Rosmarinic acid, a plant-derived compound with antiangiogenic activity, can be applied for the treatment of ocular diseases related to neovascularization, such as diabetic retinopathy, macular edema, and age-related macular degeneration. These diseases represent the leading causes of blindness worldwide if they are not properly treated. Intravitreal devices allow for localized drug delivery to the posterior segment, increasing the drug bioavailability and promoting extended release, thus, reducing side effects and enhancing the patient’s compliance to the treatment. In this work, rosmarinic acid-loaded poly lactic-co-glycolic acid intraocular implants were developed with a view for the treatment of ocular neovascularization. Physical-chemical, biocompatibility, and safety studies of the implants were carried out in vitro and in vivo as well as an evaluation of the antiangiogenic activity in a chorioallantoic membrane assay. Data obtained showed that rosmarinic acid released from the implants was quantified in the vitreous for 6 weeks, while when it was in the solution formulation, after 24 h, no drug was found in the vitreous. The delivery device did not show any sign of toxicity after clinical evaluation and in electroretinographic findings. Histological analysis showed normal eye tissue. Rosmarinic acid released from implants reduced 30% of new vessel’s formation. The intravitreal implant successfully allowed for the prolonged release of rosmarinic acid, was safe to rabbits eyes, and demonstrated activity in vessel reduction, thus demonstrating potential in preventing neovascularization in ophthalmic diseases.
which can cause serious complications such as intraocular hemorrhage, retinal detachment, endophthalmitis, and cataracts in addition to great discomfort to patients [6, 12, 13].

In order to allow the prolonged release of therapeutic levels of drugs in the vitreous, retina, and choroid, increased bioavailability, reduced systemic adverse effects, and intravitreal delivery systems are great choices [14]. They can reduce the complications of intravitreal injections and increase patients’ comfort and adherence to treatment [14, 15]. The use of biodegradable polymeric matrices of PLGA in solid implantable devices have been extensively investigated for ocular administration due to their biocompatibility, predictable kinetics of biodegradation, and mechanical resistance [15–17].

In this work, a biodegradable PLGA implant containing rosmarinic acid was developed and its potential for the prolonged intravitreal release of the drug was evaluated as well as its antiangiogenic activity and biocompatibility in vitro and in vivo.

**Results and Discussion**

The treatment of diseases affecting the posterior segment of the eye is limited and challenging once the conventional forms of drug administration fail to provide therapeutic levels of drugs to the vitreous, retina, and choroid [18]. Biodegradable implants are able to release drugs directly to the vitreous and maintain a long-term concentration in the therapeutic range [15].

In order to study the potential to promote a prolonged release of rosmarinic acid, PLGA implants loaded with the drug were developed. They were rod-shaped, homogeneous systems approximately 0.45 mm in diameter and 6 mm in length (Fig. 1). The mean weight was 1.60 ± 0.15 mg and they contained approximately 400 μg of the drug.

Differential scanning calorimetry was carried out to investigate the drug-polymer interaction after implant preparation. The curve (Fig. 2a) of raw PLGA shows an endothermic event at 57.5°C corresponding to the glass transition temperature of the polymer [19], and other endothermic events observed between 330 and 365°C are attributed to the thermal decomposition of the polymer [20]. Thermal analysis of raw rosmarinic acid (Fig. 2b) shows an endothermic event at 65.8°C, which could be related to the rosmarinic acid glass transition, indicating that part of the drug was in the amorphous state. The drug melting point was observed at 167.3°C and an endothermic event starting at 220°C indicates rosmarinic acid thermal decomposition [21]. Since no thermal events suggestive of degradation are observed in the temperature range of 70–90°C, the technique used to mold the implants, the hot molding technique, is appropriate to prepare rosmarinic acid-loaded implants. The curve of the lyophilized mixture of rosmarinic acid and PLGA (Fig. 2c) shows endothermic events attributed to PLGA glass transition and decomposition as well as the drug melting point. However, a shift in the PLGA glass transition temperature and rosmarinic acid melting point was observed, probably due to a physical interaction between them [22]. Thus, further analysis by FTIR was performed to investigate this possible interaction.

