International Journal of Pharmaceutics 570 (2019) 118662

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Chitosan/hydroxyethyl cellulose inserts for sustained-release of dorzolamide for glaucoma treatment: *In vitro* and *in vivo* evaluation

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ARTICLE INFO

Keywords:
Glaucoma
Dorzolamide
Chitosan
Hydroxyethyl cellulose
Ocular inserts
Sustained-release drug delivery systems

ABSTRACT

Eye drops containing hydrophilic drugs are commonly used to reduce intraocular pressure (IOP) in glaucoma patients, but compliance to the treatement is commonly reduced by frequent dosing and eventual systemic side effects. Sustained-release drug delivery systems, such as ocular inserts, can reduce dosing, limit systemic exposure, reduce side effects, and, then, improve patient adherence to therapy. Here, we developed and evaluated chitosan/hydroxyethyl cellulose-based ocular inserts for sustained release of dorzolamide, a hydrophilic drug. Dorzolamide inserts (DI) were produced by solvent/casting method and characterized by various physicochemical techniques. Pharmacokinetics studies were performed using scintigraphic images and *ex vivo* biodistribution. The effectiveness of inserts was tested in glaucomatous rats. Characterization studies showed that the drug strongly interacted with the polymeric matrix, but *in vitro* results showed that DI took only 3 h to release 75% of dorzolamide entraped. However, scintigraphic images and *ex vivo* biodistribution studies revealed that more than 50% of ^{99m}Tc-dorzolamide remained in the eye after 18 h of DI administration, while only about 30% of the drug remained in the eye after drops instilation. DI exerted significant hypotensive effect for two weeks, after single administration, while IOP values remained high in placebo and untreated groups. Eye drops were effective only during the treatment period. Only DI treatment prevented retinal ganglion cells death. Altogether, these findings evidenced the potential application of polymeric-based inserts for sustained release of dorzolamide in glaucoma management.

1. Introduction

Glaucoma is a group of eye diseases that damage the optic nerve and retinal ganglion cells (RGC). These damages usually result from elevated intraocular pressure (IOP) (Davis et al., 2016). According to the National Eye Institute, glaucoma remains as the leading cause of blindness in adults over 60 years old (Tandale and Wagh, 2011).

Among other options, dorzolamide eye drop solution is currently being used to lower IOP in glaucomatous patients (Mincione et al., 2008; Tandale and Wagh, 2011). Nevertheless, the eye presents unique challenges when it comes to delivering drug molecules and even eye drops often lead to low ocular bioavailability (Gulsen and Chauhan, 2005). Overflow from the cojunctival sac expel a great portion of the drop, while the liquid remaining on the ocular surface can be washed away through the nasolacrimal duct, thereby decreasing the amount of the drug that reaches the targeted ocular structures (Ludwig, 2005). So, in general, less than 5% of an applied dose is absorbed into the eye and, more typically, less than 1% is absorbed (Irimia et al., 2018a; Sigurdsson et al., 2005). Moreover, patients tend to present inadequate adherence to their daily therapeutic regimen. In this case, forgetfulness, uncomfortable sensations, difficulty of instillation, and other practical issues are cited as important factors for low compliance and low persistence when eye drops are prescribed (Kulkarni et al., 2008).

Controlled drug release devices are systems which can provide continuous drug release at predetermined rates. Such systems eliminate frequent dosing, which results in better patient compliance (Bourges et al., 2006). Furthermore, therapeutic drug levels are achieved without ocular tissues exposure to toxic drug levels. Moreover, those devices are

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https://doi.org/10.1016/j.ijpharm.2019.118662
Received 21 May 2019; Received in revised form 7 August 2019; Accepted 3 September 2019
Available online 03 September 2019
0378-5173/

more economic because smaller amounts of drugs are required to achieve the same effect as eye drops, since these systems release drugs over extended periods of time (Natu et al., 2011a,b).

