF or 20% Inact-PRGF) in quintuplicate for 24 h. After this period, the different culture mediums were removed and cells were incubated with 1/500 Hoechst 33342 in PBS for 10 min. To quantify the percentage of wound healing in the case of HCE and the number of migratory cells in the case of HK and HConF, phase contrast images of the central part of the septum before treatment and phase contrast and fluorescence photographs after 24 h were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area and the migratory cells found in this gap after the 24 h of treatment were measured using the Image J Software (NIH, Bethesda, Maryland, USA). The results were expressed as the number of cell migrated per mm^2 of area in the case of HK and HConF, and as percentage area of wound healing in the case of HCE cells.

2.7. Statistical analysis

Significant differences among defined groups were tested using the non-parametric Kruskal–Wallis procedure followed by

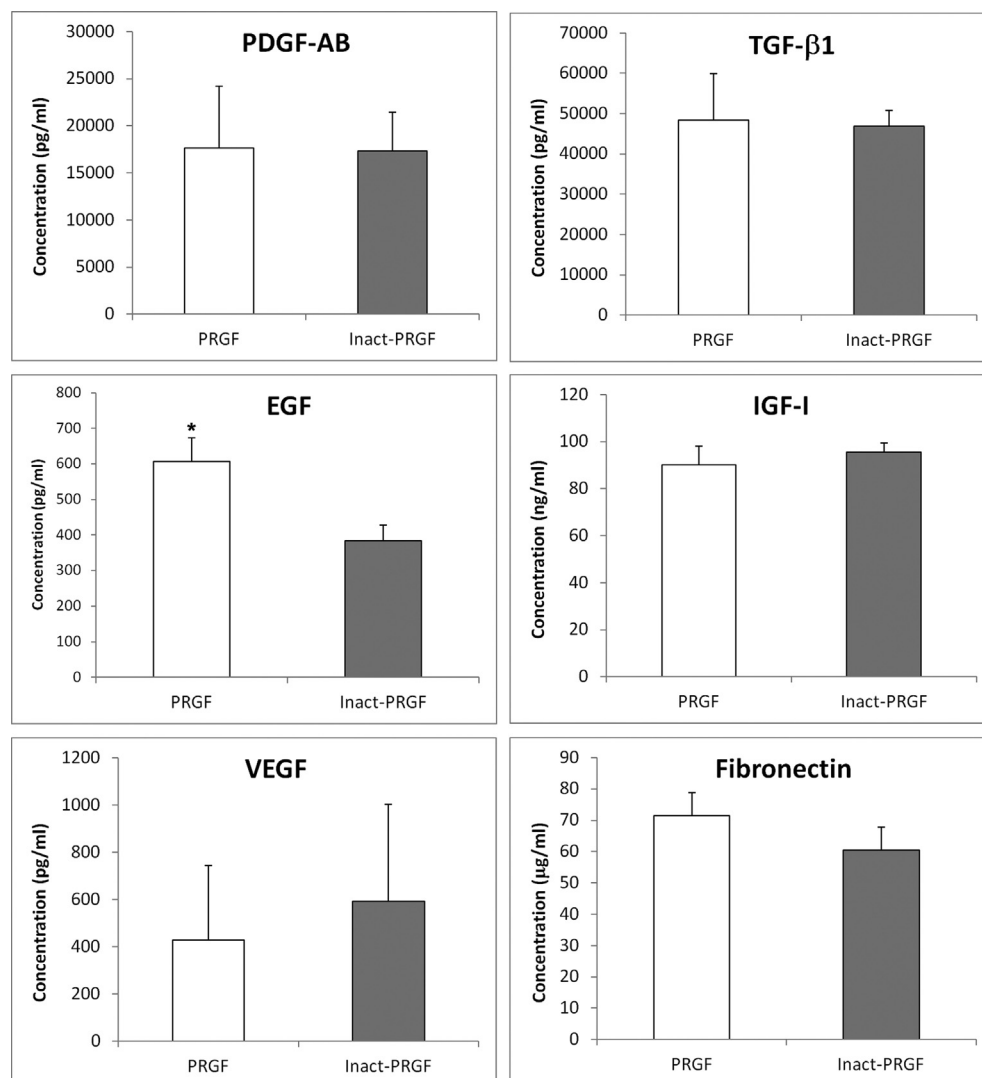


Fig. 1. Several growth factors and proteins involved on corneal wound healing were measured on non-inactivated and heat-inactivated PRGF samples.