The FTIR spectrum of raw PLGA 75:25 (Fig. 3a) showed a characteristic band at 1748 cm⁻¹ corresponding to C=O stretching from the ester groups. Absorption bands at 2947 and 2995 cm⁻¹ were also identified, as well as bands at the 1460–1000 cm⁻¹ region, characteristics of C=O and O–H stretching. Similar spectrums for PLGA 75:25 have been reported in the literature [20, 23]. The rosmarinic acid spectrum (Fig. 3b) showed bands at 1607, 1515, and 1464 cm⁻¹, related to C–C stretching vibrations in the aromatic ring. A band at 3165 cm⁻¹, typical of C–H stretching in aromatic compounds, was observed. In the same region, bands related to O–H stretching from carboxylic acid and a phenol group were also identified. The presence of a phenol group was also evidenced by bands at 1348 and 1180 cm⁻¹ due to O–H bending and C–O stretching, respectively. Bands at 1724 and 1706 cm⁻¹ are attributed to C=O stretching from ester and carboxylic acid groups, respectively. This spectrum is in accordance with the ones described in other studies for raw rosmarinic acid [21, 24]. The analysis of the solid mixture of PLGA and rosmarinic acid showed that their main groups were preserved (Fig. 3c). Similarly, the spectrum of a lyophilized mixture of PLGA and rosmarinic acid (Fig. 3d) did
not show modifications in FTIR bands from functional groups present in PLGA and rosmarinic acid. The different shape observed for an O⁻H stretching band at the region of 3451 cm⁻¹ might be due to intermolecular interactions of this group, which superimposed the C–H stretching occurring in this region [22, 25]. Both mixtures presented less intense bands for the rosmarinic acid groups, which could be explained by drug dispersion throughout the polymeric matrix. The absence of major changes in the FTIR bands of PLGA and rosmarinic acid suggests there is no chemical interaction between them after lyophilization.

Scanning electronic microscopy photomicrographs showed that rosmarinic acid-loaded implants presented a smooth and homogeneous surface before incubation, without visual pores and channels (▶ Fig. 4). After incubation, pores and channels were observed, probably due to polymer degradation that involves breaking ester bonds of the PLGA chain by hydrolytic attack of water molecules of the media [26]. This facilitates drug release by diffusion throughout the polymeric matrix [27, 28]. Rosmarinic acid is a small compound with slight solubility in water (1.3 g/L; 3.6 × 10⁻³ mol/L). Its small size and linear chain facilitated the passage through the formed porous in PLGA implants during the release study. After 8 days of incubation, more pores and channels were observed (▶ Fig. 4), which matches with the peak of drug release. The accumulated in vitro release of rosmarinic acid was approximately 92.7% for 21 days, with 80% of the drug released within the first 10 days (▶ Fig. 4).

The safety of rosmarinic acid, blank implants, and rosmarinic acid-loaded implants was assessed in vitro in human retinal pigmented epithelial cells. The assay of rosmarinic acid showed that the drug, even at high concentrations, did not affect the viability of the cells. The increase of treatment time did not demonstrate a significant difference in cell viability after 24 and 72 h (▶ Fig. 5 a). For blank PLGA implants and those containing 25% w/w of rosmarinic acid, after 72 h of treatment, the viability of retinal cells was not affected, as observed by the absence of inhibition halos that are caused by the rupture of the cell membrane due to the necrotic process when the material is harmful to the cells (▶ Fig. 5 b).

