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Title page

Potential effect of PRGF-Endoret in stromal wound healing in additive surgery

Short title: PRGF enhances wound healing in additive surgery

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ABSTRACT

PURPOSE: To compare the clinical and histological outcomes after intrastromal corneal ring segment (ICRS) implantation with and without plasma rich in growth factors (PRGF) in an experimental animal model.

MATERIAL AND METHODS: First, the toxicity of PRGF was stablished in hen´s keratocyte cultures. Then, an animal model with 18 hens…..18 hens as an animal model were randomly divided into two groups. In first group one ICRS was implanted in each eye and in the second group ;the ICRS was firstly immersed 30 minutes in PRGF-Endoret solution, then implanted and finally, PRGF-Endoret was inoculated into the channel. Animals of each group were also separated into three groups regarding the time they were sacrificed and corneal tissue was fixed for histological analysis at 2, 7, and 30 days. Cell death was detected by TUNEL assay. Proliferation was labelled by BrdU incorporation and myofibroblasts differentiation by alpha-smooth muscle actin (αSMA) immunodetection. Clinical examination, analysing epithelial wound closure, deposits and stromal haze, was carried out at the different study times.

RESULTS: No toxic effect was observed by PRGF in hen´s stromal cell cultures. Clinically, in PRGF-ICRS corneas at 7 days there were more deposits with higher intensity than in ICRS group. Histologically, at day 2 there were less epithelial damage over the segment in PRGF-ICRS group, corneal edema around the segment disappeared earlier and, at day 7, there were also double number of cells around the segment than in the ICRS group displaying different morphologies. The number of BrdU-positive cells was statistically higher in PRGF group at all analyzed times and TUNEL positive cells at 7 and 30 days. There were no differences in the number of αSMA-positive cells at 30 days between both groups.

CONCLUSIONS: The ICRS immersion in PRGF-Endoret prior and after to its corneal implantation, in an experimental animal model, enhances clinical deposits and histological cell turnover without increasing myofibroblast differentiation reducing stromal wound-healing time after surgery.

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Introduction

Plasma rich in growth factors (PRGF) represents a new technology that uses autologous proteins and growth factors derived from plasma and platelets as therapeutic formulations for different regenerative purposes. Under a strict pharmaceutical development, it is possible to elaborate biological stable eye-drops that can be used for the treatment of several diseases of the ocular surface.[1-9] Recent data suggest that the cocktail of molecules playing a biological function in PRGF eye-drops is extensive, including trophic factors,[10] anti-inflammatory agents [11] and bacteriostatic/bactericide molecules.[12,13] Furthermore, it has been shown that significantly enhances proliferation and migration of both keratocytes and conjunctival fibroblasts.[1,2] It also prevents and inhibits transforming growth factor beta-1 (TGFβ1) induced myofibroblast differentiation,[1-4,14] concluding that it may have a role in myofibroblast modulation in the stroma of the ocular surface tissues. Recently, we observed in a mice model of corneal lesion that PRGF promoted wound healing after excimer laser photoablation, reducing corneal haze formation.[2,5] In another intriguing study, PRGF eye drops enhanced significantly the biological outcomes of both corneal stromal keratocytes and conjunctival fibroblasts, and reduced TGFβ1-induced myofibroblast differentiation in contrast to autologous serum eye drops. [1]

Intrastromal Corneal Ring Segments (ICRS) are described as an additive refractive and orthopaedic technique for ectatic corneal diseases (keratoconus, pellucid marginal degeneration and ectatic diseases).[15] After their implantation in deep stroma is described a fast closure of epithelial incision and deposits around the segment with the aim to close the void between the ICRS and the stroma where is implanted. These deposits are related with new cells and extracellular matrix.[16,17] The cells implicated have been described as fibroblast and myofibroblast (from 1 month to 3 months). Once the wound healing process is finished most of these kind of cells recover their keratocyte phenotype and it is thought that they are responsible of the corneal morphological changes which explain the flattering effect, their effect over ectatic disease progression.[16-19] These effects are described to stabilize between 3 and 6 months after implantation.[20-22] However, in some studies there is a slight movement of the segments over time and others did not control the disease.[20,23,24] Furthermore, other complications described like keratitis or neovascularization were related to problems in wound closure and no total implant tolerance.[25-27] Moreover, new different types of segments have been proposed in order

to have better stromal tolerance and avoid these problems.[28,29] Controlling wound healing with growth factors could drive also to this purpose.