Our long-term goal in this work was preparing a formulation designed to provide a localized and sustained release of dorzolamide hydrochloride (a hydrophilic drug) to be used in the treatment of open-angle glaucoma. From all the formulations developed for ophthalmic drug delivery, only inserts and implants are capable of sustained release of drugs during several days (Natu et al., 2011a,b). Given the fact that implants must be surgically placed in the eye, we aimed to develop a formulation pharmaceutically characterized as an insert, that could be placed on the eye surface without further elaborated procedures. Since the inserts will be degraded, they will slowly release the drug at the eye. This procedure should be highly efficient, decreasing side effects and increasing patient compliance. Inserts should also reduce treatment costs.

Chitosan is a biodegradable and nontoxic biomaterial, which has excellent mucoadhesive strength and has been routinely explored for controlled drug delivery at various mucosal sites of the body, including eye (Ludwig, 2005; Ways et al., 2018; Song et al., 2018; Zambito and Di Colo, 2010). Because of its polycationic properties, chitosan interacts with the polyanionic surface of ocular mucosa through hydrogen bonding and ionic interactions enhancing the mucoadhesive effect of the formulation (Wadhwa et al., 2009). It also widens the tight junctions of membranes (Han et al., 2004), enhances the drug residence time (Irimia et al., 2018b) and mucoadhesiveness (George and Abraham, 2006; Valenta, 2005) of formulations and serves as permeation and absorption enhancer (Caramella et al., 2010; Zambito and Di Colo, 2010). Due to these interesting features, chitosan seems to be an appropriate base for the development of ocular formulations (Irimia et al., 2018a).

As such, the hydroxyethyl cellulose is a non-ionic, water-soluble, odorless, tasteless, and non-toxic carbohydrate polymer used extensively in pharmaceutical areas. Hydroxyethyl cellulose exhibits a pH-independent release due to its non-ionic nature (Angadi et al., 2010) and is better tolerated on the eye surface than other cellulose derivates (Ludwig, 2005). Moreover, chitosan-hydroxyethyl cellulose hydrogels were tested by Li et al. (Li and Xu, 2002) for ophthalmologic applications. It was found that the polymers could interact by means of hydrogen bonds, thus leading to sustained release of pilocarpine.

Previously, we described the use of chisotan-based inserts for sustained realize of hydrophobic drugs, such as dexamethasone (Rodrigues et al., 2009), bimatoprost (Franca et al., 2014) and diminazene aceturate (Foureaux et al., 2015), in the eye. In this current study, we aimed to formulate chitosan-hydroxyethyl cellulose-based inserts for sustained realease of a hydrophilic drug (dorzolamide hydrochloride). Since controlling the realease of a hydrophilic drug from the matrix would be more challenging, the matrix of the insert was changed by the addition of hydroxyethyl cellulose, as it will be expected that the interaction between the polymeric matrixes could lead to better drug delivery profile.

2. Experimental section

2.1. Materials

Hydroxyethyl cellulose was obtained from Galena (Campinas, Brazil) and medium molecular weight chitosan was purchased from Sigma-Aldrich (St. Louis, USA). Dorzolamide hydrochloride was supplied by Fluka (Steinheim, Germany). Technetium-99 metastable (^{99m}Tc) was obtained from a molibdenium-99/technetium-99 metastable (^{99}Mo/^{99m}Tc) generator acquired from *Instituto de Pesquisas Energéticas e Nucleares* - IPEN/CNEN (São Paulo, Brazil). If not mentioned otherwise analytical grade reagents were used.

2.2. Preparation of inserts

Inserts were prepared by the solvent/casting technique, as described elsewhere (Franca et al., 2014; Rodrigues et al., 2009). Monolayer films (5 mm diameter; 40–70 μm thickness) were prepared by adding acetic acid (150 μL) to 10 mL of an aqueous solution containing dorzolamide hydrochloride (equivalent to 200 mg of dorzolamide). Next, hydroxyethyl cellulose (30 mg) and chitosan (500 mg) were added to the solution and the viscous dispersion was magnetically stirred overnight to ensure drug and polymers homogeneity. Hereafter, the content was casted in circular silicone-molded trays, containing individual 5 × 2 mm wells (Franca et al., 2014; Perumal et al., 2008), and allowed to dry at room temperature to produce dorzolamide-loaded inserts (DI). Finally, inserts were gently removed from the silicone-molded trays and stored under light and air humidity protection. Placebo inserts (PI) were similarly produced, without adding dorzolamide hydrochloride into de solution. For *in vivo* studies, inserts were cut in pieces of 1 × 2 mm.

