

Effect of regenerative factor rich plasma, P substance and fetal calf serum on the growth of epithelial cells in the cornea. Comparative experimental study

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Summary. Purpose. The goal of this study was to evaluate the experimental effectiveness of Regenerative Factor Rich Plasma (RFRP) of human blood versus Fetal Bovine Serum (FBS) and neuropeptide Substance P (SP) on corneal epithelium cell proliferation. Method. Rabbit corneal epithelium cell (CCL-60) growth was compared between different RFRP fractions, FBS and with the neuropeptide Substance P. The ability of the RFRP fractions and SP to revert the inhibitory effect of the CsA was also evaluated. Results. All groups showed an increase ($p < 0.001$) in corneal epithelial cell growth compared with the control group. The maximum capacity of cell growth was obtained with dilutions of 50% in the FBS, RFRP-I, RFRP-II, RFRP-III groups and with 100nM of SP. The highest growth was observed with 50% FBS, RFRP-I and RFRP-II. The group with SP and RFRP-III had significantly lower growth ($p < 0.001$). When the NK1 receptor antagonist CsA was added at a dose of IC₅₀, we found a significant decrease in cell growth ($p < 0.001$) in all culture conditions, including the control group. The decrease was similar in all groups, but was especially pronounced in RFRP-II. RFRP I, II and III promoted growth more than FBS 10%. Conclusion. The RFRP of human blood promotes the growth of corneal epithelial cells in a significantly more efficient manner than FBS and SP. RFRP can be effective both in cell cultures and stem cell cultures.

Key words: Plasma, Cornea, Culture, Regenerative factor rich plasma, Neuropeptide, Fetal calf serum

Introduction

Autologous serum has been used to treat disorders of the corneal epithelium thanks to its antigenic effect (Chaumeil et al., 1994; Geerling et al., 1998) and epitheliotropic effect (Fox et al., 1984; Tsubota et al., 1999b), and is more effective than other non-biological treatments (Geerling et al., 2001; You et al., 2001; Noble et al., 2004; Kasper et al., 2008)

The efficacy of the serum depends on the plasma concentration and the length of treatment (Tsubota et al., 1999a; Goto et al., 2001). Serum has been used in the development of various hemoderivates, such as platelet concentrates (Hartwig et al., 2004), platelet-rich plasma (PRP) (Frechette et al., 2005) or growth factor rich plasma (GFRP) (Alio et al., 2007), which use a particular fraction of the plasma, and whose active principle is based on the action of platelet growth factors.

Particular fractions of serum and plasma accelerate the growth, migration and differentiation of the corneal epithelium (Hartwig et al., 2005). In addition to platelets and growth factors, other factors such as neuropeptides (Nakamura et al., 2003; Matsumoto et al., 2004), fibronectin (Gordon et al., 1995), vitamin A (Nelson and Gordon, 1992), antimicrobial proteins (Lagnado et al., 2004), lactoferrin and immunoglobulin (Fukuda et al., 1996) are also involved in these processes. The clinical application of these elements in an isolated approach has

failed to produce positive results (Nelson and Gordon, 1992; Gordon et al 1995). For this reason, regenerative factor rich plasma (RFRP) was chosen, since includes all these elements and has been clinically shown to speed up the healing of wounds and corneo-conjunctival burns (Márquez de Aracena et al., 2007; Márquez de Aracena and Montero, 2009), and to facilitate the adaptation of transplants and histocompatible grafts (Márquez de Aracena et al., 2006; Márquez de Aracena, 2012a,b).

On the other hand, Substance P (SP) has an important effect on plasma, since it promotes migration (Nishida et al., 1996) and mitogenesis, is involved in platelet aggregation and plays a role in regulating thrombus formation, and platelets have specific receptors for SP NK1 (Graham et al., 2004). High concentration levels of SP are needed to trigger total platelet precipitation (Graham et al., 2004). This might at least partially account for the clinical efficacy of the topical and subconjunctival application of hemoderivates like RFRP, which add no artificial substances, and lead to gradual platelet activation when they come into contact with the neuropeptides released by the ocular surface. The presence of SP receptors (NK1) in platelets might lead us to think that the potential effect of blood derivatives on the ocular surface has something to do with the presence of this neuropeptide.