Animal models are the best method to study *in vivo* these clinical and histological events described and assuming the bioethical limitations of these experiments in humans. Hens could use as animal models because have a corneal anatomy and wound healing response similar to humans.[30] ICRS wound healing in hens is a well described process by our group [17,28,31] and could be used as an example of a stromal corneal wound to test different new drugs to modulate it. Control this process with growth factors could be interesting to early closure of the wound or in other situations to stimulate the process when it is stabilized or promoting a new synthesis of corneal tissue in cases of corneal melting.

The aim of this study is to analyse whether there is any modification of tissue reaction in corneal stroma after implantation of intrastromal corneal ring segment with and without PRGF.

Methods

Preparation of plasma rich in growth factors (PRGF-Endoret)

Blood sample from one healthy young male donor was collected after informed consent into 9-mL tubes with 3.8% (wt/vol) sodium citrate. The study was performed following the principles of the Declaration of Helsinki. Samples were centrifuged following the manufacturers protocol at room temperature in a PRGF-Endoret system centrifuge (BTI Biotechnology Institute, S.L. Miñano, Álava, Spain). The plasma was drawn off to avoid collecting the buffy coat containing leucocytes. The collected PRGF was incubated with PRGF activator (BTI Biotechnology Institute, S.L. Miñano, Álava, Spain) at 37º for one hour. After filtration, the PRGF-Endoret supernatant was not diluted in order to obtain maximum concentration in growth factors. At the end, the final PRGF eye-drops were aliquoted under laminar air flow conditions to be either used at that precise moment or stored at -80ºC until use. [6,14] All procedures were carried out under sterile conditions. Segments in ICRS-PRGF group were immersed in PRGF-Endoret solution for 30 minutes prior to implantation. After implantation, 0.1 ml of PRGF-Endoret was gently inoculated inside the channel with an anterior chamber cannula.

Cell culture

Hen corneal keratocytes were obtained from fresh corneal samples. After removal of the epithelium and endothelium using a sterile technique under a dissecting microscope, the keratocytes were released from the stroma by incubation with collagenase type II (2 mg/mL; Gibco, Carlsbad, CA, USA). Cell number and viability were determined using trypan blue exclusion. The culture medium was a 1:1 mixture of Dulbecco´s modified Eagle´s medium and Ham´s nutrient mixture F-12 (DMEM-F12, Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma; St. Louis, MO, USA) and a 1% antibiotic-antimycotic solution (Sigma). Cells were seeded in 35-mm plates (2 X 10^5 cells/2 mL/plate) and maintained at 37ºC in 5% CO_2 until grown to near confluence. The culture medium was changed every 2 days, and the cells were amplified by culturing for one passage. The passage was made by trypsinization (bovine pancreatic trypsin, Sigma).

Cell Viability Assay

One-passage hen's corneal fibroblasts were seeded onto 96 well-plate (3 X 10^3 cells/200 µL/well), 5 wells for each PRGF-Endoret concentration analyzed, until the cells reached 80% confluence. At this moment, and to test the response to different concentrations of PRGF-Endoret in the cells, the medium was replaced with fresh FBS alone (control) or supplemented with different concentrations of PRGF (10%, 20% and 40%) or 10% doxorubicin as positive control of cell toxicity.

The toxic response were determined with the Cell Proliferation Reagent WST-1 (Roche, Madrid, Spain). Conversion of WST to formazan was measured at 450 nm by microplate spectrophotometry Sunrise RC (Tecan). This reaction showed the reductive capacity of cells and hence, their viability. It was measured by changes in the optical density of the medium at 48 hours post-treatment.

