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ORIGINAL ARTICLE

In vivo release and retinal safety of intravitreal implants of thalidomide in rabbit eyes and antiangiogenic effect on the chorioallantoic membrane

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Abstract

Purpose: To evaluate the *in vivo* release, retinal safety and antiangiogenic effect of a thalidomide-loaded poly-lactide-co-glycolide intravitreal implant.
Methods: New Zealand white rabbits, divided into two groups, I and II, received an intravitreal implant containing or not thalidomide, respectively ($n = 12$). Intravitreal drug levels were determined during a six-week study period. The potential for toxicity associated with the implants was evaluated by electroretinography and light microscopy ($n = 8$). Twelve chorio-allantoic membranes (CAMs) from chicken eggs were incubated with thalidomide dispersion, implants containing or not thalidomide and vitreous samples and analyzed after two days regarding the percentage of vessels regression.
Results: Intravitreal concentrations of thalidomide (ng/ml) were 690.21 ± 177.95, 372.51 ± 185.56, 240.59 ± 133.48, 327.54 ± 169.71, 294.26 ± 142.41 and 465.18 ± 157.51 at 1, 2, 3, 4, 5 and 6 weeks, respectively, after implantation in group I rabbits. No drug was detected in group II samples. Electroretinography and histological evaluations did not show any sign of retina toxicity. There was significant regression of vessels in CAM incubated with thalidomide dispersion, thalidomide-loaded implants and vitreous samples from group I when compared to control.
Conclusion: The intravitreal implants delivered safe doses of thalidomide that were also effective to induce vessels regression in CAMs.

Keywords

Biomaterials, controlled release, drug delivery systems, ophthalmic drug delivery, PLGA, toxicity

History

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Introduction

Ocular neovascularization is among the most common worldwide causes of blindness. Increased vascular endothelial growth factor (VEGF) levels in the vitreous of eyes with these diseases appear to be the major stimulus of intraocular neovascularization. In the last years, there has been intense research for the development of new therapeutic approaches with potential antiangiogenic effects to retinal degenerations and others diseases of the posterior segment of the eye [1–3]. The success of the treatment mainly depends on the delivery of effective doses of drugs directly to the target site [4]. The poor penetration of drugs in the intraocular tissues reduces the number of medicines indicated for ophthalmological use and requires caution about those that are available due to the possibility of adverse effects [5,6].

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Thalidomide was introduced in 1957 as a sedative drug of low toxicity and relatively free of undesirable effects such as a hangover. However, the use of thalidomide by pregnant women led to severe infant limb defects which were probably the result of the antiangiogenic effect of the drug [7]. Despite its limited use for many years, thalidomide has shown remarkable value in the control or treatment of several diseases and is currently considered to be a potent inhibitor of angiogenesis. The antiangiogenic effect of thalidomide in a rabbit corneal neovascularization model and its inhibitory effect on TNF-α synthesis were discovered in later years [8]. For these reasons, the interest in its reintroduction for the use as antitumor agent and also as inhibitor of ocular neovascularization has increased nowadays [9].

In recent years, anti-VEGF agents became the standard of care for many retinal and choroidal diseases related to ocular neovascularization [10–12]. Despite the excellent results regarding best-corrected visual acuity and improvement in retina architecture, these agents are expensive and have to be injected repeatedly, usually in a monthly fashion. For this

reason, the development of slow delivery devices of less expensive drugs is welcome.

In the present study, a biodegradable implant containing thalidomide was developed and evaluated. Very few studies regarding the use of thalidomide in ocular neovascularization have been reported in the literature [8,13–15] and no study has been published thus far describing the intraocular use of thalidomide in a solid implantable biodegradable device that permits drug release at the site of action for a prolonged period of time, with small possibilities of systemic adverse effects.

Materials and methods

Preparation of the devices containing thalidomide

The implants were developed according to the technique previously described by Fialho et al. [16]. Thalidomide (racemic form, purity 99.49%; Microbiológica Química e Farmacêutica Ltda, Rio de Janeiro, Brazil) and the polymer poly-lactide-co-glycolide (PLGA 50/50, PURASORB® PDLG 5004, inherent viscosity midpoint of 0.4 dl/g, Purac Biomaterials, São Paulo, Brazil) at a ratio of 1:4 were dissolved in a mixture of distilled water and acetonitrile and the resulting solution was lyophilized. The powder obtained was molded into rods using a hot plate. The intravitreal implants weighed on average 1.0 ± 0.1 mg, were 4.0 ± 0.1 mm in length and 0.60 ± 0.05 mm in diameter (Figure 1A). The final concentration of thalidomide dispersed in the polymeric matrix was 25% w/w.

Animals

Thirty-two female New Zealand white rabbits weighing 2–3 kg, 10–12 weeks of age, were used. The study was approved by the Ethics Committee in Animal Experimentation of Ezequiel Dias Foundation (Protocol 028/2011, Brazil). 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.

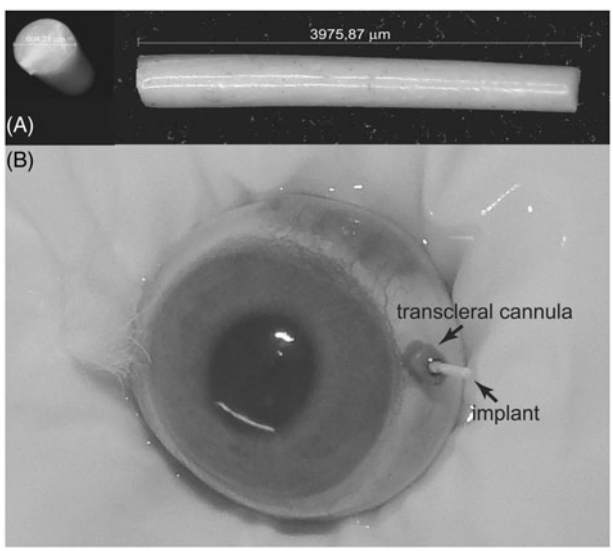

Figure 1. (A) Macroscopic picture of the thalidomide-loaded PLGA device before implantation. (B) Photograph showing the insertion of the developed implant in a rabbit eye through the 25G transcleral cannula trocar.