The antiangiogenic activity of rosmarinic acid released from the implants was evaluated using the CAM assay, where they were placed over. Treatment with bevacizumab (positive control) at concentrations of 250 and 500 µg/mL significantly reduced the percentage of blood vessels 29.93 ± 5.41% (p < 0.0001) and 31.51 ± 5.04% (p < 0.0001), respectively, when compared with...
the negative control group (treated with PBS). The application of rosmaricin acid solutions at concentrations of 250 and 500 µg/mL on the membrane promoted a significant reduction of 34.58 ± 3.70% (p < 0.0001) and 45.70 ± 4.76% (p < 0.0001) in blood vessels, respectively, when compared with the negative control group. The percentage of reduction in blood vessels for bevacizumab and rosmaricin acid groups, at the same concentrations, was not significantly different (p = 0.5787 and p = 0.2475 for 250 and 500 µg/mL, respectively), which was expected (Fig. 6). A significant reduction in blood vessels for the rosmaricin acid-loaded implants group (71.22 ± 11.50%, p = 0.0087) was observed when compared with the negative control and blank implants group (p = 0.0087). These results suggest that rosmaricin acid was released from the implants at effective concentrations. The CAM assay is an intermediate step between in vitro and in vivo studies and offers advantages to study vascular functions of drugs and formulations. In this study, bevacizumab was used as a positive control since it has well-known antiangiogenic activity and is clinically used in intravitreal injections for the treatment of ocular diseases causing neovascularization [29]. The reduction of vessels with rosmaricin acid alone was similar to bevacizumab and confirms its potential in the treatment of ocular neovascularization, as previously reported [5]. Rosmaricin acid antiangiogenic activity was previously reported to be related to its ability to inhibit proliferation, migration, adhesion, and tube formation of endothelial cells [30]. It has also been reported that rosmaricin acid suppresses the secretion of angiogenic factors such as VEGF, TGF-β, IL-6, and TNF-α, partly via the inhibition of NF-κB and β-actin [31]. The drug was delivered by the implant in an effective concentration without any sign of inflammation, neovascularization, or vascular lysis. Intravitreal injection of an aqueous solution of rosmaricin acid (400 µg/mL) in rabbits suggests a low bioavailability of the drug alone, which is better visualized when the AUC values are compared (Table 1). This is probably due to its fast dissolution and consequent elimination from the vitreous. Rosmaricin acid loaded in implants demonstrated an AUC more than 10 times higher than that of the drug in solution. The Cmax of rosmaricin acid was observed 1 h after injection (Table 1) and then the levels started to decrease, reaching 16.23 ± 5.54 µg/mL at 18 h. After 24 h, only a small amount of rosmaricin acid was detected in the vitreous of the animals, below the quantification limit of the method (Fig. 7a). Differently, the rosmaricin acid-PLGA implants inserted in the vitreous demonstrated a prolonged release profile of the drug (Fig. 7b). An initial peak of 20.47 ± 5.21 µg/mL of
Before incubation 4 days 8 days

RA released concentration (%) 10 20 30 40

RA cumulative released concentration (%) 0 10 20 30 40

Time (days) 15 20 25

112.5 µm 112.5 µm 22.5 µm 22.5 µm

Fig. 4 In vitro degradation and release profile of rosmarinic acid implants. Top: images of rosmarinic acid/poly lactic-co-glycolic acid implants obtained by scanning electronic microscopy. Bottom: in vitro release profile of rosmarinic acid (RA) from implants during 6 weeks. a % of RA released in medium. b Cumulative concentration (%) of RA released in medium (values are shown as the mean ± SD, n = 6).

ARPE-19 viability (%)

24 h 72 h

Fig. 5 Cell viability assay using RA solutions and implants at 24 and 72 h. a RA solution in ARPE-19 (values are shown as the mean ± SD, n = 3). b Intravitreal implants in ARPE-19 (diffusion method in agar) showing the absence of halos: control (top), PLGA implant (middle), PLGA/RA implant (bottom). ARPE: retinal pigmented epithelial cells, PLGA: poly lactic-co-glycolic acid, RA: rosmarinic acid.