Animals

This study was approved by the Animal Research and Welfare Ethics Committee of the University of Valladolid (Spain) in agreement with European (Council Directive 2010/63/UE) and Spanish regulations (RD 53/2013). Iber Braun adult hens (n=18), *Gallus gallus domesticus*, weighing about 2.5 kg each, were cared for following the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

The hens were randomly divided into two groups. ICRS implantation group (n=9 hens, both eyes) in which one ICRS was implanted in each eye and PRGF-ICRS group (n=9 hens, both eyes) in which one ICRS was implanted in each eye immersed previously in PRGF and PRGF-Endoret was inoculated in the channel after surgery. For surgery, the hens were anaesthetized with an intramuscular injection of ketamine hydrochloride (37.5 mg/kg; Ketolar, Parke Davis SA, Barcelona, Spain) and xylazine hydrochloride (5 mg/kg; Rompun, Bayer, Leverkusen, Germany) followed by topical application of 0.5% tetracaine hydrochloride and 1 mg of oxybuprocaine (Colircusi Anestésico Doble, Alconcusí SA, Barcelona, Spain). [17] Animals from each group were also divided into three groups regarding the time of sacrifice at 2, 7, and 30 days (6 eyes by group).

ICRS implantation

One Ferrara Ring® segment (AJL Ophthalmics, Vitoria, Spain), made of polymethyl methacrylate (PMMA), was implanted in each eye mimicking followig the manual technique adapted to chickens.[17,31]

Clinical course

The anterior segment of the eyes was evaluated under a surgical microscope (Leica M220 F12, Leica Microsystems, Nussloch, Germany) before and after ICRS implantation. The animals were observed at 1, 2, 3, 7, 15, and 30 days. Epithelial wound closure was measured by sodium fluorescein staining (Fluotest®, Alcon, Cusi, Barcelona) until epithelial closure was completed. Deposits and haze were measured using a predetermined scale as described by Ruckhofer et al. (2000b) and Fantes et al. (1990) respectively.[32,33]

Tissue processing and light microscopy

Animals were euthanized by an intracardiac injection of sodium pentobarbital (Dolethal® 0737-ESP Vetoquinol, Madrid, Spain) while under general anesthesia. Three time points after ICRS implantation were examined histologically: 2, 7, and 30 days (6 eyes by group and time point). Enucleated eyes were fixed with 10% buffered formalin, and embedded in paraffin. Sections 7-µm thick were stained with hematoxylin-eosin (H-E). The sections were examined under an Olympus BX41 microscope (Olympus Life Science, Hamburg, Germany) and photomicrographs were obtained with an Olympus DP20 Digital Camera. Quantitative measurements of the photographs were taken using the program Cell A (Olympus Soft Imaging Solutions GmbH, Münster, Germany). In two images of each cornea, measurements of corneal full thickness, where the ICRS was implanted (Fig.1; D1), were taken at 40x magnification. Measurements of the epithelial thickness

over the segment (Fig.1; D2 *) and, of the area left by the segment (**), were also measured at 40x magnification (Fig. 1). Stromal cells were counted at 100x magnification using the Touch Count function in a total area of 371,162 μm^2 around the ICRS. All of these measurements were carried out using methods described in a previous study.[17]

Cell death

To detect DNA fragmentation associated with apoptosis, terminal uridine nick end labelling (TUNEL) assays were performed in deparaffinized sections according to the manufacturer´s instructions (TUNEL, G3250, Promega Corp., Madison, WI, USA). Nuclei were counterstained with 4´, 6-diamino-2 phenylindole (DAPI, D9542, Sigma-Aldrich, Munich, Germany). Sections were examined under an Axiophot fluorescence-incorporated microscope (Zeiss Axiophot HB0-50, Carl Zeiss, Germany) and photomicrographs were captured using the AxioCam HRc Digital Camera and Axiovision release 4.8 software (Carl Zeiss, Germany). Using 200x magnification micrographs, epithelial and stromal TUNEL positive cells were evaluated as in a previous work by Ibares-Frias L et al., 2015. [17]

Cell proliferation

One hour before euthanasia, the animals received an intramuscular injection of 5-bromo-2´-deoxyuridine (BrdU, Sigma-Aldrich), a DNA synthesis marker (10 mg/ml, 5 ml/kg). Sections were deparaffinized and treated with 2N HCl for 1 hour, then incubated with mouse monoclonal IgG anti-BrdU (Dako, Cytomation Carpinteria, CA, USA) for 30 minutes. The secondary antibody was fluorescein goat anti-mouse IgG (1:100, Molecular Probes, Leiden, The Netherlands) in Tris-buffered saline. Control slides were prepared by omission of the primary antibody. Sections were examined under an Axiophot fluorescence-incorporated microscope (Carl Zeiss, Germany) and photomicrographs were captured using the AxioCam HRc Digital Camera and Axiovision release 4.8 software (Carl Zeiss, Germany). Using 200x magnification micrographs, epithelial and stromal BrdU-positive cells were counted around the segment.[17]