2.3. Characterization of the inserts

2.3.1. Swelling studies

Swelling studies of the inserts were conducted as previously described (Eroğlu et al., 2007; Rodrigues et al., 2009). After weighing, inserts were placed in phosphate buffered saline (PBS, pH 7.4) for a time interval ranging from 5 to 90 min ($n = 3$). After immersion, the inserts were removed from PBS and, then, filter paper was used to eliminate the exceeding surface water. Finally, the hydrated devices were weighed and the swelling degree was calculated as expressed in Eq. (1) (Dobaria et al., 2009), in which W_t is the weight of the swollen insert and W_0 represents the weight before insert immersion.

$$\text{Swelling index} = (W_t - W_0)/W_0 \quad (1)$$

2.3.2. ATR-FTIR analysis

A PerkinElmer FTIR Spectrometer, Model Spectrum One (USA) was used to record the attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-FTIR) spectra of DI and PI. The inserts were gently cut with a scalpel blade and the pieces were directly put over the crystal of the equipment. Four scans were taken for each sample and the force over the sample was adjusted to achieve satisfactory transmittance results (at least 50% in the main peak).

2.3.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements of DI and PI (packed in an aluminum crucible) were performed in a Shimadzu DSC50 (Japan) apparatus. Heating and cooling rates were 10 °C·min^{-1}. Nitrogen (50 mL·min^{-1}) was used as purge gas. Inserts were heated from −50 to 200 °C (run 1) in a nitrogen environment. Finally, inserts were cooled to −50 °C under nitrogen environment and, then, reheated to 400 °C (run 2).

2.3.4. Determination of dorzolamide

The amount of dorzolamide presented in the inserts was assessed by UV spectroscopy (254 nm), using a Shimadzu Ultraviolet spectrometer (Japan). DI was placed in a glass flask and glacial acetic acid (0.5 mL per insert) was added to the flask. After 30 min, purified water (9.5 mL per insert) was added and the dispersion was stirred overnight. In the next day, the dispersion was filtered through a 0.45 μm cellulose acetate and the amount of dorzolamide was quantified in the filtrated. This method was validated in agreement with ICH guidelines (ICH Harmonized Tripartite Guideline, 2005). A standard dorzolamide solution in PBS (pH 7.4), at a concentration range of 5–40 μg·mL^{-1}, was used ($y = 0.403568x + 0.0277201$, $r^2 = 0.9990$; $n = 5$).

2.3.5. In vitro drug release

In vitro dorzolamide release was assessed using the Franz cell system, as described before (Foureaux et al., 2015; Franca et al., 2014). A cellulose acetate membrane (0.45 μm pores) was employed to split the insert compartment and the compartment containing the receptor liquid (PBS; 6.0 mL). Glass cells were incubated at 37.0 ± 0.5 °C. At appropriate time intervals, PBS was collected from the respective compartment and, to maintain a constant volume, an equal volume was added. Dorzolamide release was determined by UV spectroscopy ($n = 5$). PI was used as negative control. One insert was placed in each Franz cell and an effort was made to ensure that the receptor liquid was in contact with the cellulose acetate membrane and hydrated the insert along the assay.

