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Brimonidine-LAPONITE® intravitreal formulation has an ocular hypotensive and neuroprotective effect throughout 6 months of follow-up in a glaucoma animal model†

M. J. Rodrigo, *[a,b,c,d] M. J. Cardiel, [c,e] J. M. Fraile, [f] S. Mendez-Martinez, [a,b,c] T. Martinez-Rincon,[a,b,c] M. Subias, [a,b,c] V. Polo,[a,b,c] J. Ruberte,[d,g,h,i] T. Ramirez,[c,e] E. Vispe,[j] C. Luna,[c,j] J. A. Mayoral[f] and E. Garcia-Martin[a,b,c,d]

Intravitreal administration is widely used in ophthalmological practice to maintain therapeutic drug levels near the neuroretina and because drug delivery systems are necessary to avoid reinjections and sight-threatening side effects. However, currently there is no intravitreal treatment for glaucoma. The brimonidine-LAPONITE® formulation was created with the aim of treating glaucoma for extended periods with a single intravitreal injection. Glaucoma was induced by producing ocular hypertension in two rat cohorts: [BRI-LAP] and [non-bri], with and without treatment, respectively. Eyes treated with brimonidine-LAPONITE® showed lower ocular pressure levels up to week 8 ($p < 0.001$), functional neuroprotection explored by scotopic and photopic negative response electroretinography ($p = 0.042$), and structural protection of the retina, retinal nerve fibre layer and ganglion cell layer ($p = 0.038$), especially on the superior-inferior axis explored by optical coherence tomography, which was corroborated by a higher retinal ganglion cell count ($p = 0.040$) using immunohistochemistry (Brn3a antibody) up to the end of the study (week 24). Furthermore, delayed neuroprotection was detected in the contralateral eye. Brimonidine was detected in treated rat eyes for up to 6 months. Brimonidine-LAPONITE® seems to be a potential sustained-delivery intravitreal drug for glaucoma treatment.

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1. Introduction

Glaucoma is the second-biggest cause of irreversible blindness worldwide and the leading cause in developed countries. According to the World Health Organization, it affects over 61 million people. The main modifiable risk factor is intraocular pressure (IOP) increase, which leads to progressive retinal ganglion cell (RGC) death and subsequent irreversible vision loss.[1] However, RGC dysfunction and death can also occur in ocular normotensive subjects. Although not fully explored yet, several studies have shown secondary degeneration of retinal cells due to the cytotoxic environment (reactive oxygen species, nitric oxide, glutamate or other free radicals) produced by surrounding affected neurons.[2]

Brimonidine is a highly selective alpha-2 adrenergic pan-agonist lipophilic drug.[3] It has been used in ophthalmological care to produce ocular hypotension since 1974.[4] The 20–30% reduction in IOP[5] is due to its effect on alpha$_{2A,C}$ adrenergic receptors in the ciliary epithelium,[6,7] which inhibit aqueous humour inflow and lead to an increase in uveoscleral

[a]Department of Ophthalmology, Miguel Servet University Hospital, Zaragoza, Spain. E-mail: mariajesusrodrigo@hotmail.es
[b]Aragon Institute for Health Research (IIS Aragon), GIMSO Research Group, University of Zaragoza (Spain), Avda. San Juan Bosco 13, E-50009 Zaragoza, Spain
[c]Miguel Servet Ophthalmology Research Group (GIMSO), Aragon Health Research Institute (IIS Aragon), University of Zaragoza, Spain
[d]RETICS: Thematic Networks for Co-operative Research in Health for Ocular Diseases, Spain
[e]Department of Pathology, Lozano Blesa University Hospital, Zaragoza, Spain
[f]Institute for Chemical Synthesis and Homogeneous Catalysis (ISQCH), Faculty of Sciences, University of Zaragoza-CSIC, C/Pedro Cerbuna 12, E-50009 Zaragoza, Spain
[g]Centre for Animal Biotechnology and Gene Therapy (CBATEG), Universitat Autònoma de Barcelona, Bellaterra, Spain
[h]CIBER for Diabetes and Associated Metabolic Diseases (CIBERDEM), Madrid, Spain
[i]Department of Animal Health and Anatomy, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain
[j]Chromatography and Spectroscopy Laboratory, Institute for Chemical Synthesis and Homogeneous Catalysis (ISQCH), Faculty of Sciences, University of Zaragoza-CSIC, Pedro Cerbuna 12, E-50009 Zaragoza, Spain

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outflow.[8,9] The peak occurs within 2–3 hours and lasts until 10–14 hours after instillation.[10] Therefore, to decrease IOP to optimal therapeutic levels, topical eye drops must be administered twice a day by the patient. This can have several disadvantages, such as therapeutic oversights, loss of drug efficacy when crossing the anterior eye structures, and a remarkable 12.7% incidence of ocular and periocular allergic reactions,[11–13] or even intraocular inflammation.[14] These drawbacks worsen patient quality of life and decrease the compliance rate, which lead to progression of the disease.[15] Meanwhile, the neuroprotective effect of brimonidine has been described by many research groups since the four criteria used to evaluate the potential role of a neuroprotective agent are widely proven:[16] (1) brimonidine has targets (alfa-$_{2A,B,C}$ adrenergic receptors for RGCs, glial cells and photoreceptors) in the retina,[7,17] (2) the neuroprotective effect was demonstrated in cell and animal studies,[18–20] mainly in the RGC body and axons, but also in bipolar cells[21] and photoreceptors,[22] (3) it reaches neuroprotection concentration on the posterior segment where the drug interacts with retinal cells,[23,24] and finally (4) it showed neuroprotective characteristics in recent clinical trials in patients with diabetes[25] and age-related macular degeneration.[26]

LAPONITE® $Na^{+0.7}$ $[(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{-0.7}$ is a biocompatible and biodegradable synthetic clay that in recent years has been used in a wide range of biomedical and biomaterial applications, particularly in nanomedicine, regenerative medicine and tissue engineering.[27] LAPONITE® is composed of two-dimensional nanoscale disk-shaped crystals (0.92 nm height, 25 nm diameter, 2.65×10^3 g cm^{-3} density) comprising an octahedral magnesia sheet sandwiched between two tetrahedral silica sheets.[28] When dispersed in aqueous media, a three-dimensional house-of-cards structure is formed. Sodium ions are released, leading to a weak negative-charge surface and further water absorption, resulting in an increase in the clay's volume.[29,30] It forms a clear colloidal dispersion with thixotropic, viscoelastic and transparent gel characteristics, suitable for administration by injection.[28] Since it is able to interact with other molecules by ion-exchange, van de Waals forces, hydrogen bonding, cation/water bridging, protonation or ligand exchange, LAPONITE® is even able to bring into solution compounds that are water insoluble.[31] It can act as a carrier for several drugs,[32] and can also release drugs in a controlled manner depending on surrounding conditions such as pH or temperature.[33–35] Degradation of LAPONITE® releases products that have, by themselves, biological roles; Reffitt *et al.*, showed an increase in collagen type I synthesis because of orthosilicic acid $[Si(OH)_4]$;[36] magnesium ions may also trigger cell responses, stabilize polyphosphate compounds in cells such as adenosine triphosphate (ATP), or be involved in enzymatic activity and signalling processes; sodium cations interfere with the generation of nerve impulses and the hydroelectrolyte balance, and lithium affects the behaviour of neurons.[37,38] Previous animal studies demonstrated LAPONITE®'s safety[39] and analysed the pharmacokinetics and pharmacodynamics up to 24 weeks of intravitreal injection of dexamethasone-LAPONITE® formulation in the vitreous humour of rabbit eyes.[40]

Intravitreal injection is the gold standard therapeutic option for posterior segment pathologies such as age-related macular degeneration, diabetic retinopathy or vascular occlusions. It has long been used in ophthalmological treatment[41] because it maintains therapeutic drug levels at the target site while avoiding ocular barriers.[42] Thus, repeated ocular injections are needed, threatening complications such as IOP elevation, intraocular inflammation, cataract formation, retinal detachment or even endophthalmitis, with a 0.02% incidence per injection in the latter.[43–45] Minimally invasive sustained drug delivery maintains therapeutic concentration for prolonged periods, enhancing the half-life and bioavailability of the drug and preventing the need for frequent administration.[46]

There is currently no intravitreal treatment focused on control of glaucomatous neuropathy that simultaneously decreases the IOP and prevents neuroretinal damage.