Myofibroblastic differentiation

Myofibroblasts were identified by staining with anti-alpha smooth muscle actin (αSMA) monoclonal antibody (mouse clone 1A4, Dako, Glostrup, Demark). The secondary antibody was Texas red goat anti-mouse IgG (Molecular Probes). Nuclei were stained with DAPI (Molecular Probes). Sections were examined under an Axiophot fluorescence-incorporated microscope (Carl Zeiss) and photomicrographs were captured using the AxioCam HRc Digital Camera and Axiovision release 4.8 software (Carl Zeiss). Using 200x magnification micrographs, stromal αSMA cells were counted around the segment.[17]

Statistical analysis

SPSS 20 Statistical software was used for statistical analysis. Measured variables were analyzed by calculating the mean and standard deviation. After defining these parameters, variances were compared with the Levene test. If the variances were equal, Student's t-test and analysis of variance were used to compare the equality of the means. If the variances were different, then the non parametric Kruskal-Wallis test or Mann Whitney U test was used to compare the equality of the medians. $P < 0.05$ was considered to be statistically significant.

Results

Cell Viability

All PRGF-Endoret concentrations tested increased the metabolic activity of the chicken stromal cells *in vitro* compared with doxorubicin-treated cells. No toxic effect was observed by PRGF treatment in any case. The doses of PRGF-Endoret at 40% induced a higher metabolic activity compared with FBS (10%) or PRGF-Endoret at 10 and at 20%.

Clinical assesment

None of the eyes in both ICRS and PRGF-ICRS groups had complications. Complete epithelial wound closure, defining by the absence of epithelial fluorescein stain was achieved at 3.2 ± 2 and 2.5 ± 1.7 days in both groups respectivelly without statistically significant differences ($p = 0.7$).

Deposits appeared earlier and with higher incidence in PRGF-ICRS group. At day 7, 83.33% of eyes in PRGF-ICRS group had deposits while in ICRS group there were deposits only in 16.66% of eyes ($*p = 0.021$) (Fig. 2).

At day 30, there were no clinical differences between both groups. Central corneas were clear without haze in all eyes.

Histological assessment

Hematoxylin-eosin stain

Differences between measurements with statistical significance in all groups are shown in Table 1.

The epithelial thickness decreased from day 2 to day 30 after surgery in both groups. In corneas treated with PRGF implanted segments, this hypoplasia was less evident at day 2 with statistically significant differences regarding ICRS implanted corneas (*p =0.007) (Table 1A; Fig. 3A-B,*). On days 7 and 30, there were no statistically differences between both groups (p =0.382 and p= 0,083) (Table 1B-C).

At day 2, in the stroma around the segment, it is possible to observe edema (Fig. 3A, 3B, 3E and 3F). In PRGF-ICRS group, the signs of edema disappeared earlier (Fig. 3F). After paraffin embedding and cutting, it was observed a triangular empty void left by the segment in medium and deep stroma. Throughout follow up, this void was decreasing its area in both groups. However, in PRGF-ICRS group eyes, this contraction happened faster, evaluated by the existence of statistically significant differences in the measurement of the area between groups (*p =0.013) at 7 days, being smaller this area in PRGF-ICRS group.

The healing around the implant was previously described [17] . Briefly, the cells approached to the segment from the limbus at day 7, when the corneal oedema started to decrease. Then, these cells filled the void starting by the both inferior edges of the implant in a singular disposition. At day 7, the number of cells was greater in PRGF-ICRS being the differences statistically significant (Table 1B; *p =0.001). The cells in PRGF-ICRS group were composed by a heterogeneous population with basophilic fusiform cells and smaller rounded cells, and some cell debris (Fig. 3H; cells not observed in ICRS group, Fig 3G). At day 30, the differences in the number of cells around the segments between both groups were not statistically significant (p =0.164) (Table 1C).

Cell death

At all analysed times, in all corneas, positive TUNEL cells were located in the superficial layer of the epithelium (physiologic). In the deep stroma, at 7 and 30 days, more TUNEL positive cells were observed in

PRGF-ICRS group than in ICRS group, being located mainly around the segment (*p =0,034 and p= 0,032 at 7 and 30 days respectively) (Fig. 4).