The animals were divided into two groups. In group I, devices containing thalidomide were implanted into the vitreous of the left eye of each rabbit. In group II, rabbits received the implant without drug in the left eye.

Procedure for device implantation

Before implantation, rabbits were anesthetized with an intramuscular injection of 50 mg/kg of ketamine hydrochloride (Dopalen®, Sespo Indústria e Comércio, Paulínia, Brazil) and 15 mg/kg of xylazine hydrochloride (Anasedan®, Sespo Indústria e Comércio, Paulínia, Brazil). Local anesthesia was obtained with 0.5% proxymetacaine hydrochloride (Anestalcon®, Alcon Brazil, São Paulo, Brazil).

A 25-gauge trocar cannula (Alcon, Fort Worth, TX) was used for device implantation. The transscleral cannula was placed through the pars plana in the superotemporal quadrant. The implants containing or not containing thalidomide were then inserted into the vitreous cavity of the rabbits through the cannula (Figure 1B). No vitreous bleeding occurred during the procedure.

In vivo release study

At different time points after implantation of the devices (1, 2, 3, 4, 5 and 6 weeks), four animals per time were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Dopalen) and 15 mg/kg xylazine hydrochloride (Anasedan) for vitreous and blood sample collection. The vitreous was completed removed using a 26G needle and a 1 ml syringe by simple puncture through the sclera behind the ciliary body and approximately 7 mm below the limbus to avoid contact with the lens and retina. Peripheral blood was drawn from the marginal ear vein of the rabbits and placed in dry heparinized tubes. Vitreous and blood samples were also retrieved from control animals for comparison. All eye samples were immediately stored at $-80\,^{\circ}$C. Blood samples were centrifuged for 10 min at $2000 \times$ g and the plasma was collected into a clean polypropylene tube and immediately stored at $-80\,^{\circ}$C.

At the end of sample retrieval at each time point, rabbits were sacrificed with an injection of a lethal dose of pentobarbital (Hypnol 3%, Syntec do Brasil, Cotia, Brazil). The eyes from group I were then enucleated for implant removal.

The release rate constant derived from the *in vivo* release of thalidomide was calculated using the Korsmeyer–Peppas model, using the equation $Mt/M\infty = kt^n$, where $Mt/M\infty$ is the fraction of drug released at time t, K is the release rate constant and n is the release exponent. The n value was estimated from linear regression of log ($Mt/M\infty$) versus log t [17].

Amount of thalidomide remaining in the implanted devices

For the determination of the amount of thalidomide remaining in the implants at six weeks, the retrieved implants were gently washed with distilled water, dried and then dissolved in a fixed volume of acetonitrile. The amount of thalidomide

was measured by high-performance liquid chromatography (HPLC).

Drug level analysis

The amount of thalidomide in the samples was measured by HPLC using the method described in the United States Pharmacopeia 32 [18] and a Merck® apparatus equipped with an autosampler model L-2200 (Merck Hitachi, Malta, NY). A pump (model L-2130 Merck Hitachi) was used at a constant flow rate of 1.0 ml/min. A C18 column (150 × 3.9 mm i.d., particle size 5 µm) filled with both inorganic (silica) and organic (organosiloxane) components (XTERRA® MS C18, Waters, Milford, MA) was used. The mobile phase was a mixture of ultrafiltered water, acetonitrile and phosphoric acid (85:15:0.1). An ultraviolet detector (model L-2450, Merck Hitachi) was used at a wavelength of 237 nm.

The samples were thawed out at ambient temperature and submitted to analysis in triplicate after homogenization. The samples retrieved from the animals of group II were also analyzed and served as control.

For the determination of the amount of thalidomide remaining in the implants, the final solution was submitted to analysis in triplicate.

Validation of the method showed the absence of interference of the vitreous and blood compounds and the polymer with thalidomide retention time, ruling out the risks of overestimation (Figure 2).

Clinical examination

Eyes were observed clinically and photographed weekly. Clinical evaluation included ocular inspection and binocular indirect ophthalmoscopy preoperatively and weekly after surgery until week 6. Applanation tonometer Tono-Pen XL (Reichert Technologies, Buffalo, NY), indirect ophthalmoscopy (Omega 500 Binocular Indirect Ophthalmoscope, Heine Optotechnik, Herrsching, Germany), a Clearview Optical Imaging System (Optibrand, Ft. Collins, CO) and slit-lamp biomicroscopy (Kowa SL 15 Slit-lamp Biomicroscope, Tokyo, Japan) were used for this evaluation.

Retinal safety

Electroretinography

Electroretinography (ERG) was performed at six weeks after intravitreal implantation in eight animals different from those used in the *in vivo* release study. The procedure was performed in both eyes, the right eye with the implant and the left normal eye used as control.