Bonferroni to discriminate among the means. A difference at a level of $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

3. Results

The mean platelet enrichment of the PRGF preparations was 2.45-fold over the baseline concentration of platelets in whole blood. None of the preparations contained detectable levels of leucocytes (data not shown).

3.1. Wound healing protein content

The concentration of some of the most important proteins related to corneal wound healing was analyzed on non-inactivated and heat-inactivated PRGF samples. The different protein concentrations tested are represented in Fig. 1. With the exception of epidermal growth factor (EGF), the remaining proteins including platelet derived growth factors (PDGF), transforming growth factor (TGF- β 1), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF) and fibronectin showed no changes on their concentrations after heat treatment of PRGF eye-drops at 56 °C for 1 h.

3.2. Soluble components of immune system

Concentration of different immunoglobulins usually involved in the development of autoimmune disorders were analyzed by ELISA (IgG and IgM) or turbidimetry (IgE) assays (Fig. 2). The concentrations of all immunoglobulin were reduced in the heat-inactivated eye-drops. Significant differences were observed in the case of IgE but not for IgG and IgM. In addition, in order to analyze the effect of heat-treatment of samples on IgE content, donors were divided in

two groups: allergic and non-allergic donors. Both groups showed significant differences ($p < 0.05$) on IgE concentration after heat-inactivation treatment (Fig. 2C and D). The mean concentration of IgE was reduced from 5 to 2.5 kU/l on non-allergic donors and from 133.5 to 27 kU/l on allergic donors.

Complement activity in non-inactivated and heat-inactivated PRGF samples was measured by the CH50 assay (Fig. 3). Complement activity underwent a significant reduction ($p < 0.05$) after the heat treatment. In fact, since values of complement activity in the heat-inactivated eye-drops were under the assay detection limits, it may be considered that the complement system was completely inactivated.

3.3. Cell proliferation

Addition of both PRGF preparations (non-inactivated and heat-inactivated PRGF) to human ocular surface cells significantly increased ($p < 0.05$) their proliferation rate after 48 h as it is shown in Fig. 4. In fact, cell density of HCE, HK and HConF cells showed a significant increase of 1.8, 3.8 and 2.2-fold respectively compared with non-stimulated group (control). In the case of HK and HConF cells no significant proliferative differences were observed after treatment with non-inactivated and heat-inactivated PRGF eye-drops. However, in the case of HCE cells, cell proliferation was significantly higher ($p < 0.05$) after treatment with the heat-inactivated PRGF eye-drops (Fig. 4).

3.4. Wound healing and migration assay

Fig. 5 shows fluorescence Hoechst images of HCE, HK and HConF cells after 24-h treatment with the different PRGF eye-drops. As it is observed non-inactivated and heat-inactivated PRGF formulations stimulated the migratory capacity of each analyzed ocular surface cell during the 24-h follow-up. Results from the in vitro wound