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rosmarinic acid observed 5 days after insertion is probably due to the drug present on the surface of the implants that is rapidly eliminated from the system to the medium. At 21 days, the maximum concentration of the drug was observed and might be related to the drug being released from the pores and channels formed during the matrix degradation (Table 1). After this period, statistical analysis demonstrated that the rosmarinic acid was released from the implants at similar concentrations (p < 0.05), which suggests a controlled release profile. The drug could be quantified in vitreous for up to 40 days. Considering the values of Tmax of the rosmarinic acid solution and rosmarinic acid-loaded implants, when the drug is incorporated in the delivery system, it is shifted to a later time, demonstrating the prolonged release (Table 1). Further, when we compare the values of the slope of the log-scale graphs (Fig. 7 c, d), we observed a higher inclination in the curve of the rosmarinic acid solution than that loaded in the implants, which shows that the drug concentration reduces faster in the first. Thus, the intravitreal device developed was able to promote a more prolonged and controlled release of rosmarinic acid in the vitreous of rabbits, suggesting a more efficient treatment, with the possibility of reduced adverse effects and higher adherence of the patient to the treatment.

Although many improvements have been made in the last years in order to enhance ocular bioavailability of drugs, reaching therapeutic doses in the posterior segment is still a challenge, and intraocular diseases are likely to emanate and prevail. The use of intraocular drug delivery systems is a potential alternative for the treatment of intraocular diseases since it represents the electrical activity generated by the retina in response to the luminous stimulus [34]. In this study, we analyzed the rod response (0.01 cd s/m²), a- and b-wave combined response (3.0 cd s/m²), and high-intensity response (10.0 cd s/m²). The a-wave (initial negative deflection) is produced by photoreceptors, whereas the subsequent positive deflection, the b-wave, reflects the response generated by other retinal cells, including photoreceptors, bipolar, amacrine, and cone cells, among others.
Muller retinal glial cells [35]. The absence of significant changes in the amplitude of a- and b-waves after insertion of rosmarinic acid-loaded implants indicates that the device or the drug released did not promote any harmful toxic effect to the retina, suggesting that it is safe for intravitreal use. Although rosmarinic acid has been previously investigated for the treatment of ocular diseases, studies demonstrating the preservation of retinal function after intravitreal injection of rosmarinic acid have not been reported [5–10].

Histopathological analysis of the retina showed the absence of inflammatory cells and hemorrhages in the areas close to the implant, as well as the integrity of the neuroretina and choroid cells (Fig. 10). Considering that the architecture of the retina was maintained, it is suggested that there was no damage to photoreceptors caused by the rupture of retinal pigment epithelial cells. Therefore, the retinal layers were not atrophied in the presence of the polymeric implants or the rosmarinic acid released, indicating that there was no toxicity associated.

In this work, we developed a PLGA and rosmarinic acid (3:1) implant to improve its bioavailability after intravitreal administration. It was verified that the implants were safe to the eye and may have potential to prevent neovascularization in ophthalmic diseases, as they showed antiangiogenic activity ex vivo and were biocompatible and safe to ocular tissues. The prolonged release of rosmarinic acid from the implants shows their suitability for the treatment of chronic ocular diseases that could increase patients’ compliance.

Materials and Methods

Preparation of intravitreal implants

The implants were prepared by dissolving rosmarinic acid (96%; Sigma Aldrich) and PLGA 50/50, (PURASORB PDLG 5004, inherent viscosity 0.4 dL/g; Purac Biomaterials) in acetonitrile (HPLC grade; Merck) at a ratio of rosmarinic acid:PLGA of 1:3. The solution was lyophilized and the powder obtained was molded into rods at a temperature between 70–90°C. The implants were prepared so that they contained approximately 400 µg of the drug. The mean weight of the implants was calculated from 10 samples randomly selected.

Thermal analysis

The thermal behavior of the materials used to prepare rosmarinic acid-loaded implants was evaluated by DSC using a DSC50 differential scanning calorimeter (Shimadzu). Samples of 4 mg of raw
rosmarinic acid, raw PLGA, and a lyophilized mixture of rosmarinic acid:PLGA (1:3) were accurately weighted in closed and pierced aluminum pans. The curves were obtained in the temperature range of 25–400 °C using a 10 °C/min heating rate and nitrogen atmosphere.