The ERG protocol is based on the International Society for Clinical Electrophysiology of Vision (ISCEV). Rabbits were kept in a dark room for at least 3 h for dark adaptation before anesthesia, which was performed by intramuscular injection of 1–2 mg/kg body weight xylazine and 10 mg/kg body weight ketamine. Pupillary dilatation was performed with one drop of tropicamide 15 min before the beginning of ERG.

ERG responses were recorded in both eyes simultaneously by means of JET contact electrodes on the corneas (Microcomponents SA). Subcutaneous needles in the skin near the lateral canthus of both eyes were used as reference; a ground electrode was placed on the back. Electrode impedance was checked before and after each measurement and was less than 5 kΩ at 25 Hz. Eyes were stimulated using a Ganzfeld LED stimulator (ColorDome; Diagnosys LLC, Littleton, MA). Flashes of white light (6500 K) with a duration of 4 ms were delivered in five steps of increasing luminance (0.0001, 0.0003, 0.001, 0.003, 0.01 and 10 cd·s/m^2)

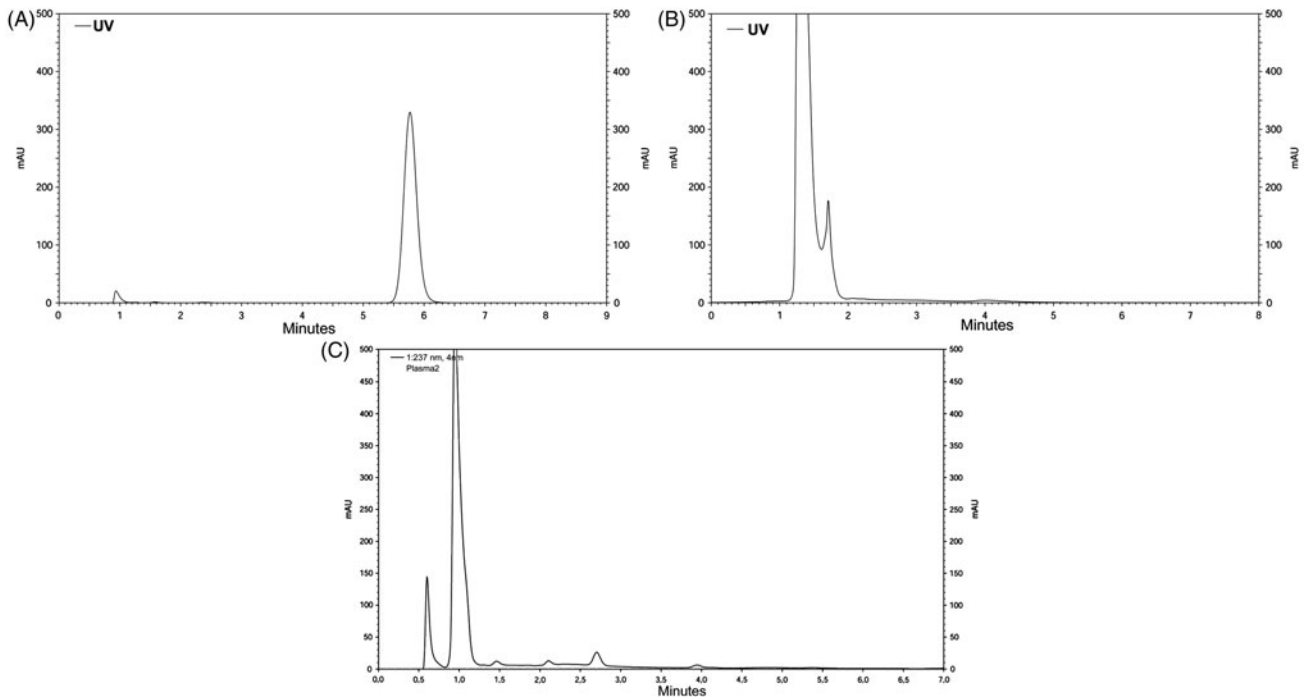

Figure 2. (A) Chromatogram of thalidomide standard. (B) Chromatogram of the vitreous humor collected from rabbits' eye that received PLGA implant without drug. (C) Chromatogram of the plasma retrieved from rabbits that received PLGA implant without drug. Chromatographic conditions: C18 column 150 mm × 3.9 mm; ultrafiltered water, acetonitrile and phosphoric acid (85:15:0.1, v/v); 1 ml/min of flow rate; wavelength of 237 nm.

with 30 s inter-stimulus interval (ISI) in the dark-adapted stage.

Oscillatory potentials (OPs) were obtained from the response elicited by the flash of 3 cds/m^2 by means of a fast Fourier transform (FFT) implemented as a band pass frequency filter (from 60 to 300 Hz). The absolute value of the area under the curve for all OP wavelets was determined between a- and b-wave implicit times.

After 10 min of light adaptation with a background light of 30 cd/m^2, light-adapted ERG recordings were performed with luminance flashes 3 cds/m^2 (ISI = 2 s) followed by a 30 Hz white flicker stimulus of 3 cds/m^2.

Responses were amplified (band pass filter: 0.3–300 Hz) and stored for off-line analysis using the Espion (Diagnosys LLC) after averaging of 6–40 individual measurements at each step depending on the signal/noise ratio.

Histopathologic study

The eight rabbits used for ERG analysis were sacrificed after the exam for histopathologic analysis. The eyes were immediately enucleated and immersed in formaldehyde 4% in 0.1 M Sorensen's phosphate buffer and stored for one day at 4 °C. The eyes were then dissected and processed for light microscopy. The samples of the posterior segment of the eye were washed, dehydrated, embedded in paraffin wax and sectioned serially. The sections were then stained with hematoxylin–eosin.