2.4. Radiolabeling of dorzolamide

The radiolabeling of dorzolamide and the radiochemical quality control were standardized based on previous works (Fuscaldi et al., 2015, 2014). First, dorzolamide hydrochloride (11 mg) was added to a sealed vial and dissolved in 500 μL of PBS (pH 7.4). Aliquots containing 30 μL of $SnCl_2{\cdot}2H_2O$ solution in 0.25 N HCl (1 mg·mL^{-1}) and 30 μL of $NaBH_4$ solution in 0.1 N NaOH (5 mg·mL^{-1}) were used as reducing agents. Next, an aliquot of 370 MBq of $Na^{99m}TcO_4$ (q.s. ad = 1 mL) was added to the vial and the solution was kept at room temperature for 5 min. Radiochemical yield was determined by thin-layer chromatography on silica gel (TLC-SG) strips (Merck, Darmstadt, Germany), using a two-solvent system: methyl ethyl ketone (MEK) and saline to determine the amount of free technetium ($^{99m}TcO_4^-$) and hydrolysed technetium ($^{99m}TcO_2$), respectively. This ^{99m}Tc-dorzolamide solution was used to prepare radiolabeled DI as described above.

2.5. In vivo studies

2.5.1. Animals

Male Wistar rats, weighing between 180 and 220 g, were supplied by the vivarium of Faculty of Pharmacy of Federal University of Minas Gerais (Belo Horizonte, Brazil). Chow and water were offered *ad libitum* to animals, which were maintained in a housing suite with controlled temperature (22–23 °C) and regulated light–dark cycle (12:12 h). All *in vivo* experiments were performed according to the local Ethics Committee for Animal Experiments of Federal University of Minas Gerais (CETEA/UFMG), in agreement with the statement of Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research.

2.5.2. Scintigraphic images and ex vivo biodistribution studies

Scintigraphic images and biodistribution assay were conducted as described elsewhere (Fuscaldi et al., 2015, 2014), with some modifications. Eye drops or inserts, containing 18.5 MBq of ^{99m}Tc-dorzolamide, were administered into the right eye in healthy male Wistar rats ($n = 5$). At 0.5, 4, and 18 h after administration, animals were anesthetized by intramuscular injection of ketamine (70 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}) and, then, placed in prone position under a gamma camera (Mediso, Hungary), employing a low-energy high-resolution (LEHR) collimator. Images were acquired using a 256 × 256 × 16 matrix size with a 20% energy window set at 140 keV for a period of 10 min.

Ex vivo biodistribution study was performed after scintigraphic imaging acquisition at 18 h post-administration. Rats, still anesthetized, were euthanized and the organs and tissues of interest were removed and weighed. Thereafter, their associated radioactivities were determined in an automatic gamma counter (Wizard, Finland). In this study, we analysed blood, stomach, small and large intestines, spleen, liver, kidneys and the eyes. Results were expressed as the percentage of radioactivity per gram of tissue (% cpm·g^{-1}).

2.5.3. In vivo efficacy

The glaucoma animal model used in this study was developed as described in the literature (Moreno et al., 2005). Briefly, male Wistar rats were anesthetized by intramuscular injection of ketamine (70 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}) and, then, two drops of a local anesthetic (0.4% benoxinate hydrochloride) were instilled directly on the cornea. An aliquot of 30 μL of hyaluronic acid (HA) (10 mg·mL^{-1}) was injected into the anterior chamber of the right eye. Injection was performed through the clear cornea, close to the corneoscleral limbus, using a hypodermic needle (22 gauge). Administrations of HA were conducted once a week, for 5 weeks, in the same calendar day and time. The left contralateral eye received no administration, being used as control. Evaluation of IOP (Moreno et al., 2005) and mean arterial pressure (MAP) (Hartman et al., 2012) were carried out at one day before the following HA injection.

Treatments were conducted after ocular hypertension establishment, immediately after the second HA injection. Before administration, DI and PI were hydrated with saline for 30 s. Only one DI was placed in both right (sick eye) and left (healthy eye) conjunctival sac. A commercial dorzolamide formulation, Trusopt™, was used as positive control of treatment. In this case, animals were treated daily, for two weeks, starting immediately after the second induction. Untreated and PI administered animals were used as negative controls of treatment. Five animals were used per group.