**Fourier transform infrared spectroscopy**

FTIR characterization was performed in a Perkin-Elmer spectrophotometer (model Spectrum 1000; Perkin-Elmer) to investigate the presence of specific chemical groups and interactions among the components. Each spectrum was obtained with a resolution of 4 cm⁻¹ and a spectral range of 4000–650 cm⁻¹. Samples of raw PLGA, raw rosmarinic acid, a solid mixture of raw rosmarinic acid:PLGA (1:3), and a lyophilized mixture of rosmarinic acid:PLGA (1:3) were used.

**In vitro degradation and release studies**

The *in vitro* release study was performed in glass vials containing 2 mL of PBS (pH 7.4), following the sink conditions, with stirring and temperature maintained constant at 30 rpm and 37 °C, respectively. rosmarinic acid-PLGA implants (n = 6) were individually placed in the vials. Implants without the drug were also evaluated (n = 6). For 21 days, at pre-established time intervals, the medium was completely withdrawn, and the same buffer solution was immediately replaced. The collected samples were analyzed by HPLC using the method described below.

Morphological changes on the surface of blank and rosmarinic acid-loaded implants retrieved from the *in vitro* release study (days 4 and 8) were evaluated by scanning electronic microscopy. After 8 days, the very fragile implants were removed and mounted for analysis. The implants withdrawn from the release media were blot dried and left in a desiccator for 72 h. Then, they were placed in stubs, dried, and gold-coated for 60 s prior to

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*Fig. 8* a Implant insertion using transscleral trocar canula 25 G. b Site of the implant immediately after insertion, showing the absence of hemorrhage. c Implant into the vitreous cavity. d Ophthalmoscopy with the normal retina vessels and the optic disc.
Fig. 9 Examples of the dark-adapted electroretinography responses of one animal at two luminance stimuli (0.01 and 3.0 cd.s/m²). RA: rosmarinic acid.

Fig. 10 Histological sections of the ciliary body and retina 6 weeks after insertion of rosmarinic acid implants (a, b) and before implantation (c, d). Region of the ciliary body with the absence of inflammatory cells (a, c) and retinal images showing the integrity of its layers (b, d).
analysis. The photomicrographs were obtained at a voltage of 15 Kv in a Zeiss DSM 950 (Carl Zeiss NTS GmbH) microscope.

**Quantification of rosmarinic acid HPLC**

The amount of rosmarinic acid in the samples from the in vitro and in vivo release studies was measured by HPLC in the isocratic mode using a Waters apparatus attached to a UV-Vis detector at 331 nm. A reverse-phase 18 column (Lichrosorb; Merck) at 25 °C, a mobile phase composed of a mixture of methanol (HPLC grade; Merck)/water (ultrapure; MilliQ) 50:50 (0.1% H₃PO₄ 85%; Sigma-Aldrich), and a flow rate of 0.8 mL/min were used. The method was selective and specific for the quantification of the drug, as well as linear (r² = 0.998) and accurate, with repeatability and intermediate precision and presenting detection and quantification limits of 0.021 and 0.068 µg/mL, respectively.

**Safety evaluation in human retinal pigment epithelial cells**

The human retinal pigment epithelial cell line (ARPE-19; CRL-2302TM) was obtained from American Type Culture Collection and maintained in a cell bank at Ezequiel Dias Foundation (Brazil) until use.

ARPE-19 cells were used to evaluate the safety of rosmarinic acid alone and incorporated in the implants as well as blank implants. Data were obtained from three independent experiments.

For rosmarinic acid alone, the MTT (98%; Sigma-Aldrich) assay was used. ARPE-19 cells were seeded in 96-well plates (1 x 10⁴ cells/well) and treated with increased rosmarinic acid solutions (2.5 to 500 µg/mL). PBS 10X was used as a death control. After 24 and 72 h, the medium was replaced with MTT solution (5 mg/mL) and fresh medium. After 2 h, the precipitated formazan crystals were solubilized with sodium dodecyl sulfate (99%; Sigma-Aldrich) and the plates were read at 595 nm using a microplate reader (ELX 800; BIO-TEK Instruments Inc.) after 18 h. Cell viability was determined as a percentage of control (untreated) viability.