Cell proliferation

From day 2, in the basal layer of the epithelium, BrdU positive cells were detected especially over the segment, and maintained until 30 days with no differences between groups (physiologic). In the stroma, were also found BrdU positive cells in both groups around the segment from day 2 to day 30, being statistically significant higher their number in PRGF-ICRS group (*p =0.023 , p=0.001 and p= 0.001 at 2, 7 and 30 days respectively) (Fig.5).

Myofibroblastic differentiation

Myofibroblasts (αSMA-positive cells) were detected only around the segment at day 30 in both groups without statistically significant differences between both groups (27 ± 10.8 and 24.25 ± 6.55 αSMA-positive cells by 200x magnification field, in ICRS and PRGF-ICRS respectively; p =0.645).

Discussion

Plasma rich in growth factors, a novel blood derivative product is a preparation which is described that enhances corneal wound healing in dry eye and some corneal surface diseases and promote proliferation over keratocytes, corneal epithelial cells and conjunctival fibroblasts *in vitro*.[1,2,6,7,9] However, there are no studies related to the effects of PRGF in stromal wounds. ICRS implantation could be a useful example of a stromal wound.

The choice of three time points for histological analysis was made based on clinical, histological and refractive events described in the literature regarding wound healing studies after different refractive surgeries,[30,34] intrastromal corneal ring implantation surgery[16,32] and after our prior results in hens.[17] At day 2 we studied apoptosis and the beginning of cell proliferation. At 7 days, we assessed the maintenance of cell proliferation (at 7 days the proliferation of epithelial cells peaked) with the disappearance of edema with cells beginning to approach the edges of the segment.[17] The objective of the study at 30 days was to study myofibroblast differentiation (in our animal model at this time point is when myofibroblast differentiation

peaked).[17] Analysing these time points we can study all the events described in a normal wound healing process. There is no other experiment showing stromal regeneration of PRGF drops in the literature.

The results of this comparative study showed that in the PRGF-ICRS group the stromal wound closed earlier, displaying bigger and more confluent deposits than ICRS group which could be related with few complications and best optical outcomes. Histologically, in PRGF-ICRS group we found a more biological compatibility of the ICRS after implantation with an earlier disappearance of intrastromal edema, faster decrease of the area of the void where the ICRS was located, and less thinning of the epithelium over the segment. Events which are correlated with the clinical ones described before. Is important to note that the central cornea remained transparent in both groups without any grade of haze.

Regarding the number and the type of stimulated cells, PRGF-Endoret induced robust proliferation of cells with different morphologies in human corneal cell cultures *in vitro*.[9] In our study, at 7 days there were statistically differences between both groups with more cells in PRGF-ICRS group. These cells were described as an heterogeneous population with cells resembling fibroblast in morphology and another more rounded which resemble cellular debris more than lymphocytes agreeing with previous manuscripts.[1,9] Certain findings support the non-inflammatory origin of these cells, firstly, the reaction is not described from the beginning when usually the inflammatory reactions begin,[35-37] secondly, there are no clinical reaction compatible with inflammation (no infiltrates, no cells in anterior chamber, no increase of the deep stromal haze and no any other complications)[38-40] and thirdly, the morphology and shape of these cells by electron microscopy (both scanning electron microscopy and transmittance electron microscopy) did not resemble lymphocytes,[38] they are similar to those described in previous studies.[17,38] This heterogeneous population could indicate a bigger stimulation by a higher release of growth factors than in a normal wound healing response due to the PRGF-Endoret immersion and intrastromal innoculation. The latter could be more undifferentiated subpopulations as described Freire et all (2012)[41].

Moreover,,at all analysed times the number of BrdU-positive cells was statistically significant higher in PRGF-ICRS than in ICRS group agreeing with the bigger stimulation described before. Also at days 7 and 30 , the number of TUNEL positive cells was higher in PRGF-ICRS group indicating cell replacement.