For IOP measurements, a topical anesthetic (0.4% benoxinate hydrochloride) was instilled to both eyes. An applanation tonometer TonoPen XL (Mentor, Norwell, MA, USA), previously calibrated, was used. Three IOP readings (with standard error less than 10%) were acquired in each eye of non-sedated animals ($n = 5$) and their average was considered the corresponding value of IOP (Moreno et al., 2005). To correct diurnal IOP variations, measurements were performed at the same time (between 11:00 AM and 12:00 PM) each day or week. The tonometrist was disguised to the treatment and an assistant performed the randomization process.

MAP was measured by the tail-cuff method, which is a noninvasive computerized system (Kent Scientific Corporation, Torrington, CT, USA) for blood pressure measurement (Hartman et al., 2012). Rats (n = 5) were acclimated to restraint and tail-cuff inflation at one day before the beginning of the assays. For each session, animals were placed in an acrylic box restraint (kept at 32–34 °C) and their tails were involved by the compression cuff that measured blood pressure 15 times to calculate the average.

For histological analysis, animals were anesthetized and euthanized. Then, both eyes were enucleated, and two small sagittal sections were made in the nasal and temporal sides. Sections were immediately immersed in Bouin's fluid for approximately 24 h and, then, they were dehydrated in increasing concentrations of ethanol (70, 80, 90, 95, and 100%), diaphanized in xylene, and included and embedded in Paraplast. Semi-serial 6 μm-sections (60 μm of interval) were obtained using a microtome (model HM335E, Microm, Minnesota, USA). Histological sections were stained with hematoxylin-eosin (HE) for for counting of the cells in the RGC layer.

2.6. Statistical analyses

Quantitative data were represented as "mean ± standard deviation (SD)" and assessed using the GraphPad Prism 5.0 software. Means of two groups were compared by unpaired Student *t* test. Means of three or more groups were compared using one-way Analysis of Variance (ANOVA), followed by Newman-Keuls test for comparison two-to-two. p-values < 0.05 were considered significant.

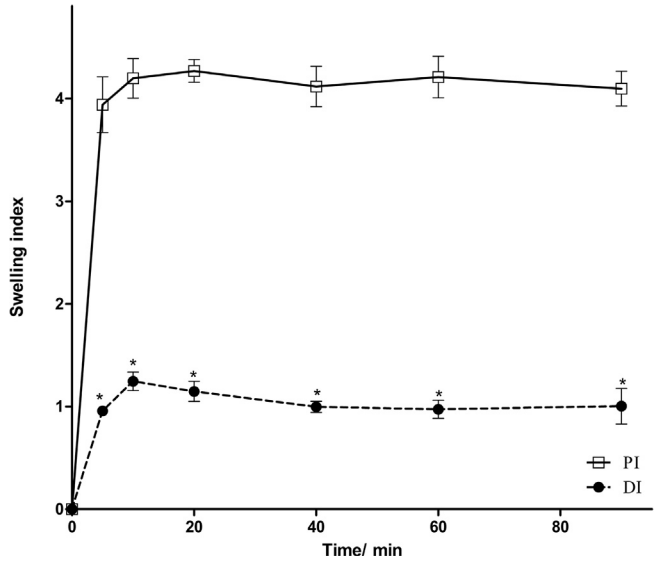

Fig. 1. Swelling index of inserts in PBS (pH 7.4). Data are represented as mean ± SD (*p < 0.05). PI: placebo inserts; DI: dorzolamide inserts. Hydrogen bonding between the polymeric matrix and dorzolamide reduced the swelling index of the formulation.

3. Results

3.1. Characterization studies

DI were produced as uniform flexible circular transparent yellowish membranes. As described in Section 2.2, the films were produced as rouded 5 mm diameter pieces and, for *in vivo* studies, they were cut in pieces of 1 × 2 mm. The thickness of the inserts was determined by scanning electron microscopy (40–70 μm thickness, data not shown). UV spectroscopy showed that each insert contained 596.32 ± 21.66 μg of dorzolamide.