On the other hand, in cell cultures, media such as fetal bovine serum (Borderie et al., 1999) or umbilical cord (Zhao et al., 2004) have been used, which present certain technical difficulties: possible risk of antigenicity, transmission of infectious diseases (Geerling et al., 1998), immune rejection, clotting disorders (Anitua et al., 2004) and excessive expense. In this context, we might assume that other hemoderivates like RFRP could be regarded as the appropriate medium, above all for autologous cell cultures in which the patient's own blood can be used, since this is easily available, low in cost, and will not give rise to problems with antigens.

For this reason, we propose to assess the experimental efficacy of the various fractions of RFRP in cell proliferation in the corneal epithelium, determine what degree of involvement neuropeptides (SP) have in this proliferative process, and evaluate the possibility of using RFRP as a cell culture medium.

Material and methods

Analysis was conducted using the methods cited below

1. To obtain the optimal growth concentration, rabbit corneal epithelium cells (CCL-60) were cultured with different fractions of RFRP and FBS at increasing concentrations (10, 25 and 50%), and SP at 5, 10, 100 and 500 nM.

2. CCL-60 cell growth with the optimal concentration of FBS, RFRP-I, RFRP II, RFRP-III and SP was compared with the control group, which was defined as the standard culture condition.

3. To calculate the maximum inhibitory concentration of the neurokinin-1 (NK-1) receptor antagonist Cyclosporin A (Cs-A), CCL-60 cells were cultured with increasing concentrations of CsA (10-50%).

4. To evaluate the ability of the RFRP to revert the inhibitory effect of the CsA, CCL-60 cells were cultured with RFRP-I, RFRP-II y RFRP-III and SP after the addition of the NK-1 receptor antagonist.

Cell line

Rabbit corneal cell line CCL-60 was purchased from American Type Culture Collection (ATCC, Manassas, USA). Cell cultures were maintained in Eagle's Minimum Essential Medium (Gibco BRL, Invitrogen Corporation, Barcelona, Spain) supplemented with 10% fetal bovine serum inactivated (FBS) by heat (Gibco) as recommended by ATCC. The cells were maintained in culture flasks of 75 cm² (Falcon, Heidelberg, Germany) at 37° C in a humidified atmosphere of 5% CO₂ and subcultivated by trypsinization at 5% using Trypsin-EDTA (Sigma-Aldrich, Madrid, Spain).

Preparation of the compounds

1. Method for obtaining the different fractions of RFRP.

Twenty mL of male peripheral blood was extracted by venipuncture in sterile tubes (4.5 mL per tube) with 5% sodium citrate base and then centrifuged at 2100 rpm for 7 minutes followed, by separation of plasma red cell with the pipette. The three fractions were defined as follows: RFRP I: 0.5 mL immediately over the red series rich in proliferating factors; RFRP-II: 0.5 mL over the previous fraction rich in migratory factors RFRP- III: the rest of the plasma that is poor in regenerative factors. Plasma was extracted from the deepest peripheral fraction. The RFRP fractions were stored at 4°C for 2 weeks or -4°C for around 4 months (26, 27, 28). The number of platelets per liter was measured.

The resulting plasma contained a high concentration of platelets and other metabolites, in such a way that the fractions closest to the red series presented the greatest number of platelets, growth factors and neuropeptides, favoring cell growth, while the most distant ones, which had fewer platelets and were richer in vitamin A and fibronectin (Márquez de Aracena, 2012b) favored cell migration, among other aspects (Liu et al., 2006).

2. Preparation of the NK1 receptor antagonist, cyclosporin A-.

The commercial preparation Neoral Sandimmum sun 100mg/ml (Novartis) was used. Each ml of oral solution contained 100 mg of cyclosporine microemulsion, 94.70 mg of ethanol and 383.70 mg of castor oil-hydrogenated polyoxyl-40. The active

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ingredient was cyclosporin A (CsA), a calcineurin inhibitor and an NK1 receptor antagonist (MW: 1202.3). It was diluted in bidistilled water. In order to determine a 50% inhibitory concentration (IC50), different concentrations (10, 25 and 50 mM) were used in at least three different assays.

3. Preparation of the NK1 receptor antagonist substance P

The neuropeptide SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) PM 1347 acetate salt (Sigma-Aldrich, Madrid, Spain) 25 mg vial, purity 98% was dissolved in bidistilled water. In order to determine cell proliferation induced by SP, different concentrations were evaluated (5, 10, 100 and 500 nM).