To our knowledge, this is the first study demonstrating that a sustained-release brimonidine-LAPONITE® formulation, administered in a single intravitreal injection, exerts a functional and structural ocular hypotensive and neuroprotective effect lasting at least 6 months in a glaucoma animal model.

2. Materials and methods

Chemicals and reagents

Brimonidine and 2-bromoquinoxaline were obtained from Sigma-Aldrich (Madrid, Spain). LAPONITE®-RD (LAP) (surface density 370 m^2 g^{-1}, bulk density 1000 kg m^{-3}, chemical composition: SiO_2 59.5%, MgO 27.5%, Li_2O 0.8%, Na_2O 2.8%) was obtained from BYK Additives (Widnes, Cheshire, UK). Balanced 0.9% salt solution (9 mg ml^{-1} NaCl) (BSS) was obtained from Fresenius Kabi (Barcelona, Spain). HPLC-grade ethanol, acetonitrile, methanol, ammonium formate, formic acid, ammonia and phosphoric acid (85% w/w) were obtained from Scharlab (Barcelona, Spain). Oasis MCX Prime 96-well μElution plates were obtained from Waters Chromatography (Barcelona, Spain).

Brimonidine-LAPONITE® formulation

Brimonidine (BRI) was loaded on LAPONITE® (LAP) following the previously described methodology for dexamethasone.[40,47] Thus, BRI/LAP was prepared by adding LAP (100 mg) to a solution of brimonidine in ethanol (10 mg per 10 ml), stirring at r. t. with solvent evaporation under vacuum to get a good dispersion of brimonidine on the surface. The BRI/LAP powder was stored at −30 °C in tightly capped single-use vials that were gamma-ray sterilized.

Drug load on the LAP was determined using the ultra-high-pressure liquid chromatography mass spectrometry (UHPLC-MS) method (see below). Sample powder (5 mg) was extracted in 5 ml of acetonitrile/ethanol (1/1 v/v). After 1 h of stirring, the sample was centrifuged at 3000 rpm for 10 min at

r.t. and the supernatant was analysed, yielding a total load of 8.98 mg per 100 mg of solid (98.8% of the initial amount).

Immediately before injection, the brimonidine/LAP powder was suspended in BSS (10 mg ml^{-1}) and gently vortexed for 10 min to yield a yellow colloidal dispersion.

Sample processing for pharmacokinetic determination

Each rat eye was cut with dissecting scissors, 1 ml of 5% formic acid solution in acetonitrile was added and the mixture was sonicated at 45 W power for 10 min with a Hielscher UP50H ultrasound processor. Next, 1 ml of 200 mM ammonium formate in 4% phosphoric acid, and 100 μλ of 50 ppm 2-bromoquinoxaline internal standard (IS) in 0.1% formic acid in acetonitrile were added to the purée and the mixture was sonicated for 10 additional minutes. The sample was then centrifuged at 3000 rpm for 10 min. The supernatant was collected and cleaned up by solid phase extraction (SPE) in an Oasis MCX μElution plate. Thus, the supernatant was passed through the adsorbent under vacuum, and the adsorbed sample was rinsed with 600 μl of methanol. The adsorbed BRI and IS were eluted from the plate with 500 μl of 5% ammonia solution in methanol. The collected extract was evaporated under vacuum and dissolved in 200 μl of 0.1% formic acid in acetonitrile. The solution was analysed by UHPLC-MS. Recovery of the analyte was determined on spiked samples of rat eye at three concentration levels (low, medium and high) and was found to be between 92% (lowest concentration level) and 98% (highest concentration level).

Analytical method

Samples were analysed using a Waters Acquity UPLC instrument coupled to a Waters Acquity QDa mass spectrometer. The chromatographic separation was achieved using a Waters Cortecs T3 column (1.6 μm, 2.1 × 75 mm) at 30 °C. The mobile phase comprised a mixture of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples (10 μl) were eluted in gradient mode (t = 0 min, 75% A; t = 3 min, 50% A, t = 4 min, 75% A) at a flow rate of 0.5 ml min^{-1}.

The MS instrument was operated in electrospray ionization (ESI) positive mode. Full scan mode (150–500 Da) was used to identify the analytes (m/z = 292 (100%) and 294 for brimonidine and m/z = 209 (100%) and 211 (92.8%) for 2-bromoquinoxaline as the IS). Quantization was carried out in single ion monitoring (SIM) mode (m/z = 292 and 209 for brimonidine and 2-bromoquinoxaline, respectively).

The method was validated according to the ICH guidelines. Selectivity was assessed by analyzing blank samples from non-treated rat eyes, and no interferences were found. Calibration curves for brimonidine were constructed in the range 1–0.025 μg mL^{-1} by plotting the brimonidine/IS peak area ratio vs brimonidine nominal concentration. A weighted ($1/X^2$) linear regression model was applied to fit the data ($r^2 > 0.999$). The measured concentration of the standard samples was found to be within 10% of the nominal concentration, showing the accuracy of the method. The limit of detection (LOD) was found to be 0.005 μg mL^{-1}, calculated by the standard error of the intercept method. The LOD was assessed with a sample of nominal concentration obtained by the method of signal-to-noise ratio of at least 10. The limit of quantization (LOQ) was determined by the standard error of the intercept method and was found to be 0.017 μg mL^{-1}.

Animals

The study was carried out on 91 4 week old Long–Evans rats (40% males, 60% females) weighing from 50–100 grams at the beginning of the study. The animals were housed in standard cages with water and food *ad libitum* in rooms kept at a controlled temperature (22 °C) and relative humidity (55%) with 12-hour dark/light cycles. The work with animals was carried out in the experimental surgery service department of the Aragon Biomedical Research Centre (CIBA). The experiment was previously approved by the Ethics Committee for Animal Research (PI34/17) and was carried out in strict accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals.

Ocular hypertension (OHT) induction and drug injection procedure

The animals were divided in two cohorts: [non-bri] and [BRI-LAP]. The [non-bri] cohort comprised 31 rats. In this cohort, OHT was induced in the right eye (RE) and the left eye (LE) was untreated and served as the control eye. The [BRI-LAP] cohort comprised 60 rats. In this cohort, OHT was induced in both eyes but the RE received an intravitreal injection with the brimonidine-LAPONITE® (Bri-Lap) formulation. The RE served as the treated hypertensive eye and the LE served as the hypertensive control eye.

Ocular hypertension was generated using the model described by Morrison *et al.* by means of sclerosis of episcleral veins[48] with a hypertonic 1.8 M solution in topical eye drops (*Anestesico doble* Colircusi®, Alcon Cusí® SA, Barcelona, Spain) and general anaesthesia by intraperitoneal (IP) injection (60 mg kg^{-1} of ketamine + 0.25 mg kg^{-1} of dexmedetomidine). To maintain OHT, animals in both cohorts were re-injected every two weeks if IOP measurements were less than 20 mmHg. At the baseline, the [BRI-LAP] cohort received 3 μl[49] of the Bri-Lap formulation (10 mg Bri-Lap per ml; amount of brimonidine injected: 2.69 μg, 0.13 mg ml^{-1} of vitreous humour, considering a rat vitreous volume of 20 μl[50]). Determination of this concentration, applying the corresponding scale correction, was based on the doses given by other authors to rats (8.8 mg of brimonidine in nanoparticles, 0.44 mg ml^{-1} of vitreous, induces neuroprotection),[51] mice (1.07 mg of Bri-tartrate in nanosponges, 0.14 mg Bri per ml of vitreous, induces ocular hypopressure)[52] and rabbits (lower dose of 0.45 mg of Bri-tartrate in microspheres, 0.20 mg ml^{-1} of vitreous, induces ocular hypopressure).[53] REs were intravitreally injected using a Hamilton® syringe (measured in μl) and a glass micropipette, which allowed visualization of the yellowish formulation being administered. After intervention, animals were left to recover at a temperature controlled by

warm pads, with a 2.5% enriched oxygen atmosphere and lubricant antibiotic ointment on the eyes.