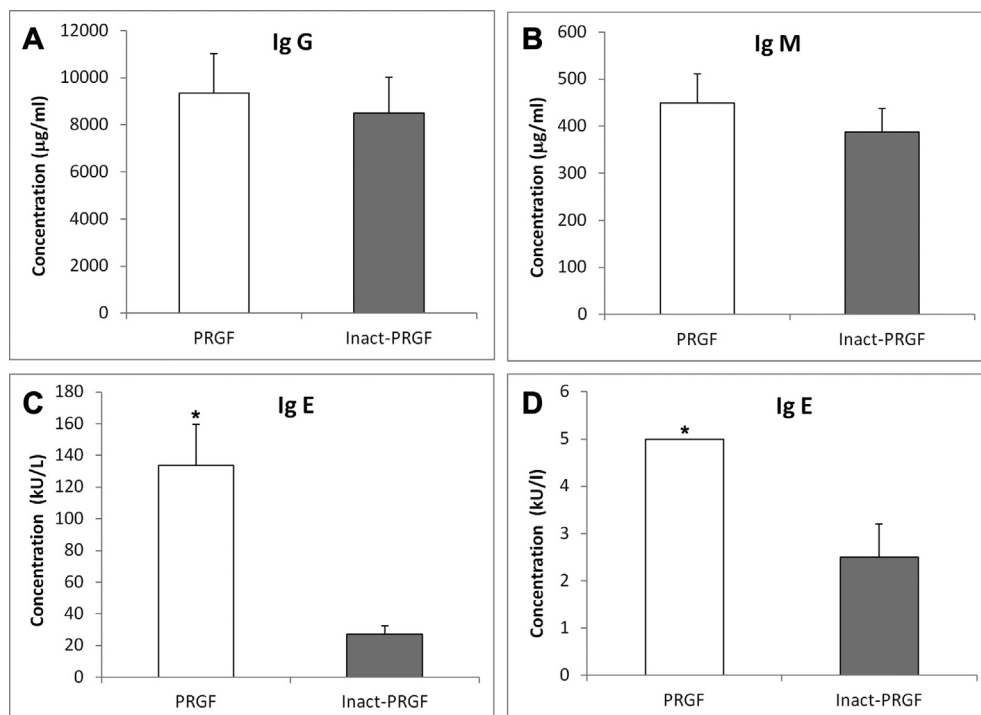


Fig. 2. Immunoglobulins detection on non-inactivated and heat-inactivated PRGF samples. Immunoglobulin G (A) and M (B) showed no significant variation after PRGF heat-treatment. The levels of immunoglobulin E were significantly reduced (*) after heat-inactivation of PRGF samples from allergic (C) and non-allergic (D) donors.

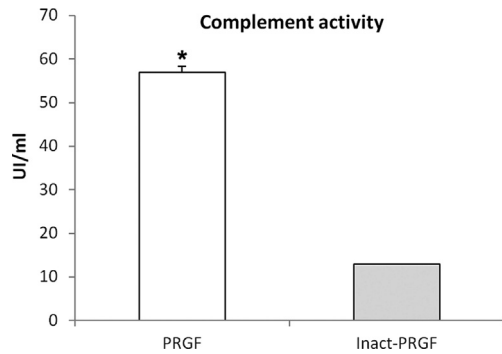


Fig. 3. Complement activity was completely reduced after heat-treatment of PRGF at 56 °C for 1 h.

healing study revealed that starting from an experimental area of 4.5 mm², epithelial cells were able to close up to 70% of the whole area in 24 h with either the non-inactivated or heat-inactivated PRGF formulation, while in the control group only partial closure was observed (22% of total area). No statistical differences were found between non-inactivated or heat-inactivated PRGF eye-drops (Fig. 6).

In addition, both PRGF formulations significantly stimulated the migratory capacity of HK and HConF cells (Fig. 6). In all the cases the migratory activity was higher when heat-inactivated PRGF treatment was used, being these differences significant in the case of HConF cells.

4. Discussion

This study describes the development and characterization of a new type of plasma rich in growth factors (PRGF) eye-drop therapy directed towards the treatment of ocular surface pathologies in patients suffering from autoimmune diseases. The new formulation has been obtained after heating the eye-drops at 56 °C. The composition of the non-inactivated and heat-inactivated PRGF samples has been evaluated and compared as well as their biological potential determined in three different cells of the ocular surface cells: corneal epithelial cells (HCE), keratocytes (HK) and conjunctival fibroblasts (HConF).

Results show that most of the proteins and morphogens measured including PDGF, TGF-β1, IGF, VEGF and fibronectin were present in the same concentrations in the heated eye-drops, confirming that the composition was not altered by the 1-h heat-treatment. Epidermal growth factor (EGF) was the only growth factor that was reduced in the heated formulations. It is well known that EGF is mainly produced by lacrimal gland and corneal epithelial cells. This morphogen exerts potent proliferative effects on corneal epithelial cells (Imanishi et al., 2000; Klenkler et al., 2007). However, the reduction of EGF concentration in the heat-inactivated PRGF eye-drops is not correlated with a decrease in cell proliferation. On the contrary, proliferative effects on HCE cells were higher in the heat-inactivated PRGF compared with non-inactivated one. This may be explained by the presence of other morphogens including the IGF-1 present in the heat-inactivated eye-drop and which exert mitogenic activity (Rocha et al., 2002; Wilson et al., 1994).