Proliferation test

The cell proliferation test was performed following the colorimetric method based on the tetrazolium compound CellTiter 96 Aqueous one solution cell proliferation assay (Promega Corporation, Madison, WI, USA). Cells were cultured for 4-5 days preceding the assay, separated by trypsin and counted in a counting chamber of the Bürker with Trypan blue type. The cells were seeded on 96-well plates, with a total of 10⁴ cells per well in a total volume of 100 mL of culture medium. The experiments were performed using the control wells with and without FBS and the various compounds evaluated in the concentrations indicated in the previous section. Once cells were added, the plates were kept in a 5% CO₂ atmosphere at 37°C for 48 hours. The

absorbance, which is directly proportional to the number of living cells, was measured at 490nm with a plate spectrophotometer (AsysHitech UVM 340. Isogen Life Science, Spain). The values were standardized by using as 100% the maximum cell growth cultured in the standard medium recommended by ATCC (10% FBS). Each plate was performed in triplicate and all experiments were performed a total of three times separately.

The research followed the tenets of the Declaration of Helsinki; informed consent was obtained from the subject after explanation of the nature and possible consequences of the study, and where applicable, the research was approved by the institutional human experimentation committee or institutional review board (IRB).

Statistical analysis

Statistical analysis was performed by a post hoc multiple comparison test (ANOVA) performing the homogeneity test of variances using the Bonferroni or Dunnett T3 test, and assuming a significance level of 0.05 using SPSS 16.0 for windows (SPSS, Chicago, IL.).

Results

The RFRP III had a platelet concentration of 257x10⁹/L; RFRP II 307x10⁹/L and RFRP I 521x10⁹/L. The three fractions were free of leukocytes.

All study groups showed an increase (p<0.001) in corneal epithelial cell growth compared with the control group (Fig. 1). The maximum capacity of cell growth

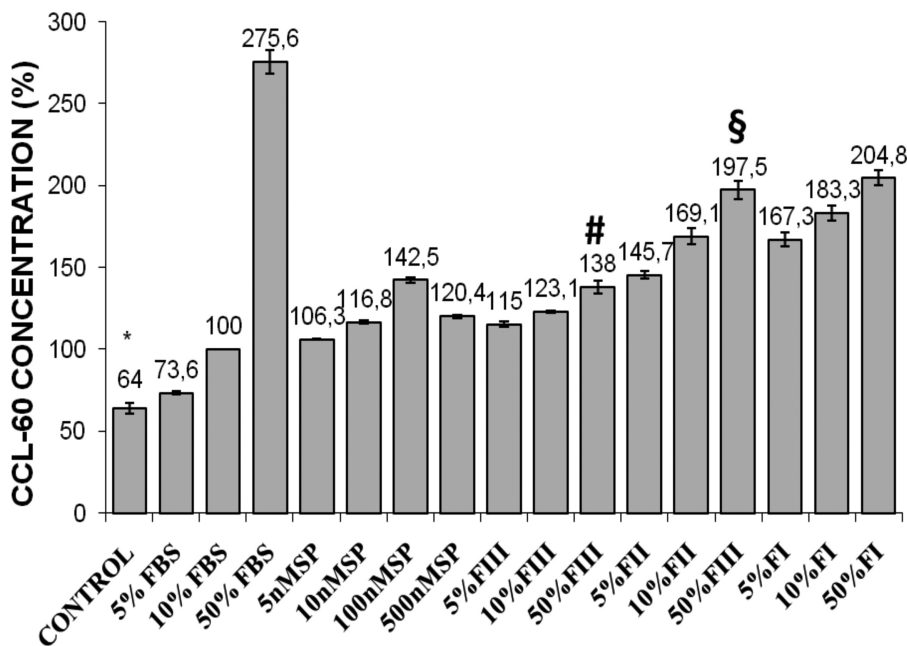


Fig. 1. Cell line CCL-60 concentration measured after the addition of different increasing concentrations of FBS, SP and platelet fractions (FIII, FII, FI). *: All groups were significantly different compared to control (P<0.001), and significantly different compared two by two, except for: # 50% RFRP-III vs. 199nM SP and § 50% RFRP-II vs. 50% RFRP-I.