Clinical, functional and structural *in vivo* ophthalmological examination

Clinical ophthalmological signs such as redness, scarring, infection or intraocular inflammation were evaluated weekly. Measurements of intraocular pressure were also recorded with the Tonolab® rebound tonometer. The IOP value was the average of three consecutive measurements resulting from the average of 6 rebounds. For this purpose, rats were sedated for less than three minutes with a mixture of 3% sevoflurane gas and 1.5% oxygen to avoid the potential effect of gas anaesthesia, as recommended.[54]

Neuroretinal structure functionality was studied using electroretinography (ERG) (Roland consult® RETIanimal ERG, Germany) with flash scotopic ERG and photopic negative response (PhNR) protocols at the baseline and 8, 12 and 24 weeks. To test flash scotopic ERG, animals were dark-adapted for 12 hours and anaesthetized with IP and topical anaesthesia. Their eyes were fully dilated with mydriatic eye drops (tropicamide 10 mg ml^{-1}, phenylephrine 100 mg ml^{-1}, Alcon Cusí® SA, Barcelona, Spain) and their cornea was lubricated (hypromellose 2%). Corneal electrodes served as active electrodes, reference electrodes were placed subcutaneously on both sides, and the ground electrode was placed near the tail. Electrode impedance was accepted if there was a difference of <2 kΩ between electrodes. Both eyes were simultaneously tested using a Ganzfeld Q450 SC sphere with white LED flashes for stimuli and seven steps were performed with increasing luminance intensity and intervals (step 1: 0.0003 cds m^{-2}, 0.2 Hz s^{-1}; step 2: 0.003 cds m^{-2}, 0.125 Hz s^{-1}; step 3: 0.03 cds m^{-2}, 8.929 Hz s^{-1}; step 4: 0.03 cds m^{-2}, 0.111 Hz s^{-1}; step 5: 0.3 cds m^{-2}, 0.077 Hz s^{-1}; step 6: 3.0 cds m^{-2}, 0.067 Hz s^{-1}; and step 7: 3.0 cds m^{-2}, 29.412 Hz s^{-1}).[55] The PhNR protocol was performed after light adaptation to a blue background (470 nm, 25 cds m^{-2}) and red LED flashes (625nm, 0.30 cds m^{-2}) were used as stimuli. Latency (in milliseconds) and amplitude (in microvolts) were studied in *a*, *b* and *PhNR* waves.

Neuroretinal structures were studied using optical coherence tomography (OCT Spectralis, Heidelberg® Engineering, Germany) at the baseline, 3 days and at 2, 4, 6, 8, 12, 24 weeks after the Bri-Lap injection. Protocols such as retina posterior pole (R), retinal nerve fibre layer (RNFL) and ganglion cell layer (GCL) with automatic segmentation were evaluated. These protocols analysed an area of 1, 2 and 3 mm around the centre of the optic disc by means of 61 b-scans, and subsequent follow-up examinations were performed at this same location using the eye-tracking software and follow-up application. The vitreous was also visualized using the en face vitreous protocol. For the scans, rats were IP anaesthetized (as mentioned above) and a flat contact lens was adapted to their cornea to obtain high-quality images.

A masked trained technician discarded biased examinations or corrected them manually if the algorithm had obviously erred.

Immunohistochemistry

Under general anaesthesia, animals were euthanized with an intracardiac injection of sodium thiopental (25 mg ml^{-1}). Eyes were immediately enucleated, fixed in neutral-buffered formalin (10%) and embedded in paraffin. A total of 44 eyes belonging to 22 rats from the [BRI-LAP] cohort were analysed (22 hypertensive REs injected with Bri-Lap and 22 control hypertensive LEs). Embedded eyes were trimmed to reach the optic nerve head. Next, 5 μm sections were deparaffined, rehydrated and washed in 10% H_2O_2 for 5 minutes (quenching) before incubation of the following primary antibodies at 4 °C overnight: mouse anti-Brn3a (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at 1 : 50 dilution; and rabbit anti-glial fibrillary acidic protein (GFAP) (DAKO, Bath, United Kingdom) at 1 : 1000 dilution. After that, sections were incubated for 90 minutes at room temperature with specific secondary antibodies: biotinylated horse anti-mouse at 1 : 50 dilution and biotinylated goat anti-rabbit at 1 : 100 dilution (Vector Laboratories, Burlingame, CA, USA). They were then incubated with ABC-HRP (Thermo Fisher Scientific, Waltham Massachusetts, USA) at 1 : 50 dilution for 90 minutes at room temperature. The sections were washed in phosphate-buffered saline before and after every incubation. Finally, the sections were stained with diaminobenzidine (DAB) for 3 minutes and counterstained with Harris haematoxylin (Sigma-Aldrich Corp., St Louis, MO, USA) for 20 minutes at room temperature. Procedural immunohistochemistry controls were performed by omission of the primary antibody in a sequential tissue section. Eye sections stained with haematoxylin/eosin were also used to analyse the general morphology of the retina.

Retinal ganglion cell count

Retinal ganglion cells were counted in radial sections of the retina along 2 mm of a linear region of the ganglion cell layer and corresponding to four areas, two on each side of the optic nerve head. Images were analysed by an operator blinded to treatment groups.

Statistical analysis

This was a longitudinal and interventionist study. All data were recorded in an Excel database, and statistical analysis was performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL). The Kolmogorov–Smirnov test was used to assess sample distribution. Given the non-parametric distribution of most of the data, the differences between the cohorts were evaluated using the Mann–Whitney *U* test and the changes recorded in each eye over the 24-week study period were compared using a paired Wilcoxon test. All values were expressed as means ± standard deviation. Values of $p < 0.05$ were considered to indicate statistical significance. To avoid a high false-positive rate, the Bonferroni correction for multiple comparisons was calculated. The level of significance for each variable was established based on Bonferroni calculations.

Statistical analysis of the number of ganglion cells was conducted in R (v. 3.6.0) using a paired *t*-test. The results are shown as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

Intraocular pressure and clinical signs

In the [non-bri] cohort, an IOP increase of >20 mmHg was found in the RE between weeks 1 and 10, peaking at week 7 (29.92 ± 7.39 mmHg). Between week 11 and the end of the study (week 24) IOP remained stable at 17.38 ± 2.87 to 23.66 ± 5.45 mmHg (Fig. 1a).

In the [BRI-LAP] cohort, REs showed normotensive levels (IOP < 20 mmHg) until week 3, after which IOP increased, ranging between 17.36 ± 4.10 and 23.99 ± 4.04 mmHg until the end of the study. LEs also showed progressive increases in IOP throughout follow-up. REs, however, showed statistically significant higher levels of IOP than LEs (Fig. 1b).

When REs from the [non-bri] and [BRI-LAP] cohorts were compared, the eyes treated with Bri-Lap always exhibited statistically lower IOP levels from weeks 1 to 10, and even Bonferroni correction for multiple comparisons (marked with **) was exceeded from weeks 1 to 8 ($p < 0.001$). However, this trend inverted from week 12 and no statistical differences were found after that (Fig. 1a).