Ayache et al. (2006) showed that heat inactivation of serum and plasma has a denaturing effect on a wide range of proteins. However, in the present study we have demonstrated that 1-h treatment of the autologous PRGF formulation does not alter some of its protein and growth factor content. The pool of proteins preserved is known to promote proliferation and

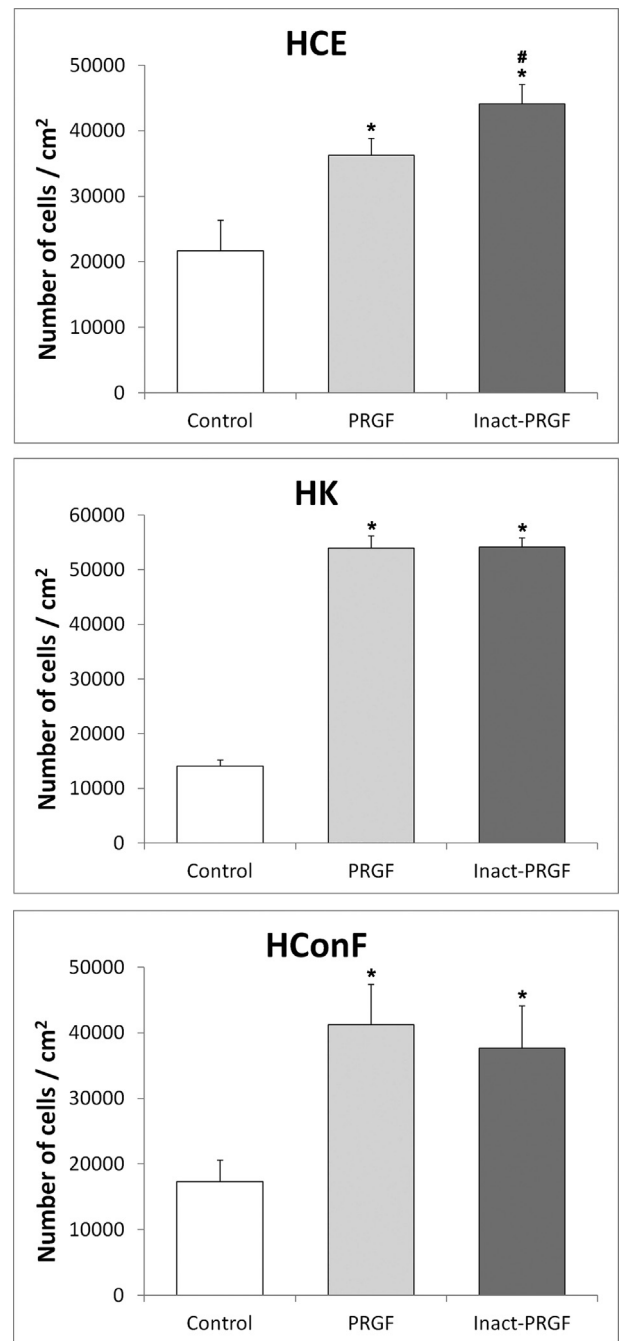


Fig. 4. Proliferation of corneal epithelial cells (HCE), keratocytes (HK) and conjunctival fibroblasts (HConF) cells after culturing with 0.1% FBS (Control), 20% non-inactivated or 20% heat-inactivated PRGF. Each ocular surface cells significantly increased (*) their proliferative index after treatment with any of the formulations. Heat-inactivated PRGF treatment significantly increased (#) the proliferation rate of HCE cells regarding PRGF treatment. No statistical differences were found between non-inactivated and heat-inactivated PRGF samples on HK and HConF cells.

migratory response on the different ocular surface cells after corneal injury (Klenkler and Sheardown, 2004; Maldonado and Furcht, 1995).