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was obtained with dilutions of 50% in the culture medium in the FBS, RFRP-I, RFRP-II, RFRP-III groups and with 100nM in the SP group. The slowest growth was represented by FBS at 10% (Figs. 1, 2).

The highest growth was observed with a concentration of 50% FBS (275±7.1%), RFRP-I (204.8±4.5%) and RFRP-II (197.5±5.8%) added to the culture medium. Fifty percent of FBS showed an increase with respect to RFRP-I and II. The group with medium enriched with SP (142.5±2.2%) had lower growth (p<0.001) than FBS50% RFRP I and RFRP -II, with no significant differences compared with RFRP-III (138.1±4.7%).

When an antagonist of NK-1 receptors (Cs-A) was added at a dose of IC50, there was a reduction (p<0.001) in cell growth in all groups (Figs. 3, 4) including the control group (p>0.001). RFRP-I and RFRP-II groups also maintained their increase over the SP group (p<0.001). The quantitative reduction caused by the CsA in absolute terms showed no differences between any of

the groups, with an average decrease of 32.56 (C: 30.31; SP: 34.94; RFRP-III: 26.97; RFRP-II: 45.4; RFRP-I: 25.2). The RFRP-II reduction was slightly greater (152.09±2.2%) with respect to the other groups, and the difference was accentuated with respect to RFRP-I (179.58±0.6%).

The culture medium most conducive to growth was FBS at 50%, followed by RFRP-I (50%), RFRP-II (50%) and finally RFRP III (50%) and SP. Regarding growth in enriched medium with SP, we found significant differences (p<0.001) in the case of the media supplemented with FBS 50%, RFRP-I and RFRP-II. There were no differences compared with the medium supplemented with RFRP-III.

When the NK1 receptor antagonist CsA was added at doses of IC50, we found a significant cell growth decrease (p<0.001) in all culture media, including the control culture. The decrease was similar in all groups, and was especially pronounced in the RFRP-II group. However, all groups continued to maintain significant

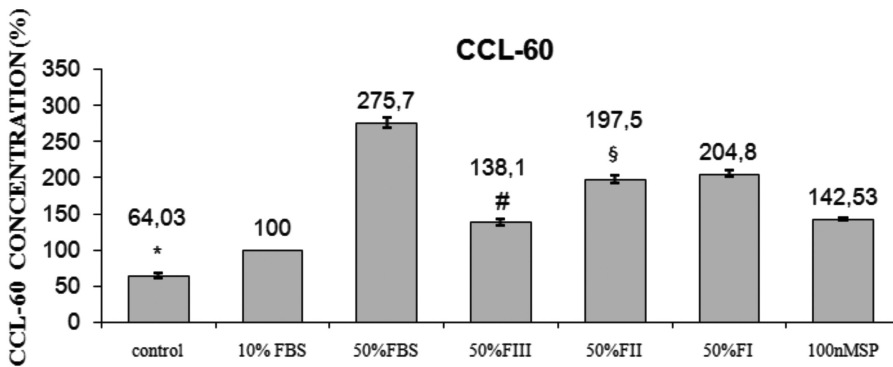


Fig. 2. Cell line CCL-60 concentration measured after the addition of: fetal bovine serum (FBS), substance P (SP) and different fractions of platelet factors: RFRP-III (FIII); RFRP-II (FII); RFRP-I (FI). *All groups were significantly different compared to control (P<0.001) and significant differently compared two by two, except for: # 50% RFRP-III vs. 100nM SP (P=0.1) and § 50% RFRP-II vs. 50% RFRP-I (P=0.1).