Evaluation of the vascular effects of angiogenesis in a CAM model

The chorioallantoic membrane (CAM) from fertilized chicken eggs was used for this study and the following samples were evaluated: thalidomide (10 µl of a 0.25 mg/ml dispersion), biodegradable implants containing or not containing thalidomide (mean weight of 1.00 mg) and vitreous samples (volume of 10 µl) from groups I and II collected from the *in vivo* release study. Twelve eggs were used for each sample evaluated.

The method previously described by Nowak-Sliwinska et al. [19] was used, with some modifications. The fertilized chicken eggs were placed in a hatching incubator and three days after fertilization, a hole of approximately 2 cm in diameter was opened in the eggshell to provide access to the CAM. Five days after fertilization, samples were applied over the CAM surface in a well-defined part. After 48 h the CAMs were removed from the eggs and analyzed with a light microscope (Leica, model DM4000B, Germany) coupled to a Leica digital CCD camera model DFC 280 (Software Leica Application Suite V 3.3.0, Germany). The images obtained were used for the quantification of the capillary features (size and quantity) using the image processing program ImageJ (version 1.44p; National Institutes of Health, USA). As a negative control (NC), the same procedure was performed and 10 µl of phosphate-buffered saline (PBS, pH 7.4) was applied over the CAM surface; as a positive control (PC), 10 µl of Avastin® (Bevacizumabe 100 mg/4 ml, Produtos Roche Químicos e Farmacêuticos S.A., Brazil) was applied over the CAM surface. The NC group was fixed as 100% for the calculation of blood vessel reduction.

Statistical analysis

The Mann–Whitney non-parametric test was used to compare outcomes in both groups. The unpaired *t*-test was used to compare outcomes of percent blood vessels in the CAM study. Values of $p < 0.05$ were considered to be statistically significant.

Interrelation between dark-adapted b-wave amplitude and stimuli luminance was modeled using the Naka–Rushton function [20] that yields the parameters: V_{max} is asymptotic (maximum b-wave amplitude); l is the light intensity; K is the necessary luminance reaching 50% of V_{max}, which is a mark of dark-adapted sensitivity; and n is the dynamic working range of photoreceptors (Equation 1).

$$V = V_{max} \frac{l^n}{K^n + l^n} \quad (1)$$

Results

Clinical examination

Intraocular pressure (IOP) did not change significantly before or during the study (17.7 ± 1.88 and 18.8 ± 1.47 mmHg for right and left eyes, respectively; mean ± SD).

Among the 24 eyes implanted with the PLGA device, only three presented minor ocular changes. In one eye from a group I rabbit a mild mucopurulent ocular discharge accumulating on the medial canthus followed by mild conjunctival hyperemia on the right eye was observed seven days after implantation, with spontaneous resolution after two weeks. Another eye from group I showed moderate conjunctival hyperemia after three weeks with no other important ocular signs. The third rabbit eye from group I showed transient hypotony, but no abnormality of the posterior segment of the eye. None of these animals were removed from the study.

The implant containing thalidomide was easily visualized in the vitreous cavity immediately after administration (Figure 3A) and at six weeks (Figure 3B). It was observed that the implant changed its shape and became shorter during the study (Figure 3C).

In vivo release study

The mean thalidomide concentrations released from the biodegradable PLGA implants in the vitreous throughout 42 days (6 weeks) of evaluation are presented in Figure 4. Mean intravitreal concentrations (ng/ml) were 690.21 ± 177.95, 372.51 ± 185.56, 240.59 ± 133.48, 327.54 ± 169.71, 294.26 ± 142.41 and 465.18 ± 157.51 at 1, 2, 3, 4, 5 and 6 weeks after implantation, respectively.

In the blood samples, no thalidomide peaks were observed in the chromatograms obtained throughout the study, suggesting that the drug did not reach the systemic circulation at levels higher than 50 ng/ml.

The amount of the drug that remained in the thalidomide-loaded biodegradable implants at six weeks was 93.79 ± 0.83 mg, corresponding to approximately 37.5% of the total amount of thalidomide in the device.

The release exponent obtained from the linear regression was 1.8086, which means that a combination of both diffusion