The percentage of eyes with OHT (>20 mmHg) in both cohorts was studied and analysis revealed a lower percentage of hypertensive eyes when treated with Bri-Lap up to week 8. This was especially remarkable during the first month (0% *vs.* 72% at week 1, 4.8% *vs.* 88% at week 2, and 28.1% *vs.* 91.7% at week 4, respectively) both in the injected RE but also in the untreated LE. Nevertheless, a higher percentage of hypertensive eyes treated with Bri-Lap intravitreal formulation from the [BRI-LAP] cohort, as compared with the LEs used as hypertensive controls, was found throughout the study (Fig. 1c).

There were no cases of allergic reaction, infection, intraocular inflammation or retinal detachment. Two animals developed cataracts during the episcleral vein sclerosis procedure, though these reverted spontaneously in the subsequent weeks.[56] One case of cataract formation after the intravitreal injection developed, probably due to surgical issues as rats have thick lenses. This animal was thus only used for histological studies. As a remarkable adverse event, fifteen early and unexpected animal deaths occurred without any obvious cause: four rats died at week 2, four rats died at week 4, six rats died at week 8 and one rat died at week 12.

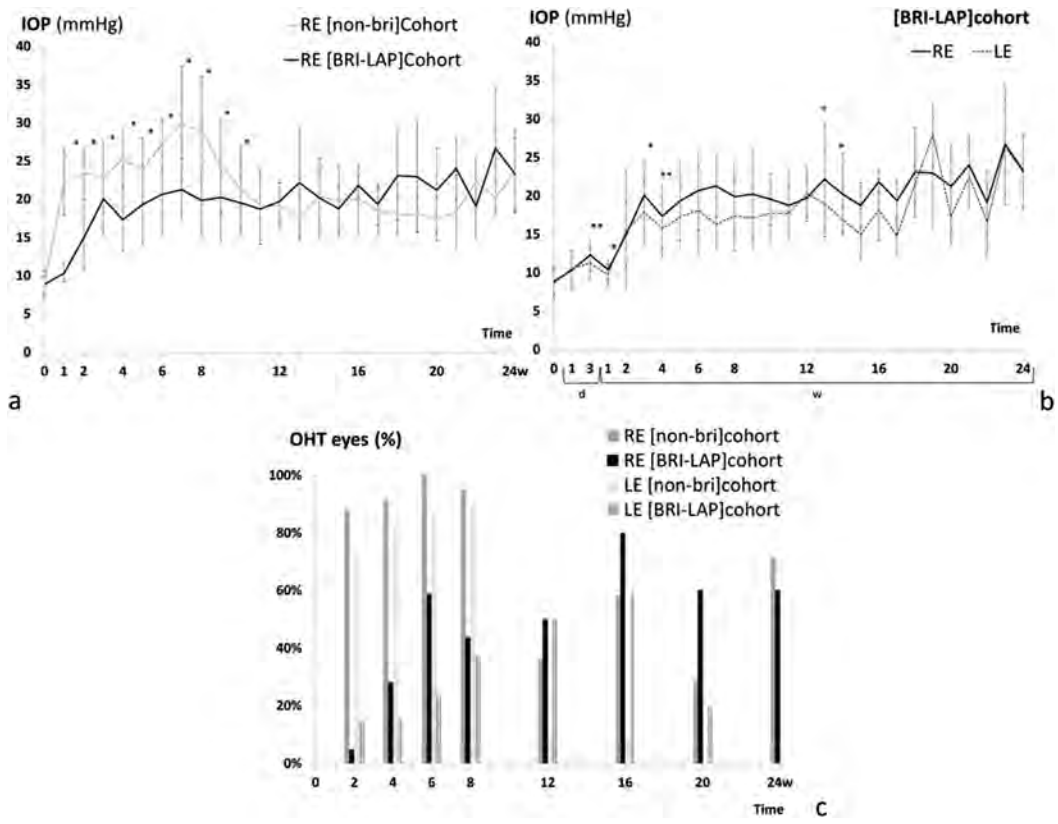

Fig. 1 Intraocular pressure curves. (a) Right eye comparison between the [non-bri] cohort (rats with ocular hypertension in the right eye) and the [BRI-LAP] cohort (rats with ocular hypertension in both eyes and an intravitreal injection of brimonidine-LAPONITE® formulation in the right eye). (b) Comparison between right and left eyes in the [BRI-LAP] cohort. (c) Percentage of ocular hypertensive eyes (>20 mmHg) in the [non-bri] cohort and the [BRI-LAP] cohort during follow-up. Abbreviations: IOP: intraocular pressure; RE: right eye; LE: left eye; w: week; d: day; *$p < 0.05$: statistical differences, **$p < 0.001$: statistical differences with Bonferroni's correction. REs from the [non-bri] cohort received an ocular hypertensive injection by sclerosing the episcleral veins. LEs from the [non-bri] cohort did not receive any treatment. REs from the [BRI-LAP] cohort received an ocular hypertensive injection by sclerosing the episcleral veins plus an intravitreal injection with brimonidine-LAPONITE® formulation. LEs from the [BRI-LAP] cohort received an ocular hypertensive injection by sclerosing the episcleral veins.

Electroretinography

REs from the [non-bri] cohort showed a decreasing tendency in amplitude in *a* (13.25 ± 16.78 *vs.* 67.53 ± 138.31 μV), *b* (44.64 ± 28.20 *vs.* 56.54 ± 53.34 μV) and *PhNR* (18.68 ± 24.77 *vs.* 25.52 ± 26.79 μV) waves when explored using the PhNR protocol at week 24 with respect to week 12, although no statistical differences were found.

The [BRI-LAP] cohort did not exhibit statistical differences in latency between REs and LEs when explored using the scotopic flash ERG protocol, but statistically significant higher amplitudes in *a* and *b* waves were found in the REs injected with Bri-Lap formulation as compared with LEs, mainly with lower intensity stimulus, at 8, 12 and 24 weeks. Similar results were obtained with the PhNR protocol, in which no statistical differences in latency were found between eyes, although a tendency to maintain this value was observed over 24 weeks. Statistically significant higher amplitudes in REs *vs.* LEs were also obtained in *a*, *b* and *PhNR* waves at 8, 12 and 24 weeks. Furthermore, a progressively increasing trend in *PhNR* wave amplitude was found in REs from weeks 8 to 12 (Fig. 2a and b).

When REs from the [non-bri] and [BRI-LAP] cohorts were compared, no statistically significant differences in latency were found using any protocol. The scotopic ERG test at week 24 showed worse statistically significant results in the amplitude and latency parameters in the [BRI-LAP] cohort, but the PhNR protocol revealed statistically significant higher amplitudes in the *PhNR* wave at week 12 and in the *a* and *PhNR* waves at week 24 in eyes treated with the Bri-Lap formulation (Fig. 2c and d).

Optical coherence tomography

In the [non-bri] cohort, REs showed a progressive loss in R, RNFL and GCL thickness measured by OCT over 24 weeks of follow-up.

In the [BRI-LAP] cohort, REs showed a trend towards greater R thickness and lower percentage loss (mainly in the inner sectors at early stages; $p < 0.05$) when compared with untreated contralateral hypertensive LEs (Fig. 3a). Higher RNFL thickness and, consequently, lower percentage loss were found in REs over follow-up with statistically significant differences in the early stages (Fig. 3a). A striking increase in thickness in most sectors was also found at day 3. When analysing the GCL protocol, LEs exhibited greater thickness ($p < 0.05$) in outer sectors in the early stages (outer nasal and outer temporal sectors at weeks 2 and 4, respectively). However, a lower percentage loss trend was observed in REs throughout the rest of the follow-up (ESI Table 1†).