Myofibroblastic differentiation occurs after 30 days of implantation, however, there were no differences between groups in αSMA positive cells number. This event could be related to that among the great number of proliferating cells at 7 days, only a small number of cells is differentiate into myofibroblast which agree

with previous works which shown that PRGF decreases myofibroblast differentiation and secondary clinical haze. [1,2,10]

All of the potential benefits described of PRGF-Endoret in wound healing were observed despite of PRGF-Endoret in our experiment were not autologous for hens. This fact was due to it is almost impossible to prepare a hens-derived PRGF-Endoret because the hen´s thrombocytes have nucleus and they are larger than those of humans. Hence their speed of centrifugation is similar to that of other blood components, making it impossible to isolate. For this reason, we used blood from healthy humans. When transferring the events of the study to humans therefore it could be considered that PRGF-Endoret exerts a more potent effect when it is used in an autologous fashion as in other studies which published results of human products over animal models.[42] On the other hand, the observation that human PRGF-Endoret stimulates proliferation in hen´s stromal cells populations "in vitro" demonstrated its biological potential and biosafety.

Another limitation of this experiment was to use both eyes of the same animal for the same treatment, this was performed for logistic organization regarding manipulation and processing of PRGF and to avoid differences between right and left eye. However, all of the characteristics of the animals were totally comparable. Moreover, our previous experiments in hens allow us to ensure that having implanted the 2 segments of the same group in the same animal will not influence the results.

To show this enhance in wound healing response is necessary to combine PRGF and ICRS implantation, due to PRGF alone in the channel is not related to any significant wound healing process (data not shown).

Conclusions

Our results indicate that PRGF, in clinical and histological response, after implantation of ICRS in an experimental animal model, enhances stromal cell proliferation reducing wound-healing time. Although are necessary more studies to explain all of the mechanism implicated in the events after PRGF-ICRS implantation, the results of this study indicate that it could improve wound healing during ICRS implantation, and in general, the stromal wound healing response after additive surgery.

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The authors declare the following competing financial interest(s): E.A. and GO are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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LEGENDS OF FIGURES

Figure 1: Figure 1. Distances (D) measured from 4X hematoxylin-eosin stained micrographs in the area where the ICRS was implanted. The following distances at the site of implantation were measured: D1, corneal thickness in the area of the segment; *2, epithelial thickness; **, Area of the triangular hole left by the ICRS. L: Edge of the preparation close to the limbus. C: Edge of the preparation close to the corneal center

Figure 2: Intrastromal deposits in ICRS group (Fig. 2A) and in PRGF/ICRS group (Fig.2B) along the inner curvature of the segment (arrows)

Figure 3: Cross-section of hen corneas stained with hematoxylin-eosin at 5X magnification (A, B, C and D) and 10X (E, F, G and H) at 48 hours (A, B, E and F) and 7 days (C, D, G and H). More edema is observed around the segment in ICRS group (see black arrows in Fig E). More epithelial hypoplasia also is evident over the segment in ICRS group (* Fig. A > than in B). The area of the void left for the segment was smaller in group PRGF/ICRS than in ICRS group (*<** in Fig 4 D and 4C). There were more cells in group PRGF/ICRS than in ICRS group (black arrows in Fig. G and H).

Figure 4: Graphic of the number of BrdU positive cells counted in a 20X field in ICRS group and PRGF/ICRS group.