The swelling indexes of the inserts are shown in Fig. 1. Both PI and

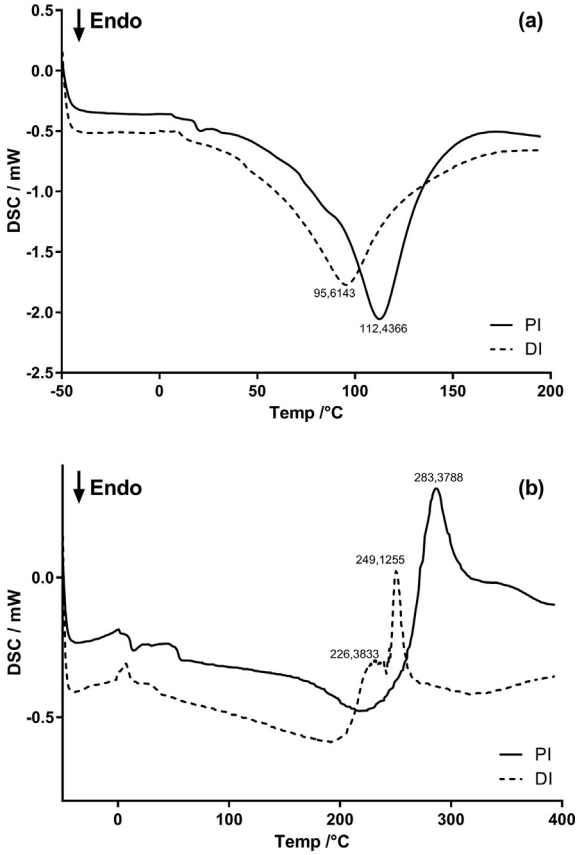

Fig. 3. Differential scanning calorimetry (DSC) curves of placebo inserts (PI) and dorzolamide inserts (DI): runs 1 (a) and 2 (b). Dorzolamide dismembered the degradation peak of the main polymeric chains and lowered the temperature in which it happens.