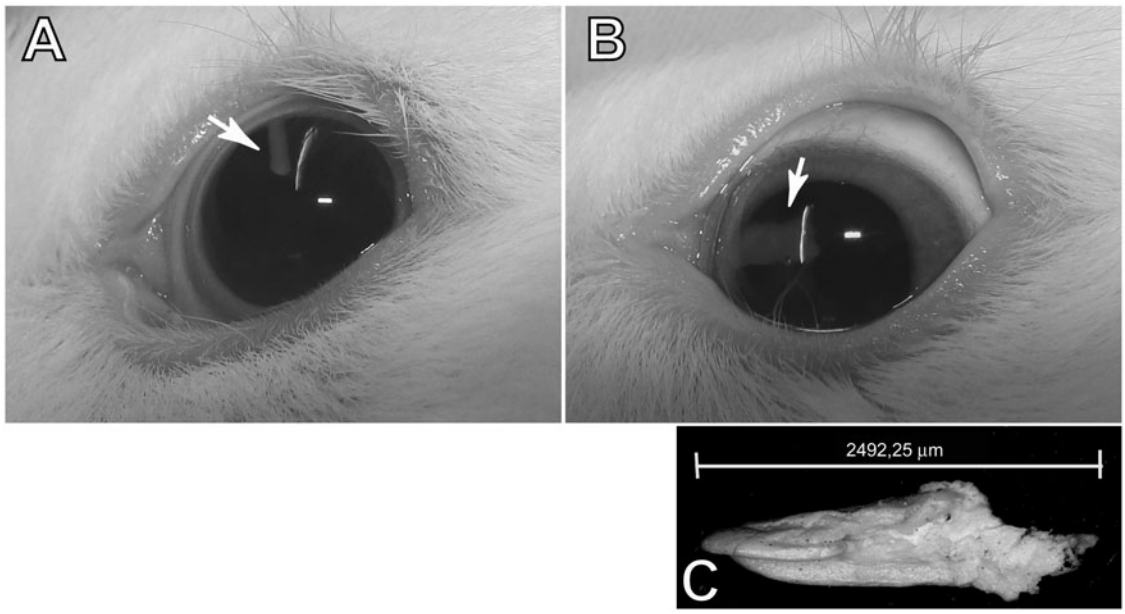

Figure 3. Photograph showing the implant in the vitreous cavity immediately after administration (A) and at six weeks after administration (B). (C) Photograph of the implant retrieved from the vitreous after six weeks.

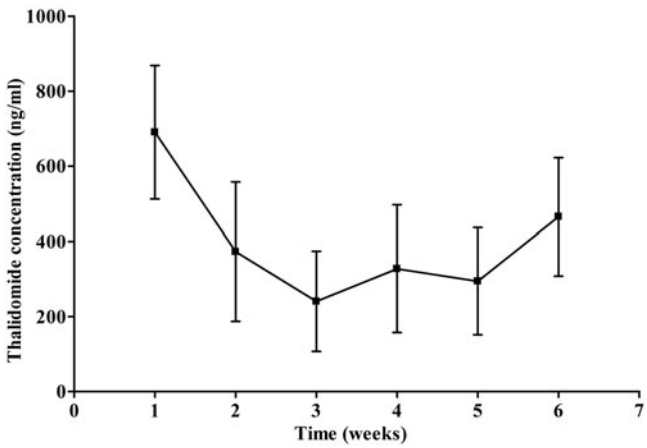

Figure 4. Thalidomide concentrations released from the drug-loaded PLGA implants in the vitreous of rabbits' eyes during the study period (mean ± SD, $n = 4$).

and erosion are involved in drug release [17]. The release rate constant derived from the *in vivo* release of thalidomide was 1.6208.

Retinal safety

One rabbit developed a total retinal detachment in the right eye and was excluded from the ERG and histological analysis.

Electroretinographic study

Dark-adapted ERG showed no statistically significant differences for b-wave amplitude or implicit time for rod (0.01 cd·s/m^2), or a- and b-wave amplitude or implicit time combined response (3.0 cd·s/m^2) between eyes that received the thalidomide implants (right) and the control eyes (left). Furthermore, no significant differences were found for the parameters K and V_{max} of the b-wave amplitude versus stimulus energy model [20] (Mann–Whitney; $p > 0.05$) (Table 1, Figure 5), indicating no influence of the thalidomide implant on the rod-driven ERG responses. OPs area under the curve evaluated from the combined responses also showed no differences between treated and untreated eyes (Table 1).

Similar results were observed for the light-adapted condition, with cone b-wave and 30 Hz flicker amplitude and implicit times showing no significant differences between eyes (Table 1), indicating no changes in cone-driven responses.

Histopathologic study

Histopathologic examination demonstrated no signs of retinal toxicity or inflammatory cell infiltration. No structural abnormalities were noted at day 42 by light microscopy and the normal anatomy of the retina was preserved in eyes that received the intravitreal implant of thalidomide (Figure 6).

Evaluation of vascular effects of angiogenesis in a CAM model

The percentage of blood vessels remaining in the CAM after application of thalidomide dispersion, PLGA implants without drug, thalidomide-loaded PLGA implants, vitreous from group I and II rabbits, PBS (NC) and Avastin (PC) are shown in Figure 7.

The mean percentage of blood vessels remaining in the CAM after application of vitreous samples obtained at 1, 2, 3, 4, 5 and 6 weeks from group I rabbits (89.56 ± 5.95, 93.48 ± 6.89, 95.36 ± 5, 94.26 ± 6.93, 93.17 ± 5.83, 89.55 ± 5.95, respectively) were significantly lower than the NC, set as 100% ($p < 0.05$), but also significantly higher than PC (68.48 ± 5.47); when thalidomide dispersion and thalidomide-loaded PLGA implants were used, the percentages (79.74 ± 4.98 and 76.36 ± 5.04, respectively) were also significantly lower ($p < 0.05$) than the NC ($p < 0.05$) but also significantly higher than PC (69.28 ± 5.41). There was no significant difference between the percentage of blood

Table 1. Dark-adapted and light-adapted ERG amplitude (μV) and implicit times (ms) in seven right rabbits eyes (thalidomide implant) and left normal eyes used as controls.