The biocompatibility of the implants was investigated by the diffusion method in agar. ARPE-19 cells were seeded in 6-well plates (2 x 10⁴ cells/well) and incubated for 24 h in DMEM-F12 (Sigma-Aldrich) medium with 10% fetal bovine serum. Next, the culture medium was removed and fresh medium containing 10% fetal bovine serum, 1.5% agar, and 0.4% neutral red was added. Rosmarinic acid-PLGA and blank implants were then placed on the prepared gel. Polyethylene was used as a negative control. After 72 h, the samples were evaluated macroscopically by the presence of an inhibition halo.

**Antiangiogenic activity**

The antiangiogenic activity of rosmarinic acid released from implants was evaluated by the chicken chorioallantoic membrane assay. Fertilized eggs (n = 12) were incubated at 37 °C and 60% relative humidity. On the 3rd day of incubation, a hole of approximately 1.0 cm in diameter was made in the eggshell. The inner membrane was removed, and the eggs were sealed with adhesive tape. On the 5th day of incubation, 20 µL of rosmarinic acid solution (250 and 500 µg/mL) blank and rosmarinic acid-loaded implants were placed on the egg membrane. Bevacizumab (Avastin, Roche Químicos e Farmacêuticos SA; 250 and 500 µg/mL) and PBS (pH 7.4) were used as positive and negative controls, respectively. On the 7th day of incubation, the membrane was extracted after previous fixation with 3.7% formaldehyde solution and photographed with a digital camera coupled to a stereomicroscope. The images were analyzed using the software ImageJ (version 1.50i; National Institutes of Health). For this, they were converted to grayscale and then the black pixels, which corresponded to the vessels of the chorioallantoic membrane, were quantified. The control group was set to 100%. One-way ANOVA followed by Bonferroni’s multiple comparison test was used to compare groups using the software Prism 5.0 (GraphPad Software Inc.).

**Animals**

New Zealand white rabbits purchased from Experimental Farm Professor Hélio Barbosa (Igarapé, Brazil), weighing 2.0 to 2.5 kg, kept in individual cages with food and water ad libitum, under controlled temperature and humidity, and a light-dark cycle were used for in vivo experiments. All animals were previously examined and those presenting any ocular abnormality were excluded. The protocol (092/2015) was approved by the Ethics Committee in Animal Experimentation of Ezequiel Dias Foundation, Brazil (approval date June 8, 2016) and all experiments were conducted in accordance with the statement of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research and with the EC Directive 86/609/EEC for animal experiments.

**In vivo release study**

The animals were randomly divided into three groups (n = 6) that received rosmarinic acid-loaded implant (400 µg/implant, group I), blank implant (group II), and an intravitreal injection of rosmarinic acid solution (400 µg/mL, group III). The left eye of each animal was used as a control. Rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (22 mg/kg; Anasedan, Ceva Saúde Animal Ltda), and one drop of oxybuprocaine hydrochloride (4 mg/mL; Oxinest, Cristalia) was topically instilled in the animals’ eyes prior to the procedures. Intravitreal injection of rosmarinic acid solution was performed using a 30-gauge needle attached to a 1-ml syringe inserted around 1 mm posterior to the limbus. After 1, 2, 4, 12, 18, 24, and 48 h, the vitreous was collected and frozen at −80 °C until drug analysis.

The implants were inserted in the vitreous cavity using 25-gauge transscleral cannula (Alcon) that was placed through the pars plana of the eye in the subtemporal quadrant. After pre-established time intervals (4, 7, 14, 21, 28, 35, and 42 days), vitreous samples were collected and frozen at −80 °C until drug analysis.

Before vitreous retrieval, all animals were euthanized with a lethal dose of pentobarbital 100 mg/kg. The released concentrations were compared with each other using the Kruskal-Wallis test followed by Dunn’s multiple comparison test (p < 0.05). Pharmacokinetic parameters of AUC, T_max, and C_max of the groups that received the intravitreal implant or the solution were calculated using the software Prism 5.0 (GraphPad Software Inc.). For better comparison, the time data was transformed from days to hours in the implant group. The results were also plotted as log scale to

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compare the pharmacokinetic pattern between immediate re-lease and loaded formulation, and the respective slopes from the curves were calculated (Prism 5.0; GraphPad Software Inc.).