No differences were observed in the biological potential of the non-inactivated and the heat-inactivated PRGF eye-drops. In fact, both formulations promote the proliferation and migration capacity of the three different cells from the ocular surface evaluated in the study. These results support that the heating process does not

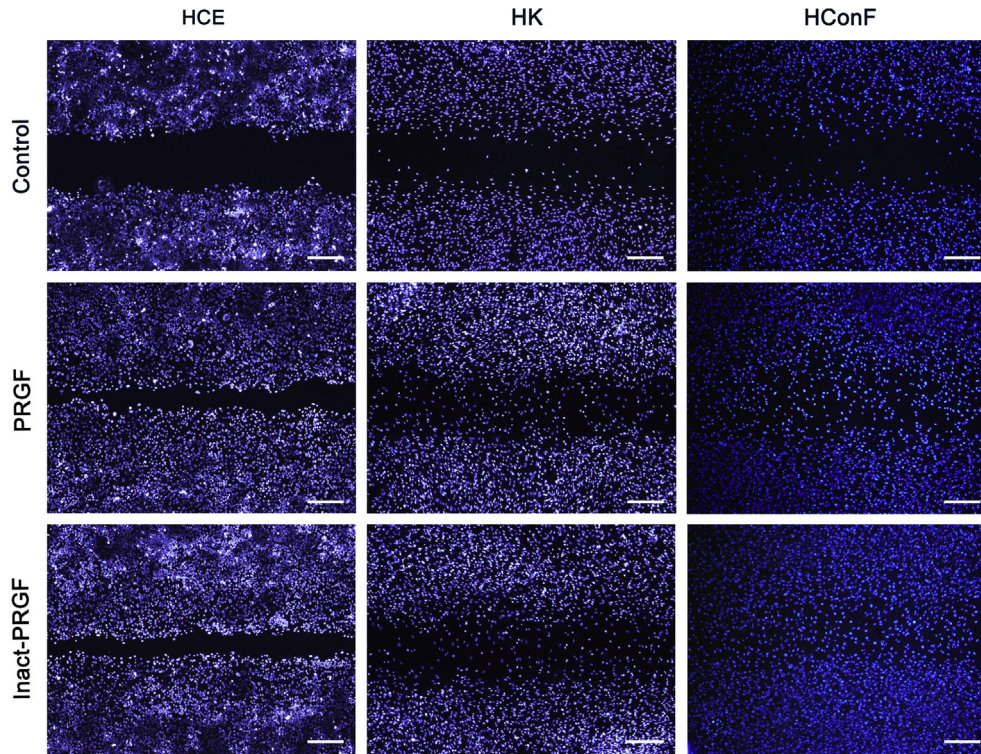


Fig. 5. Fluorescence Hoechst photomicrographs illustrating the percentage area of wound healing of HCE cells and the migration rate of HK and HConF cells after treatment with 0.1% FBS (Control), 20% non-inactivated PRGF or 20% heat-inactivated PRGF. Scale bar: 200 μ m.

alter the biological and theoretically therapeutic potential of the heated PRGF formulation, and they can be correlated with the results obtained by Freire et al. (2012).

The most relevant achievement of the study is related to the components of the immune system that have been reduced or eliminated from the autologous eye-drop after the heating process. In fact, in the case of allergic donors a reduction of more than 75% in the content of IgE was obtained while in the case of non-allergic donors this reduction was approximately of 50%. These results suggest that PRGF inactivation could be effective to treat ocular disorders of allergic patients, where immunoglobulin IgE has a main role in the pathogenesis of allergic diseases (McGill et al., 1998).

Denaturation of immunoglobulins have been demonstrated to be at higher temperatures than 56 °C, showing that the variable fragment of immunoglobulins (Fab fragment) is denatured at 61 °C, while the constant fragment (Fc fragment) is denatured at 71 °C (Garbett et al., 2008; Vermeer and Norde, 2000; Vermeer et al., 2000). These studies correlate with the results obtained in this work, where no variation on IgG and IgM concentrations were observed after heat inactivation of PRGF eye drops.

The role of autoantibodies in several ocular autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome and graft versus host disease has been highly suggested. In addition, several studies have described the complement activation involvement in the development of these ocular autoimmune diseases through binding to the autoantibodies Fc receptors. Inoculation of serum or purified immunoglobulins from autoimmune disease mouse model to nude mice maintained the ability to transfer ocular surface disease; on the contrary, these diseases did not occurred when wild type serum was inoculated (Daha et al., 2011; Patriarca et al., 2006; Read, 2004; Stern et al.,

2012). Several studies have demonstrated the leadership role of complement system in serum or Ig-mediated ocular autoimmune diseases when using complement-gene knockout mice, or after depleting the complement system with cobra venom factor (Cochrane et al., 1970; Nguyen et al., 2007; Vogel et al., 2004). It is important to underline that in the present article blood was obtained from healthy patients, without autoantibodies. Future studies should be done to confirm that heated PRGF eye-drops (inactivated, without complement) act in a different way to the autoimmune non-heated PRGF eye-drops with corneal cells in those patients with autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome and graft versus host disease.