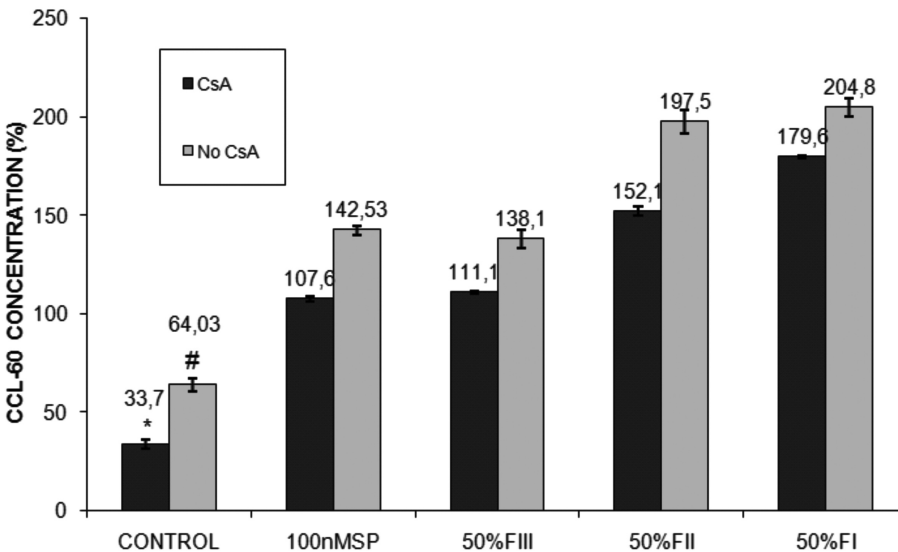


Fig. 3. Concentration cell line CCL-60 measured after the addition of: NK1 receptor antagonist cyclosporin A (CsA) at doses of IC50 alone and with various platelet fractions (RFRP-III, RFRP-II, RFRP-I) or the SP. All groups were significantly different compared to control in the group without CSA* (P<0.001) or with CsA # (P<0.001).

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growth in comparison with the control group both with and without the NK-1 antagonist. The differences were similar to those in the previous study with no NK1 antagonists. The RFRP-I group continued to show a greater increase, whereas the RFRP-II group decreased more and the RFRP-III and SP groups remained at the same level. There were no significant differences in the decline in growth in any of the groups treated with NK1 antagonist. It was also noted that the group cultured with CsA and SP simultaneously had a significant increase compared to the control group. Cell growth in the groups treated with FIII and SP was very similar, even after adding NK1 antagonists. There was also a further decline in growth in the FII group compared to I and III.

Discussion

It is known that the fractions that are richest in platelets favor cell proliferation most, thanks to the presence of growth factors and neuropeptides, whereas those that are poor in platelets (and rich in vitamin A and fibronectin) promote cell migration more (Liu et al., 2006). The aim of treatment is to increase cell proliferation, migration and differentiation. For this reason, depending on the particular requirements of the situation, the technique explained here can be used to obtain the plasma fractions that are most useful, without the need for adding precipitants like calcium chloride to the GFRP (Anitua et al., 2004).

In this study, it was noted that all groups (FBS, RFRP and SP) experienced increased cell growth: all showed significant growth compared to the control group ($p < 0.001$). Overall, they were dose-dependent, meaning that a higher concentration produced higher growth. One exception to this rule was SP: when amounts over 100 nM were used, the growth was lower. The FBS at 50% had significant growth compared to the rest ($p < 0.001$), followed by the RFRP-I and RFRP-II at 50% groups; SP and RFRP -III groups had a lower

growth, although it was still significant, with similar behavior. The slowest growth, 10%, was represented by FBS; this is a normal concentration of serum-supplement medium (Bray et al., 2012).

These results showed that the most appropriate culture medium to culture cells would be that enriched with FBS, although the technical problems identified previously have encouraged our search for alternative means. In this sense, as a consequence of the results obtained, hemoderivates could be considered as an appropriate medium, especially in the case of autologous cell cultures in which the blood of the individual favors growth due to its zero antigenicity, decreasing the chances of transmission of disease.

The usefulness of RFRP was observed in all the fractions employed here, especially in RFRP-I and RFRP-II, while the RFRP-III group produced less growth, as did the SP group. These data confirmed the effectiveness of its clinical application (Márquez de Aracena et al., 2006, 2007; Márquez de Aracena and Montero, 2009). In our experience, RFRP's effectiveness has been demonstrated in treatment for postoperative pterygium and recurrent corneal erosions. It has been shown to shorten the healing time of post-traumatic keratitis, promote epithelization of eyelid burns, and improve and enhance subjective epithelization in a diagnosis of dry eye (Márquez de Aracena, 2012b).

To evaluate an objective and real response in a similar pattern to normal conditions, blood was obtained from a healthy subject with an average number of platelets. Since we found significant efficacy regarding cell growth using different concentrations of RPRF, it seems probable that its effectiveness would be improved in those patients with a higher platelet concentration.