Clinical evaluation

Ocular examinations were conducted at baseline and weekly dur-ing the 6 weeks following implantation. The presence of any sign of inflammation, bleeding, or the occurrence of retinal detach-ment was verified by slit lamp biomicroscopy (Kowa SL 15 Slit-lamp Biomicroscope) and indirect binocular ophthalmoscopy (Omega 500 Binocular Indirect Ophthalmoscope; Heine Opto-technik). For evaluation of the eye fundus, the Clearview Optical Imaging System (Optibrand) was used.

Intraocular pressure of both eyes of each rabbit was measured weekly using a TonoPen (Tono-Pen XL; Reichert Technologies). Three consecutive measurements were taken for each eye and an average value was used for comparison. To minimize circadian oscilla-tion, the measurements were taken at the same hour in all rabbits. All assessments were randomized by the same veterinary ophthalmologist. The values were compared using the Kruskal-Wallis test followed by Dunn’s multiple comparison test (p < 0.05).

Functional evaluation

ERG recordings were carried out in compliance with the Interna-tional Society for Clinical Electrophysiology guidelines [36] in each eye before inserting the implant and 6 weeks after. Initially, rab-bits were allowed to adapt to the dark for at least 3 h to obtain maximum amplitudes and stable parameters. Afterwards, they were anesthetized, and their pupils were dilated with 0.5% tropi-camide (Mydriacyl; Alcon). The eyes were topically anesthetized with 0.5% proparacaine hydrochloride (Anestesalon; Alcon) im-mEDIATELY before the recordings. Each rabbit was placed facing the light stimulus at a distance of 15 cm. Stainless steel needle refer-ence electrodes were placed subcutaneously in the skin near the lateral canthus of the eyes, and a ground needle electrode (model ES; Grass Technologies) was placed subcutaneous on the back. Bipolar contact lense ERGJet electrodes (Fabrinal SA) were placed on both corneas with 2% w/v methylcellulose. Impedance was set to less than 5 kΩ at 25 Hz in each electrode. A white light (6500 K) stimulus was generated by a Ganzfeld LED stimulator (ColorDome desktop Ganzfeld; Diagnosys LLC). The intensity of the light stim-ulus at a distance of 15 cm. Stainless steel needle reference electrodes were placed subcutaneously in the skin near the lateral canthus of the eyes, and a ground needle electrode (model ES; Grass Technologies) was placed subcutaneous on the back. Bipolar contact lense ERGJet electrodes (Fabrinal SA) were placed on both corneas with 2% w/v methylcellulose. Impedance was set to less than 5 kΩ at 25 Hz in each electrode. A white light (6500 K) stimulus was generated by a Ganzfeld LED stimulator (ColorDome desktop Ganzfeld; Diagnosys LLC). The intensity of the light stim-ulus was confirmed by slit lamp biomicroscopy (Kowa SL 15 Slit-lamp Biomicroscope) and indirect binocular ophthalmoscopy (Omega 500 Binocular Indirect Ophthalmoscope; Heine Opto-technik). For evaluation of the eye fundus, the Clearview Optical Imaging System (Optibrand) was used.

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Histopathological evaluation

After ERG analysis, the animals were euthanized with a lethal dose of pentobarbital and the eyes were immediately enucleated and fixed for 48 h in Davidson’s fixative solution: 2% formaldehyde (37%; Sigma-Aldrich), 35% ethanol (absolute; Merck), 10% glacial acetic acid (99%, Sigma-Aldrich), and 53% water (ultrapure; Milli-Q). Next, tissue samples were dehydrated with increasing concen-trations of ethanol, diaphanized in xylene (99%; Sigma-Aldrich), and embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (Sigma-Aldrich). The morphology of the retina, presence of inflammatory cell infil-tra-tion, and hemorrhages in the ocular tissues were evaluated by light microscopy.

Contributors’ Statement


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Conflict of Interest

The authors declare that they have no conflict of interest.

References