With the aim of finding out the total effect exerted by the Bri-Lap formulation, REs from the [non-bri] and [BRI-LAP] cohorts were compared. Retina scans from the [BRI-LAP] cohort showed a tendency towards greater thickness in the early stages (statistically significant sectors are detailed in Fig. 3b). Analysing the RNFL protocol, a tendency to greater thickness in eyes injected with Bri-Lap formulation was observed from early/intermediate stages through to the end of the study, and the glaucomatous superior/inferior axis showed higher statistical significance (Fig. 3b). According to GCL examinations in the [BRI-LAP] cohort, all sectors (except the temporal sector) exhibited greater thicknesses with statistical significance in the superior/inferior axis at earlier stages (Fig. 3b); at the end of the study (week 24) every single sector from the group injected with Bri-Lap formulation had greater thicknesses, reaching statistically significant differences in the inner inferior and outer temporal sectors (ESI Table 2†).

To evaluate if the Bri-Lap formulation could exert an effect on the contralateral eye, LEs from the [non-bri] cohort (control eye without any treatment, but with contralateral OHT eye) *vs.* LEs from the [BRI-LAP] cohort (OHT eye without treatment but with Bri-Lap injection in the contralateral OHT eye) were compared. No statistical differences were found in most sectors at any of the stages analysed, except at week 24, when greater RNFL thickness was measured in the [BRI-LAP] cohort explored using the RNFL protocol in the nasal superior sector (18.57 ± 6.24 *vs.* 34.60 ± 10.55 μm, $p = 0.023$), and in GCL thickness explored using the GCL protocol in total volume (0.14 ± 0.01 *vs.* 0.15 ± 0.01 μm, $p = 0.030$) and in the inner inferior sector (21.71 ± 1.60 *vs.* 26.20 ± 1.09 μm, $p = 0.004$). LEs from both cohorts were >20 mmHg at week 24. However, by that stage LEs from the [BRI-LAP] cohort had statistically higher axonal and ganglion thickness than LEs from the [non-bri] cohort.

Images from vitreous scans showed hyperreflective aggregates of Bri-Lap formulation dispersed in the vitreous body as floaters, with a tendency to move to the vitreoretinal interface. A progressive decrease in the number and size of Bri-Lap aggregates was also detected over time (ESI Video 1†).

Immunohistochemistry

In experimental glaucoma, accurate measures of the number of RGCs are essential to evaluating the efficacy of novel therapeutic agents.[57] However, because there are around 30 different types of RGCs with different morphologies, gene expression and physiological properties,[58] it is necessary in experimental glaucoma to use a marker that identifies all the different types of RGCs. In this study, we have used an antibody against transcription factor Brn3a that is considered the most reliable pan-marker of RGCs in retinal sections.[59]

As expected,[60] simple visual examination revealed that the central areas of the retina showed greater density of RGCs marked with anti-Brn3a than the peripheral areas (Fig. 4a). Furthermore, the count of positive Brn3a cells along 2 mm of the retina showed that the mean number of RGCs was significantly higher in hypertensive eyes injected with the Bri-Lap formulation than in untreated contralateral hypertensive eyes (REs 23 ± 0.39 *vs.* LEs 20.66 ± 0.98; mean number of RGCs per linear mm of retina, $p = 0.040$) (Fig. 4b and c), confirming the neuroprotective effect of brimonidine during glaucoma.

Although it is generally accepted that glaucomatous damage is a consequence of axonal degeneration that leads to RGC death, glial activation is also present in glaucoma.[61]

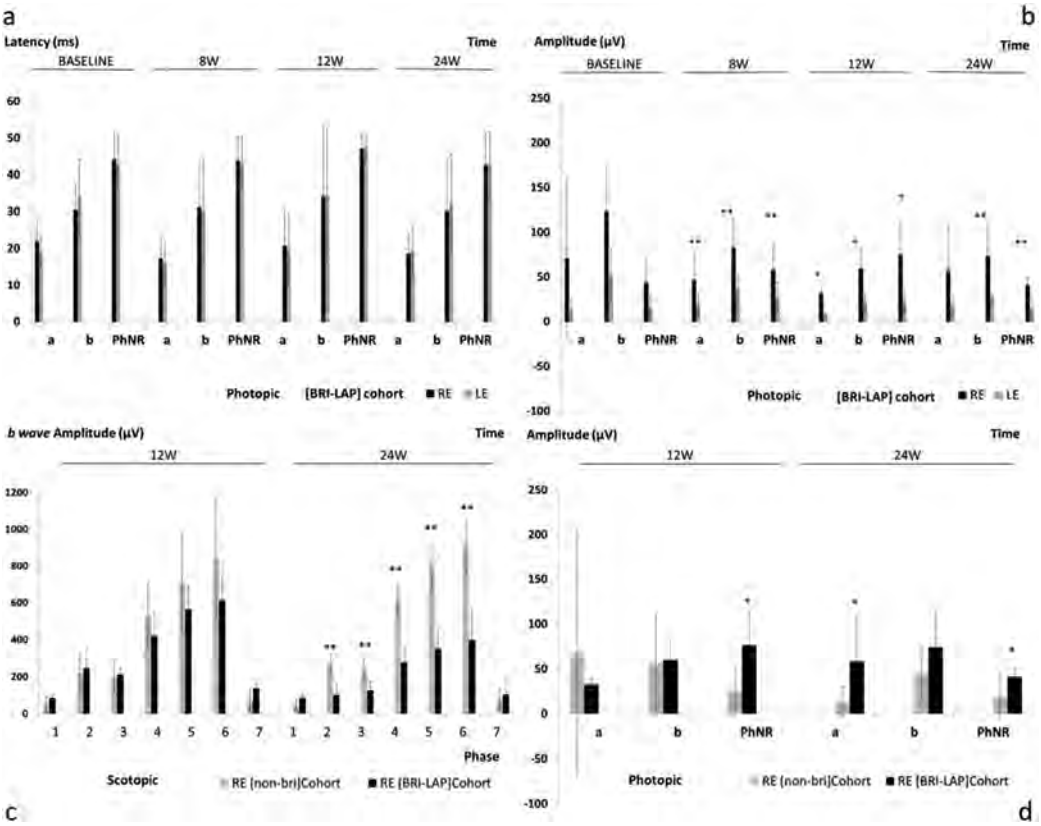

Fig. 2 Functional examinations using electroretinography (ERG). (a) PhNR (photopic negative response) latency from the [BRI-LAP] cohort (rats with ocular hypertension in both eyes and an intravitreal injection of brimonidine-LAPONITE® formulation in the right eye), maintained over 24 weeks. (b) PhNR amplitude, (*a*, *b* and *PhNR* waves) increased and was statistically significantly higher in eyes treated with the Bri-Lap formulation in comparison with contralateral left eyes. (c) The scotopic ERG test showed lower (*b* wave) amplitude at 24 weeks in eyes treated with the Bri-Lap formulation. (d) PhNR amplitude (*a* and *PhNR* waves) was statistically significantly higher in eyes treated with the Bri-Lap formulation in comparison with hypertensive and untreated eyes in the [non-bri] cohort. Abbreviations: RE: right eye; LE: left eye; *a* wave: signal from photoreceptors; *b* wave: signal from intermediate cells; *PhNR* wave: signal from retinal ganglion cells; Phases 1 to 7: multistep procedure with increasing intensity of luminance and different intervals from 0.0003 cds m^{-2} to 3.0 cds m^{-2}; w: week; ms: milliseconds; μV: microvolts; *$p < 0.05$: statistical differences, **$p < 0.001$: statistical differences with Bonferroni's correction.

Experimental IOP triggers GFAP upregulation in astrocytes and Müller cells.[62] To test if the hypotensive effect of brimonidine had any effect on GFAP expression in hypertensive eyes, GFAP immunohistochemistry was performed in the radial eye sections of the [BRI-LAP] cohort. The results obtained showed increased GFAP expression in the ganglion cell layer of the central retina two weeks after injection of a hypertonic solution into the episcleral veins (Fig. 5). In contrast, there was not an obvious increase in GFAP expression in the optic nerve head of glaucomatous eyes (Fig. 5). No differences in GFAP expression were observed between the glaucomatous eyes injected with Bri-Lap (REs) and untreated control eyes (LEs) (Fig. 5), suggesting that the Bri-Lap formulation does not have a beneficial effect on the gliosis produced by the increased IOP.