Adaptation	Stimulus	ERG parameter	Thalidomide (right) eye	Control (left) eye	*p* (Mann–Whitney)
Dark-adapted	Rod (0.01 cd.s/m^2)	b-wave amplitude	237.6 ± 17.0	262.4 ± 46.9	0.4063
		b-wave implicit time	63.4 ± 3.2	63.0 ± 3.1	0.5000
	Combined response (3.0 cd.s/m^2)	a-wave amplitude	107.7 ± 13.1	118.7 ± 20.2	0.2891
		a-wave implicit time	13.4 ± 0.4	13.1 ± 0.6	0.6250
		b-wave amplitude	307.4 ± 30.9	336.7 ± 54.6	0.2344
		b-wave implicit time	63.4 ± 1.4	63.9 ± 1.6	0.4063
		OP	327.6 ± 32.3	359 ± 49.3	0.2344
	V_{max}	b-wave amplitude	286.0 ± 25.9	303.3 ± 46.0	0.3438
	K		−2.6 ± 0.1	− 2.7 ± 0.1	0.5938
Light-adapted (30 cd/m^2)	30 Hz Flicker	Amplitude	67.0 ± 9.9	76.4 ± 14.5	0.2344
		Peek time	61.0 ± 0.5	61.1 ± 0.9	0.5000
	Cone ERG (3.0 cd.s/m^2)	Amplitude	102.3 ± 10.7	109.2 ± 15.9	0.2344
		Implicit time	31.7 ± 1.0	30.6 ± 0.2	0.8672

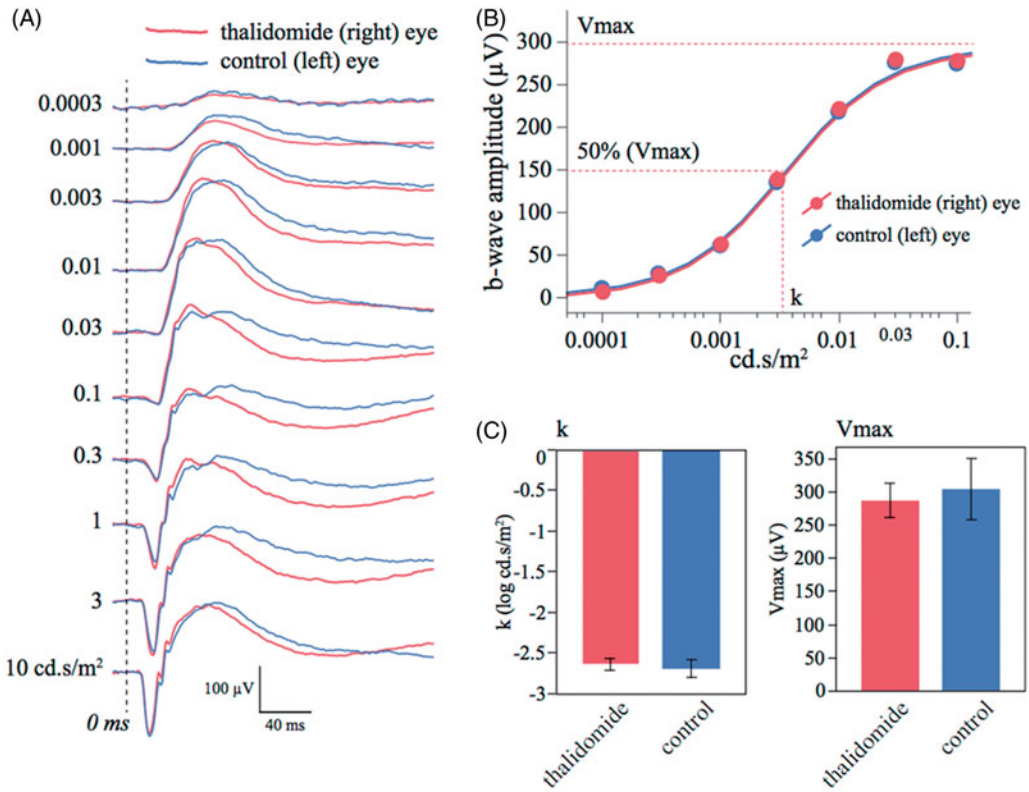

Figure 5. Examples of dark-adapted ERG responses of one animal to increasing luminance stimuli. (A) Red traces are from the eye receiving the thalidomide implant and blue traces from the untreated control eye. (B) Circles represent the b-wave amplitude plotted against stimulus luminance (red for thalidomide and blue for control) and the lines are the best fit for the Naka–Rushton function. Dashed lines highlight the Naka–Ruston parameters V_{max} and K. (C) Bars represent the mean and standard error for K and V_{max} from treated and untreated eyes.

vessels in CAM incubated with PLGA implants without drug (98.67 ± 4.87), the vitreous samples from group II (98.87 ± 4.79) and the NC ($p < 0.05$).

Discussion

In the last decade, intravitreal anti-VEGF therapy has become a widespread treatment option for retinal and choroidal diseases characterized by neovascularization. Thalidomide has important antiangiogenic effect and modulates neovascularization and angiogenesis through inhibition of basic fibroblast growth factor (bFGF), cytokine-induced nuclear factor B and tumor necrosis factor α, and also depletion of VEGF membrane receptors and direct inhibition of VEGF secretion [8,15,21]. Based on this rationale, an intravitreal thalidomide implant may be a reasonable alternative to intravitreal injections of humanized antibodies against VEGF.

Orally administered thalidomide is an inhibitor of ocular angiogenesis [13]. However, this route is associated with several adverse effects caused by the drug, including potential limb defects in fetuses of pregnant women and it was also observed that at least 10% of patients, including men and non-pregnant women present adverse effects such as neutropenia, leukopenia, lymphopenia, anemia, thrombocytopenia, peripheral neuropathy, tremor, dizziness, paresthesia, dysesthesia, somnolence, constipation and peripheral edema [9]. Topical (eye-drop) delivery is not effective because of the limited intraocular penetration [22]. Intravitreal injection has