In summary, in the present preliminary article we have shown a complete inactivation of complement system after heating PRGF eye-drops at 56 °C for 1 h may avoid the translation of immune disorders to ocular surface through PRGF eye-drops.

5. Conclusions

In summary, this study describes the development and characterization of a new type of heat-inactivated autologous PRGF eye-drops. The latter has preserves the content of most of the proteins and morphogens involved in its wound healing effects while reduces drastically the content of IgE and complement activity. Although the present work represents only a preliminary study and additional in vivo studies are needed to effectively evaluate the potential of the new formulation, this is the first time that a heat-inactivation treatment of a platelet-rich plasma system is suggested to treat ocular surface diseases in patients in which immune system is involved in their disease etiology.

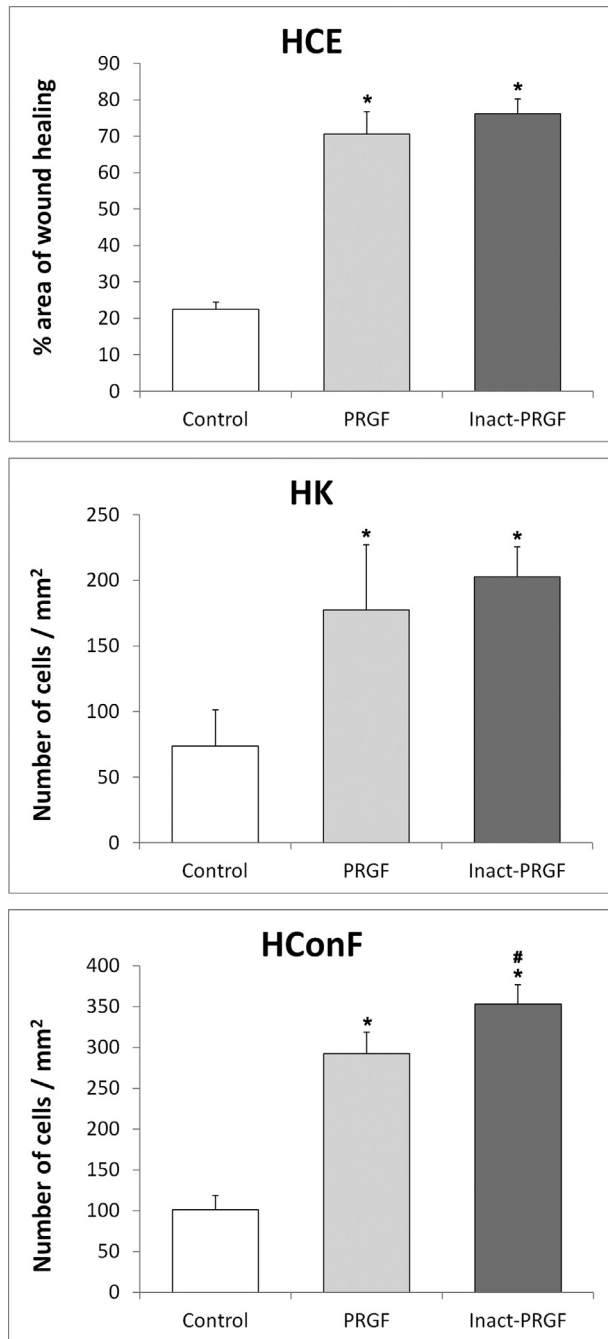


Fig. 6. Percentage area of wound healing of HCE cells and the migration rate of HK and HConF cells showed a significant increased (*) after treatment with PRGF or Inact-PRGF samples regarding control group. Significant differences (#) between PRGF and Inact-PRGF treatment was only observed on the migration rate of HconF cells, showing no differences between PRGF formulations on the migration activity of HCE and HK cells.

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