Concentration of brimonidine in rat eyes

In contrast to our previous study using dexamethasone release in rabbits,[40] this study on rats precluded analysis of the brimonidine content in the different ocular tissues, and total content in the rat eyes was determined after appropriate homogenization and subsequent fractionation by SPE. Fig. 6 shows the brimonidine concentration curves *vs.* time over the course of the study following IV administration of the Bri-Lap formulation. Concentration is expressed in nanograms of brimonidine per ml of the final solution.

Brimonidine concentration remains nearly constant in the first week after IV administration (121.0 ± 25.6 ng ml^{-1}), showing a steady decrease until a plateau was reached at 6 weeks and achieving a value of 62.8 ± 9.0 ng ml^{-1} 24 weeks after administration. Brimonidine levels in contralateral eyes were always below the detection limit.

4. Discussion

In our previous paper, we showed that the release of dexamethasone from LAPONITE® was sustained for up to 6 months in the vitreous body of healthy rabbit eyes.[40] In this

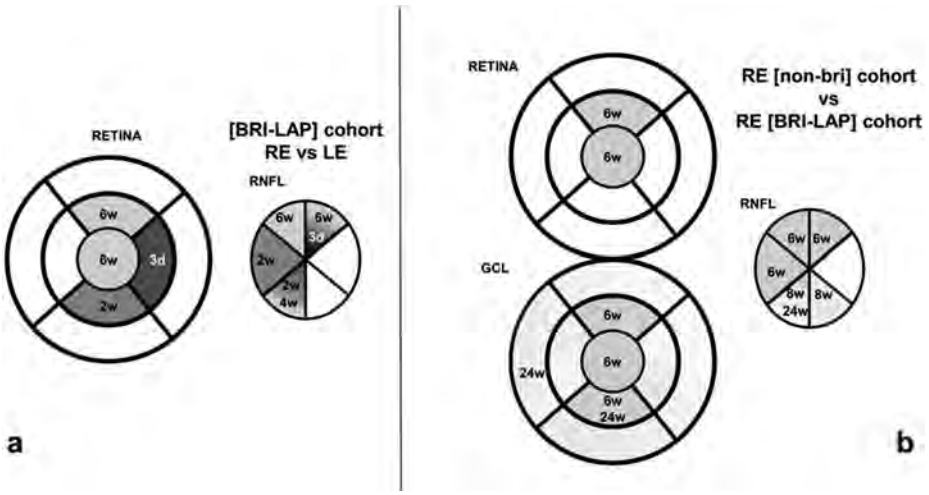

Fig. 3 Neuroretinal analysis using OCT. (a) OCT sectors with increased thickness in right eyes injected with Bri-Lap formulation as compared with untreated left eyes. (b) OCT sectors with statistically significant increases in thickness in right eyes from the [BRI-LAP] cohort as compared with right eyes from the [non-bri] cohort. Dark to light greyish sectors indicate OCT neuroretinal sectors exhibiting greater thicknesses with statistical significance, from earlier to later stages, respectively. Abbreviations: GCL: ganglion cell layer; RNFL: retinal nerve fibre layer; RE: right eye; LE: left eye; w: week; d: day; $*p < 0.05$: statistical differences. REs from the [non-bri] cohort received an ocular hypertensive injection by sclerosing the episcleral veins. LEs from the [non-bri] cohort did not receive any treatment. REs from the [BRI-LAP] cohort received an ocular hypertensive injection by sclerosing the episcleral veins plus an intravitreal injection with brimonidine-LAPONITE® formulation. LEs from the [BRI-LAP] cohort received an ocular hypertensive injection by sclerosing the episcleral veins.

study, we decided to make three important changes: (1) use of brimonidine, a different drug, to demonstrate the generality of the release method; (2) tests in another animal (rats), required before translational trials; and (3) application in a disease model (chronic glaucoma) in order to confirm not only the absence of side effects but also the therapeutic effect over an extended period, the goal being to use the treatment in future glaucomatous patients.

At present, there is no effective intravitreal hypotensive and neuroprotective treatment for glaucoma or other optic neuropathies in daily ophthalmology practice. The results of this study show that a single intravitreal injection of the Bri-Lap formulation, producing sustained release of brimonidine from the LAPONITE® carrier clay for at least 6 months, had a functional and structural hypotensive and neuroprotective effect. As it is administered intravitreally, this formulation would ensure treatment compliance and satisfactory control of the disease over extended periods of time, with administration being necessary perhaps twice yearly.

This paper shows that the Bri-Lap formulation has a net hypotensive effect (decrease of approximately 9 mmHg) when injected into an eye with ocular hypertension (compared to an untreated hypertensive cohort [non-bri]) lasting for 8 weeks. This is twice the time described when using intravitreal nanosponges.[52] The greatest hypotensive effect was observed in the early stages and coincided with the greatest release of brimonidine (approximately 120–80 ng ml^{-1}). It disappeared in later stages when the release of brimonidine plateaued (approximately 60 ng ml^{-1}).

Meanwhile, the fact that in the eye injected intravitreally with the Bri-Lap formulation IOP did not increase until week 3 of the study suggesting that the volume (3 microlitres) did not cause hypertensive iatrogenesis and that the greatest hypotensive effect (14.96 ± 4.16 *vs.* 23.34 ± 3.53 mmHg) occurs within the first two weeks of administration. However, from week 3 onwards the REs injected with Bri-Lap showed higher IOP than the contralateral hypertensive left eyes ($p < 0.05$). This finding may be because of both the variability described for the Morrison technique[56] for hypertensive induction (although all injections were administered by the same experienced ophthalmologist) and the intrinsic characteristics of the clay. LAPONITE® becomes hydrated and expands in volume in aqueous media.[29] However, the brimonidine deposited on the surface produces a hydrophobic effect, which gradually dissipates as release occurs, which would delay hydration and expansion until the surface of the LAPONITE® recovers its hydrophilic characteristics, which appears to occur from week 3 or 4 onwards.

Neuroretinal examinations using OCT technology showed that Bri-Lap formulation enhanced structural protection in axonal (up to week 6) and ganglion structures in intermediate (weeks 6 and 8) and late (weeks 12 and 24) stages. These results support the previous ones, in which the greater hypotensive effect observed in the early stages of the study protected the axons from IOP-dependent damage while the subsequently inferior concentrations of brimonidine (in the order of nanograms) detected in the plateau stage later provided neuroretinal protection by interacting with the retina's adrenergic receptors.[7,17]

Our findings also showed a protective functional effect, as explored with the PhNR ERG and mainly applicable to the RGCs (greater amplitude at week 12) and the axons (main-