However, neuropeptides such as SP have been shown to be an important factor in stimulating the corneal epithelial migration (Liu et al., 2006). Given that in some ocular surface disorders such as neurotrophic ulcers these substances are decreased, they may be

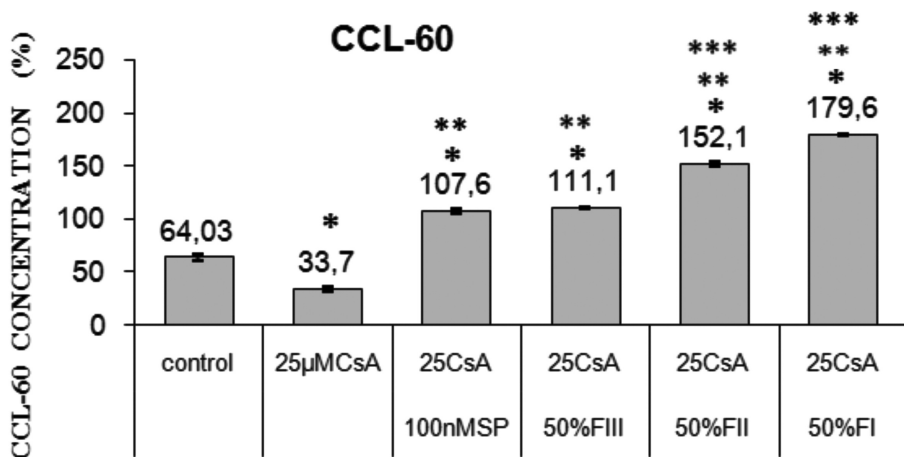


Fig. 4. Cell concentration of the CCL-60 line measured after addition of the receptor NK1 Cyclosporin A (CsA) at a dose of IC50 alone and with the different platelet fractions (FIII, FII, FI) or SP. * $P < 0.001$ compared with control. ** $P < 0.001$ compared with 25µM CsA. *** $P < 0.001$ with respect to 25µM CsA+100nMSP.

substituted by the application of serum (Nishida et al., 1996; Liu et al., 2006). In this study we observed that SP, separately administered, is less effective than RFRP hemoderivates. However, neuropeptides cannot be disregarded, given that the addition of SP antagonist (CsA) produces a reduction in all study groups in a similar manner. So the increased growth of corneal epithelial cells is not only due to the neuropeptide (SP), but other pathways or factors are also involved. Furthermore, CsA-related cell growth inhibition in the SP group was also limited, given that it used other ways to promote cell growth.

It was evident that the culture medium with SP acted in a similar way to poorer plasmatic fractions (RFRP -I), and as the plasma concentration is higher (RFRP-II and III), the differences are more significant. Because of this, SP enhanced cell growth on the epithelium of the cornea to a limited extent.

CsA could be considered to be a factor which causes an overall decline in corneal epithelial growth, which can be situated at 32.56 in all groups. This also indicates that it had a determined and limited mechanism of action as well as specific inhibition.

The decrease in growth was slightly lower in the RFRP-II group than in the rest. This fact could mean that, in the fraction retained, a higher proportion of certain elements could well be affected by CsA. The fact that until now we have considered that SP receptors are located in the platelets, with the RFRP-III fraction as the richest one, is curious. We could perhaps deduce that the inhibitory function of the CsA acted on cell growth in a limited way and not just at the level of substance P and platelets.

The effectiveness of RFRP on corneal epithelial cell culture led us to consider it as a suitable factor for cell culture, especially in autologous cultures of both adult and pluripotential cells for later grafts and transplants. This confirms the results of our preliminary studies (Márquez de Aracena and Pérez, 2012a,b; Márquez de Aracena and Montero, 2012; Márquez de Aracena, 2012a,b)

Conclusion

This study confirms the results of previous research concerning the effectiveness of RFRP on the regeneration of the ocular surface to promote cell growth.

RFRP was effective as an additive factor for culture cell medium, especially fractions I and II. RFRP is more effective than FBS at the usual dose.

SP promoted cell growth in a limited way, less than RFRP did. They may therefore have different mechanisms of action. RFRP, which is a simple, economical, effective and ambulatory technique, can be very useful for cell and stem cell culture, especially autologous culture for later grafts. It can avoid potential problems caused by using FBS or the umbilical cord (transmission of diseases, infections and immunity).

Acknowledgements. Supporter by Señores de la Casa Real de los Godos.

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Accepted March 13, 2013