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Figure 1

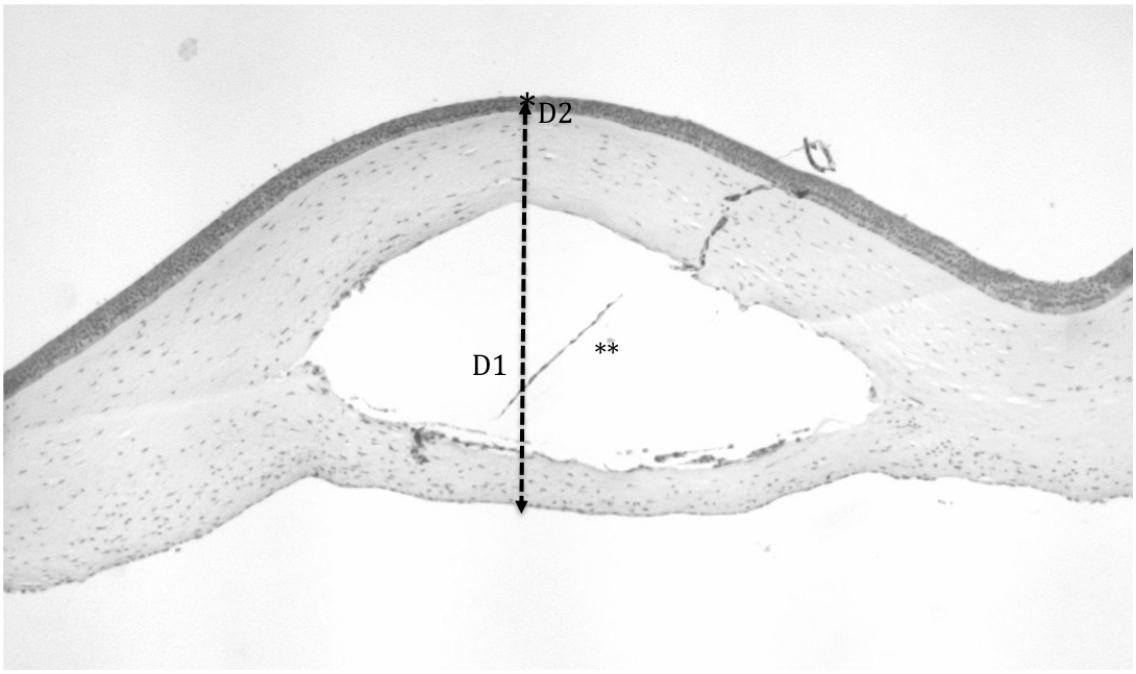

Figure 2

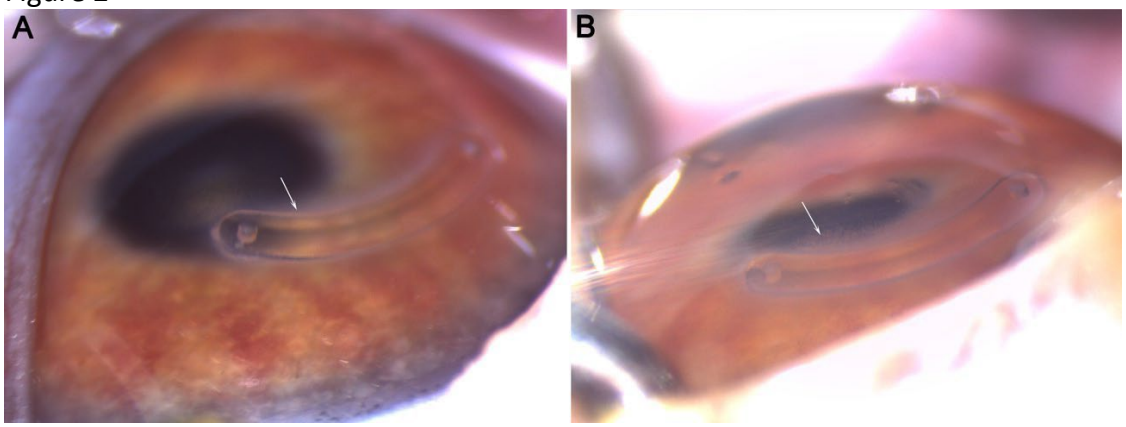

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Figure 3

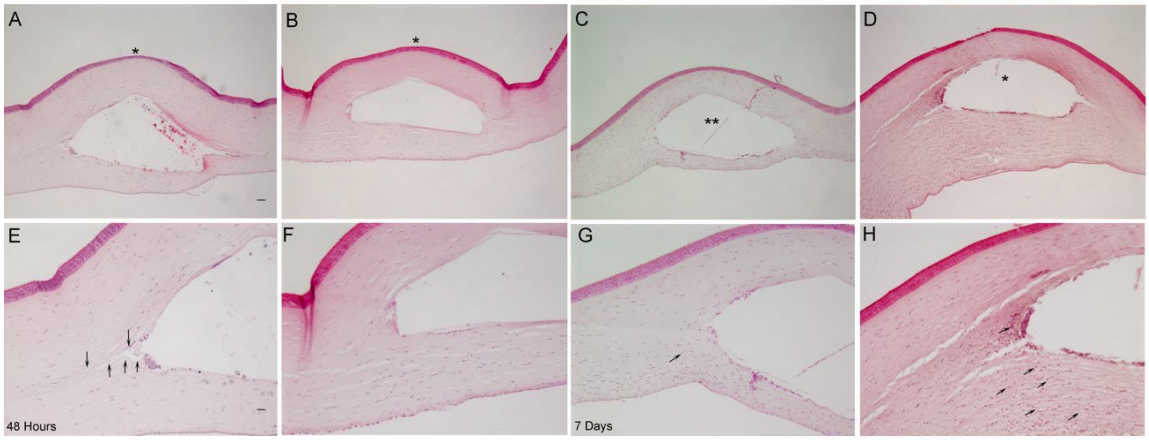
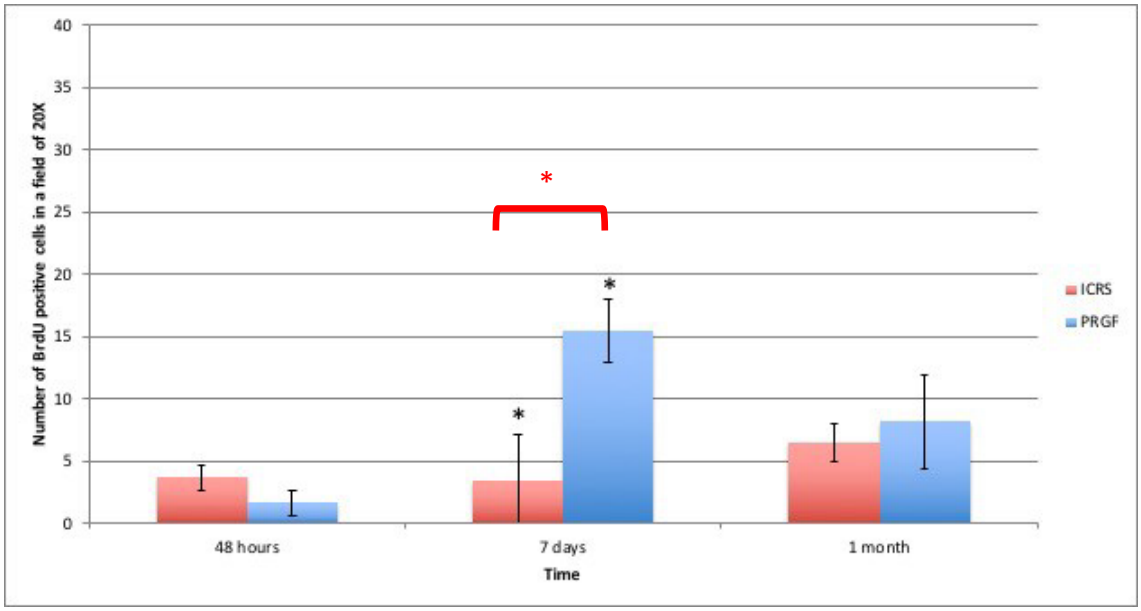


Figure 4



TABLES

Table 1: Statistically significant differences of the measurements made in the hematoxylin-eosin stained preparations at 2, 7 and 30 days after ICRS implantation.

A

2 days	ICRS (N= 6)	PRGF-ICRS (N= 6)	*p* value
Corneal thickness-D1 (µm)	650.96± 98.19	567.22±50.95	0.093
Epithelium over ICRS-D2 (µm)	**19.66±3.29**	**26.76±43.92**	**0.007***
Area (µm²)	137626.9±26942.75	112312.61±23367.39	0.113
Cells/µm²	0.00132±0.00021	0.00108±0.00018	0.066

B

7 days	ICRS (N= 6)	PRGF-ICRS (N= 6)	*p* value
Corneal thickness-D1 (µm)	614.68±101.22	633.94±38.17	0.699
Epithelium over ICRS-D2 (µm)	17.83±5.24	20.14±2.08	0.382
Area (µm²)	**168990.46±21179.01**	**136954.71±5551.57**	**0.013***
Cells/µm²	**0.00133±0.00048**	**0.00325±0.0051**	**0.001***

C

30 days	ICRS (N= 6)	PRGF-ICRS (N= 5)	*p* value
Corneal thickness-D1 (µm)	554.61±100.25	501.95±38.22	0.287
Epithelium over ICRS-D2 (µm)	18.90±4.55	23.66±4.42	0.083
Area (µm²)	116567.59±25659.29	99080.12±26992.43	0.253
Cells/µm²	0.00302±0.00072	0.00433±0.00066	0.164