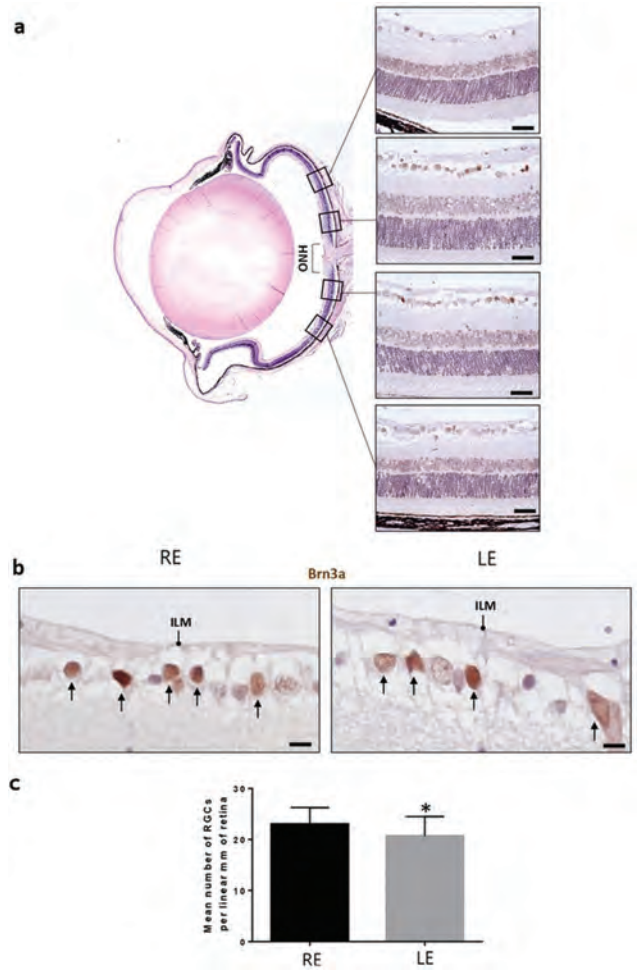

Fig. 4 Retinal ganglion cell analysis in glaucomatous eyes. (a) Retinal ganglion cells were counted in radial sections of the eye along 2 mm of a linear region of the retina, corresponding to four areas, two on each side of the optic nerve head (ONH). (b) Two representative images of the ganglion cell layer marked with anti-Brn3a corresponding to a right eye (RE) and a left eye (LE) of the same animal. Arrows mark the positive nuclei to Brn3a. (c) The mean number of retinal ganglion cells per linear mm of retina was significantly higher in hypertensive eyes injected with Bri-Lap formulation than in the untreated contralateral hypertensive eyes (RE 23.00 ± 0.39 *vs.* LE 20.66 ± 0.98, $p = 0.040$). Abbreviations: RE: right eye; LE: left eye; ILM: internal limiting membrane. Scale bars: (a) 22.72 μm, (b) 5.8 μm.

tained latency). This suggests that the Bri-Lap formulation has a mainly protective effect on the soma of the RGCs, which was corroborated in the histological studies' finding of a higher RGC count using the specific Ac Brn3a. Kim *et al.*,[51] also reported a neuroprotective effect after intravitreal injection of brimonidine-loaded nanoparticles that improved RGC survival in an optic nerve crush model, though this did not last longer than 14 days. The axonal protection provided by intravitreal brimonidine, associated with better anterograde and retrograde transport, has also been demonstrated by other groups.[63,64]

The Bri-Lap intravitreal formulation also has a protective structural and functional effect on the retina in the early stages of treatment (up to week 8; $p < 0.05$). This was maintained in photoreceptors under photopic stimulation (but not under scotopic stimulation) in the later stages (week 24) (see Fig. 3d). Similar results were reported by Ortín-Martínez *et al.*,[22] where topical brimonidine had a protective effect on the cones, and by Yukita *et al.*,[63] who observed conservation of RGC function under photopic conditions but without effect on the *a* and *b* waves of the scotopic ERG. Intraocular injection of brimonidine has also been shown to preserve outer nuclear layer thickness as measured by OCT[65] and even to reduce geographic atrophy secondary to age-related macular degeneration in a phase 2 study with a brimonidine drug delivery system.[26]

The difference found between the protective functional effect on RGCs (observed throughout the study) relative to photoreceptors with photopic stimulus (found at a later stage) may be due to the time required for the formulation to pass through the different layers of the retina and approach the photoreceptors. For instance, week 12 was the earliest that intraretinal Bri-Lap formulation was observed using OCT imaging.

OCT studies of GCL thickness detected a smaller percentage loss of thickness in the REs of the [BRI-LAP] cohort from week 4 onwards (*vs.* LE). However, a higher number of RGCs were counted from the start of the study, indicating that BRI-LAP also had an early neuroprotective effect on the injected eye. This finding seems to suggest underestimation of the neuroprotective effect on the GCL as measured by OCT. Before week 4, the left eyes of the [BRI-LAP] cohort exhibited greater GCL thickness (as measured by OCT) but nonetheless showed a lower number of RGCs (histological studies). This may be due to the increase in the size of the soma prior to ganglion death[66] because, as in the case of other authors who used the same glaucoma model,[48,67,68] RGC death was observed in the early stages of the study (before week 4). Another thickness confusion factor may have been glial infiltration and activation.[69] This was ruled out, however, as glial activation with no statistically significant differences between REs and LEs was detected in the [BRI-LAP] cohort (with induced bilateral glaucoma). Another fact to consider is that the astrocyte and Müller cell reaction (detected using GFAP) occurred at a very early stage of the study (from week 2 onwards) in both OHT-induced eyes, even when IOP was on average less than 20 mmHg. This shows that an upward fluctuation in IOP (albeit in a range considered normotensive: <20 mmHg) triggered a premature immune response resulting in consequent cell death, as also described in ref. 70 and 71.

These analyses seem to suggest a possible error or deviation with regard to considering—in the early stages of the disease—greater GCL thickness, as measured by OCT, as indicative of better condition or protection, and lesser thickness as neurodegeneration. This long-term longitudinal study has demonstrated the dynamism, and therefore change in thickness, that can be quantified by OCT. The authors of this study consider that the results measured by OCT in the early stages should be

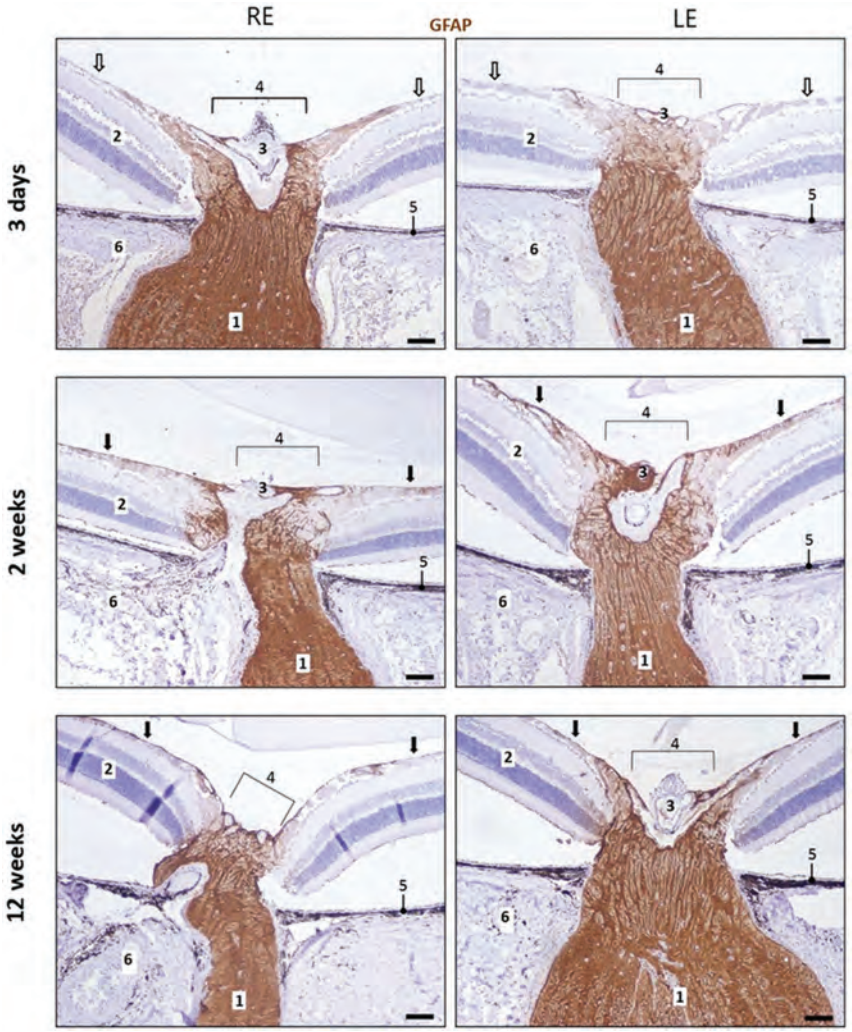

Fig. 5 Increased GFAP expression was observed in the ganglion cell layer of the central retina two weeks after injection of a hypertonic solution into the episcleral veins (black arrows). The Bri-Lap formulation does not induce any obvious change in GFAP expression in the retina or in the optic nerve head in glaucomatous eyes. 1: Optic nerve; 2: Central retina; 3: Central vessel of the retina; 4: Optic nerve head; 5: Retinal pigment epithelium; 6: Sclera; Arrows: central retina without overexpression of GFAP. Scale bars: 82.2 μm.