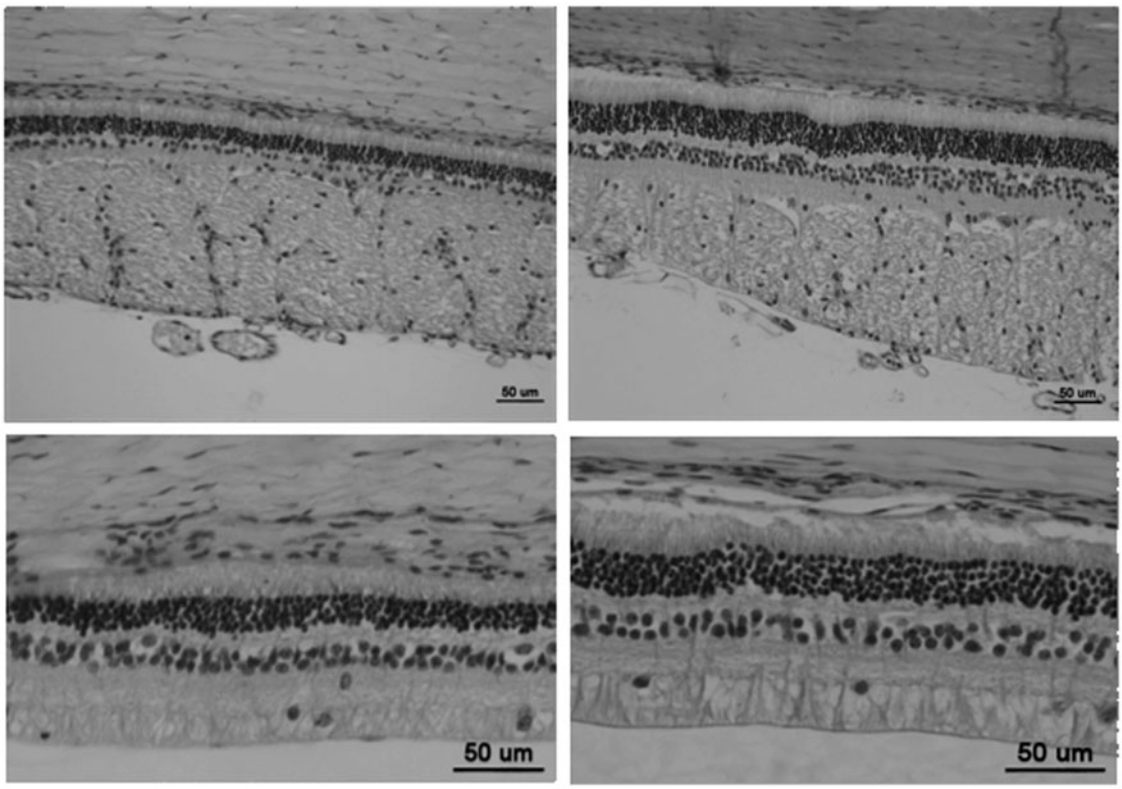

Figure 6. Semi-thin retina sections of a rabbit eye six weeks after intravitreal thalidomide implantation (top right: central retina; bottom right: peripheral retina) and from its contralateral eye (top right: central retina; bottom right: peripheral retina) used as control. No histological changes were detected in any retinal layers from implanted and contralateral control eyes.

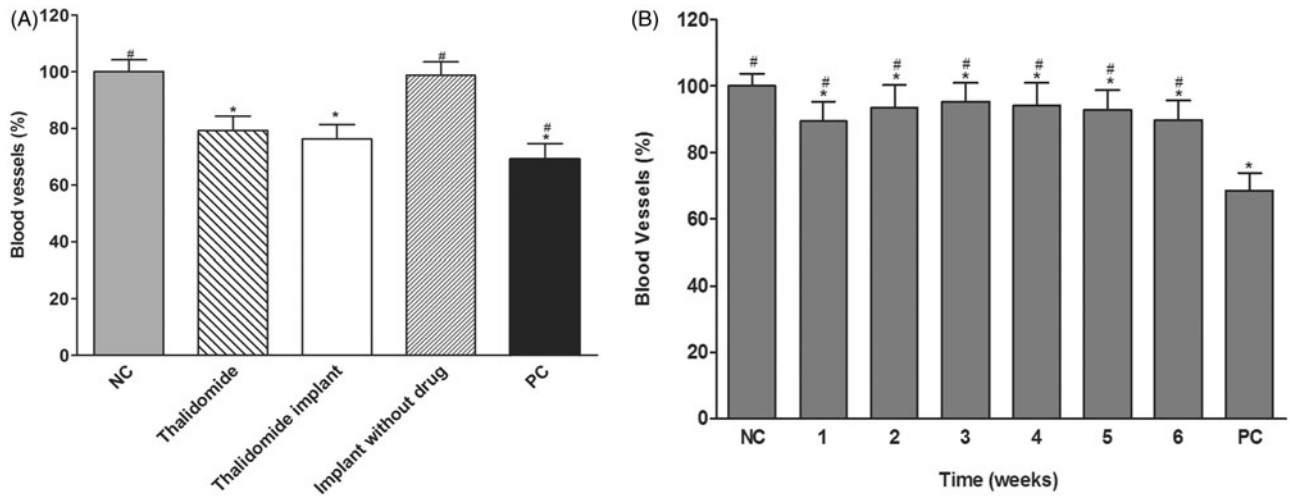

Figure 7. Percent of blood vessels in the CAM after application of: (A) thalidomide alone, thalidomide-loaded PLGA implants and PLGA implants without drug; (B) retrieved vitreous. NC: negative control; PC: positive control. The values are expressed according to the NC group, which was fixed as 100%. The values are shown as mean ± standard deviation ($n = 12$). *Values are significantly different from the NC group ($p < 0.05$; unpaired t-test); #Values are significantly different from the PC group ($p < 0.05$; unpaired t-test).

to be repeated to maintain the therapeutic level of the agent, which may lead to patient discomfort and noncompliance and involve potential risks of cataract, retinal detachment and endophthalmitis [22,23]. In a study realized by our group, the intravitreal administration 0.1 ml of a 10 mg/ml thalidomide suspension showed that the drug was quickly eliminated and only a small amount was detected on the second day after injection (0.09 mg/ml) (unpublished work). Considering the administered amount of thalidomide suspension is the same of that of the intravitreal implants, it is clearly demonstrated the viability of the developed biodegradable systems in the maintenance of the drug concentration for a prolonged period.