considered as a whole and not from the simplistic assumption of greater thickness/protection and lesser thickness/damage, as other authors have shown in other inflammatory neurodegenerative diseases.[72]

Interestingly, starting treatment with the Bri-Lap formulation in one eye could also control IOP in the contralateral eye, even though brimonidine levels were below the detection limit. In this regard, it was observed that when LEs from the [BRI-LAP] cohort were compared with LEs from the [non-bri] cohort the percentage of eyes with OHT was lower in the [BRI-LAP] cohort. Even in the later stages of the study (week 24) OCT detected greater thicknesses in the untreated hypertensive LE in the [BRI-LAP] cohort when compared with a healthy normotensive eye (left eye of the [non-bri] cohort) that undergoes the physiological process of ganglion death and is affected by the harmful agents in its OHT-induced contralateral eye.[73] These optimal results may have been a consequence of retrograde and anterograde contralateral substance dissemination *via* the visual pathway[74–76] and of improvement of axonal transport by brimonidine.[77] In addition, brimonidine may spread through the blood vessels. Communication and propagation of molecules to the opposite eye affecting the retina has also been suggested.[78] As brimonidine in the blood was not quantified in this study, neither of these routes can be ruled out.

It is a remarkable hallmark that intravitreal injection of Bri-Lap produces neuroprotection, even with higher IOP ($p < 0.05$), in the treated eye very soon after injection and over a period of six months. Furthermore, to the best of our knowledge, this paper is the first to demonstrate a neuroprotective effect on the eye contralateral to the one treated, which even shows an improvement in degeneration over time.

Brimonidine concentration in the vitreous of treated patients stands at 185 nM.[79] The amounts analysed in the rats

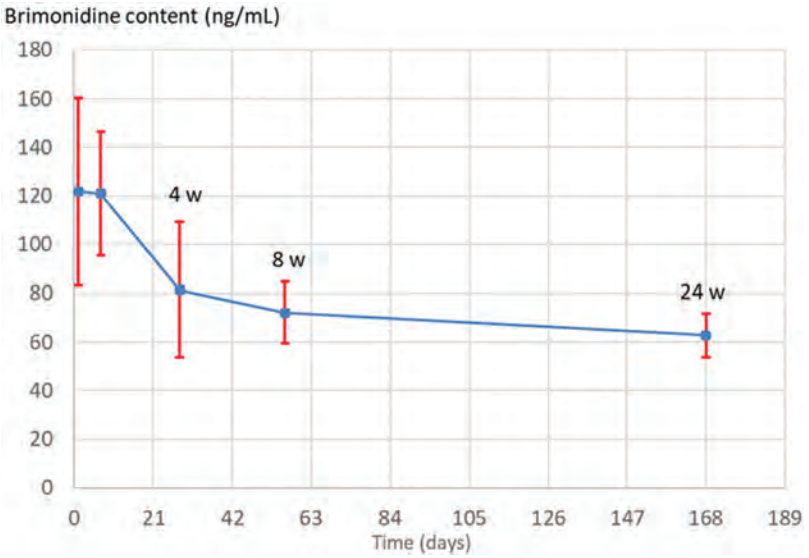

Fig. 6 Mean brimonidine concentrations in rat eyes after intravitreal administration of the Bri-Lap formulation.

in our study reveal an apparent concentration (considering a vitreous volume of 20 μl) ranging from 4.1 μM (week 1) to 2.0 μM (week 24). The decrease in drug levels in the eye throughout the study coincided with the degradation (smaller aggregates over time) of the Bri-Lap formulation observed using OCT (unpublished data). It is an order of magnitude greater than the concentration observed in patients and three orders of magnitude greater than that required for receptor activation, since only 2 nM of alpha-2 agonists are required for maximum receptor activation.[16] This suggests that formulations with lower doses could also be effective while incurring lower risk of side effects.

Brimonidine, in neutral form, shows very low solubility in the aqueous phase. This is one of the reasons why it is usually administered in cationic form (as tartrate). The slow release of Bri-Lap can therefore be explained by two factors: the low solubility of brimonidine and the retention ability of LAPONITE® due to hydrogen bonds and van der Waals interactions, as we previously observed with dexamethasone.[40,47] All brimonidine content (associated or not associated with LAPONITE®) was measured in the eye over the study. As mentioned in the introduction, brimonidine has a short half-life (12 hours) and rapid clearance in the eye.[10] It is probable that at 6 months (60 ng ml^{-1}) most of the analysed brimonidine is associated with LAPONITE®. The very low (unknown) quantity of non-associated brimonidine would not control IOP efficiently in the final stages of the study. This observation concurs with previous authors, showing an initially higher ocular hypopressure effect[52] that decreases later (up to 4 weeks).[53] Drug resistance cannot therefore be ruled out. However, up to the end of the study it showed a neuroprotective effect (as found when using nanoparticles[51]). In addition, the basicity of the Bri-Lap formulation could counteract the acidosis of glaucoma[80,81] and enhance the benefits.

This study demonstrates that using LAPONITE® as a drug carrier for intraocular delivery has several advantages. From a chemical perspective, the Bri-Lap formulation is easy and simple to prepare and drug release is not associated with carrier degradation, unlike other drug delivery systems (DDS).[82] From a clinical perspective, the clear, thixotropic and nanoscale gel formulation allows it to be injected into the vitreous body through smaller-gauge needles, in contrast to Brimo DDS®—which requires applicators[26]—or other devices and implants.[83] To the authors' knowledge, this is the first *in vivo* study of a brimonidine DDS that shows longer sustained reduction of IOP[53,84] and a neuroprotective effect, even in a disease model in which degradation is assumed to occur more rapidly.

Limitations and future studies

It should be mentioned that there was a striking and unexpected level of early death among the rats. This may have resulted from both repetition of intraperitoneal anaesthesia with dexmedetomidine (deaths decreased drastically or disappeared in the later stages when anaesthetic interventions were less frequent), and by the depressant effects of brimonidine on the central nervous system. It may also have been due to potentiation of the effects of both alpha-2 agonists simultaneously.[3] Brimonidine is able to cross the blood–brain barrier,[4] and can cause sedation, bradycardia, hypotension and subsequent death.

Based on all the above, the authors consider that blood analysis and future pharmacodynamic adjustment and scaling studies would be advisable and necessary before exploring potential transferability to clinical practice. It would also be interesting to co-insert[85] different agents in the clay carrier to combat various neurodegenerative pathways using microcapsule systems as described by Arranz-Romera *et al.*[86] Prieto

et al.[40] demonstrated sustained release and tolerance of intraocular dexamethasone with LAPONITE®; this powerful anti-inflammatory drug could combat gliosis and even improve the results achieved in this study, as it appears that the Bri-Lap formulation has no effect on gliosis as there are no differences between eyes or cohorts with respect to GFAP.

5. Conclusions

This paper presents, for the first time, a study in which intraocular administration in a rat eye of a brimonidine-LAPONITE® formulation was well tolerated and had an early functional and structural hypotensive and neuroprotective effect. It acted mainly on retinal ganglion cells and the sustained-release mechanism enabled a single intravitreal injection to last for at least 6 months. The study presents a formulation with potential for transfer to clinical treatment of glaucoma[79] and other optic neuropathies.

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Conflicts of interest

There are no conflicts to declare.

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