For the reasons mentioned above, the development of a biodegradable delivery system of thalidomide to target the posterior segment of the eye, such as the biodegradable implant from the present study, may overcome most of these drawbacks. In addition, the implant has a small diameter that allows its placement through any 25-gauge cannula, allowing its extensive use by any retina specialist without the need for further expertise with specific implantation devices.

The *in vivo* release profile obtained from the thalidomide biodegradable implants showed that in the first week a higher amount (690.21 ± 177.95 ng/ml) of the drug was released in comparison to other study periods due to the presence of the drug on the surface of the implants. Then, a slow and controlled release was observed throughout the next five weeks of the study that may be attributed to the diffusion of the drug through the pores and channels in the polymeric matrix that usually appear during the degradation process, which could be clearly demonstrated by our group in a previous work [16]. At the sixth week, the implant was almost completed degraded, which explains the increase in the concentration at this time and represents the final burst commonly verified the pharmacokinetics of PLGA implants in the vitreous cavity [16].

The *in vitro* release profile obtained from these implants, previously realized by our group (data not shown), showed that biodegradable implant released approximately 60% of thalidomide in 20 weeks under sink conditions. Different to this profile, the developed device releases approximately 62.5% of the drug in the rabbits' vitreous in six weeks. At that time, it was observed that the implants were highly degraded and we assume that it would not be possible to evaluate if the amount of thalidomide probably found in the vitreous after that time would be represented by the residual amount of thalidomide in the implant or by the free drug released from the implant and still not eliminated. The implant released the drug *in vivo* faster than it did *in vitro*, a fact that can be attributed to the environment surrounding the implant in the vitreous, which is not the same as the *in vitro* environment. According to Fialho et al. [16] drug movement in the vitreous body and the elimination profile of the drug in the rabbit eye contribute to the faster drug release. No drug was detected in rabbit blood samples, suggesting that the drug was released mainly in the posterior segment of the eye. For this reason, we suppose that no potential systemic adverse effects derived from the drug might occur by the application of biodegradable implants.

As observed in a previous study [24], in which thalidomide exhibited dose-dependent inhibition of *in vivo* angiogenesis in the CAM model, the biodegradable implants containing thalidomide developed in this study also could reduce blood vessel formation in the CAM model at a concentration 10 times lower than that of the PC. Furthermore, the concentration of thalidomide in the rabbits' vitreous at each time point of the *in vivo* release study was sufficient to significantly reduce blood vessel in the CAM when compared to NCs, confirming that thalidomide released from the implants has antiangiogenic activity. The robustness of the CAM assay was confirmed by the PC, bevacizumab, which also induced significant reduction in the percentage of CAM vessels, and by NCs, PBS and normal vitreous, that did not induce any significant regression in CAM new vessels. Besides the CAM model, thalidomide has also been effective in inhibiting human umbilical vein endothelial cell proliferation, retinal pigment epithelial cell proliferation and migration, and also suppressed HUVEC tube formation in a concentration-dependent manner [25,26]. *In vivo*, it has been shown that thalidomide has an antiangiogenic effect against neovascular growth induced by VEGF in the rabbit cornea, as well as against neovascularization induced by bFGF or VEGF in the mouse cornea [25]. More studies are being realized by our group to evaluate the efficacy of the developed system in a rabbit model of ocular neovascularization.

The PLGA polymer degrades completely *in vivo* into its components, lactic acid and glycolic acid, which are converted to carbon dioxide and water which can be easily eliminated by ocular tissue. Despite the acidic characteristics of PLGA degradation products, it is widely described in the literature that intravitreal PLGA implants were not able to cause any inflammation process related to polymer degradation [27,28]. For example, a biodegradable dexamethasone drug delivery system made with PLGA was approved by the Food and Drug Administration (FDA) and it is already in the market (Ozurdex®, Allergan, Inc, Irvine, CA). This system shows a six-month effect and it was not observed any sign of inflammation that could be due to the degradation of PLGA [29–31].

The implant was well tolerated by the rabbit eye. Only minor ocular changes were detected in the present study such as mild mucupurolent discharge, conjunctival hyperemia and transient hypotony, probably secondary to the implantation process, with no ocular fundus changes verified by indirect ophthalmoscopy at any time point. In addition, ERG photopic and scotopic analysis did not show any significant change in wave amplitude when the eyes that received the implant were compared to the contralateral ones. Finally, no histological change was found in any retinal layer of rabbits that received the implant and no difference in retinal microscopic morphology was found when compared to contralateral control eyes.

A biodegradable thalidomide implant delivered antiangiogenic levels of the drug throughout the six-week period of the study. The antiangiogenic effect of the implant was confirmed in the CAM model and no important posterior segment change was detected by ERG and histological analysis. Taken together, these data allow us to conclude that the developed device may represent an interesting therapeutic tool for posterior segment diseases related to neovascularization and warrant further preclinical and clinical studies.

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Declaration of interest

The authors report no declarations of interest.

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