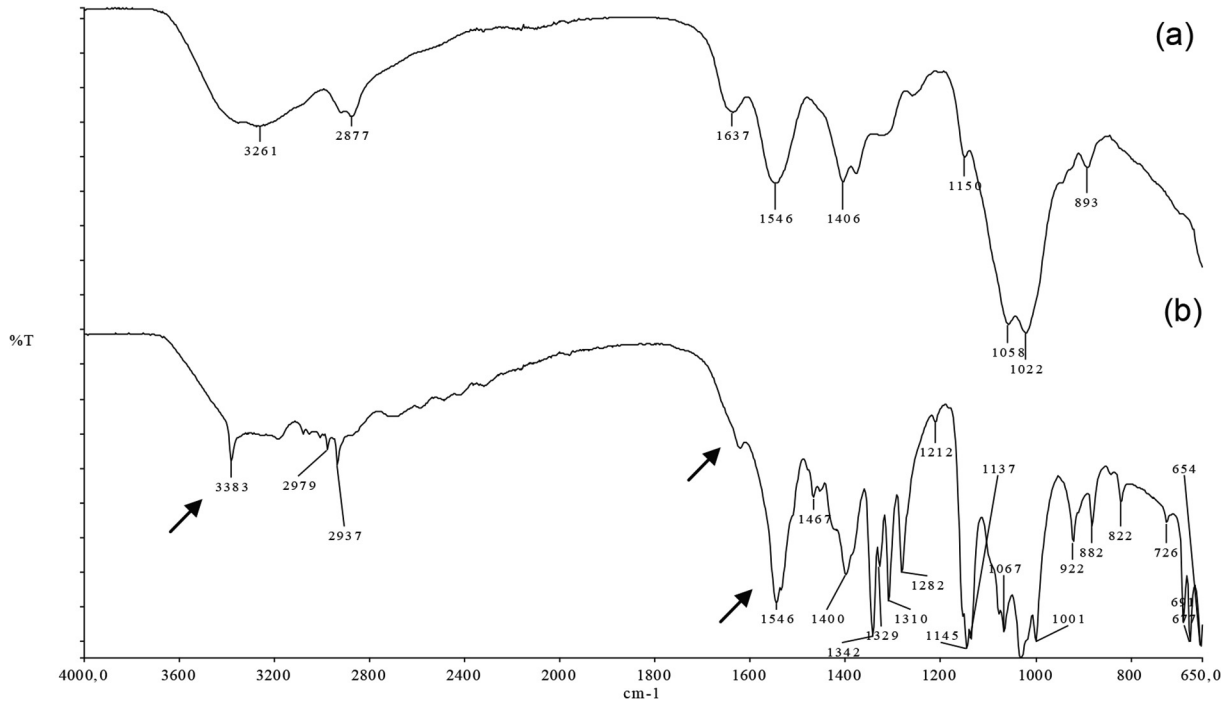

Fig. 2. ATR-FTIR spectra of (a) placebo and (b) dorzolamide inserts. Dorzolamide first band was shifted to a higher frequency (from 3372 cm^{-1} to 3383 cm^{-1}), while amide I (1637 cm^{-1}) and amide II (1546 cm^{-1}) chitosan bands decreased and increased, respectively (black arrows).

DI hydrated very quickly, but it was observed that the presence of dorzolamide decreased water uptake by the insert. The Fig. 2 depicts the ATR-FTIR spectra of the PI and DI. In the DI spectrum, the characteristic band of the dorzolamide, i.e. N–H bonds of secondary amide, shifted from 3372 cm^{-1} (Papadimitriou et al., 2008) to 3383 cm^{-1}. Other characteristic bands of the dorzolamide [NH_2^+ stretching at 2900 cm^{-1}; SO_2 asymmetric stretching (sulphone and sulphonamide) at 1342 cm^{-1} and 1310 cm^{-1}; SO_2 symmetric stretching (sulphone and sulphonamide) at 1145 cm^{-1} and 1137 cm^{-1} and the thin bands at 1400 cm^{-1} and 650 cm^{-1}) were also (Natu et al., 2011a,b; Papadimitriou et al., 2008). In the PI spectrum, two characteristic absorption bands of chitosan were detected at 1637 cm^{-1} and 1546 cm^{-1} and they were attributed to amide I (C═O stretching) and to N–H (amine) vibration overlapped to amide II (N–H vibration), respectively. From the FTIR spectra of DI, it is possible to observe that the first band was widened. Moreover, the N–H vibration band at 1546 cm^{-1} significantly increased.

DSC curves of PI and DI are presented in Fig. 3. PI exhibited a broad endothermic peak at 112.44 °C, as well as a broad exothermic peak at 283.37 °C in the first and second scan curves, respectively. This second peak was dismembered and a significant decrease in the degradation temperature from 283.37 °C to 249.12 °C was observed when dorzolamide was added to the inserts. DI released approximately 75% of the drug during a 3-h period (Fig. 4).

3.2. Scintigraphic images and ex vivo biodistribution studies

The radiolabeling yield of ^{99m}Tc-dorzolamide was over 93%, which is suitable to perform scintigraphic images with high quality. Images examination revealed that ^{99m}Tc-dorzolamide remained in the right eye of the rats at leats for 4 h after both eye drops and inserts administration. However, scintigraphic images obtained at 18 h post-administration revelead that a portion of ^{99m}Tc-dorzolamide from eye drops was drained to the body (mainly to the abdominal region), while ^{99m}Tc-dorzolamide from inserts basically remained in the eye (Fig. 5).

In order to quantify the extent of drainage and to identify the destination of ^{99m}Tc-dorzolamide, *ex vivo* biodistribution studies were performed (Fig. 6). At 18 h post-administration, 30.0 ± 5.4% of ^{99m}Tc-dorzolamide from eye drops and 53.6 ± 17.2% of ^{99m}Tc-dorzolamide from inserts remained in the site of administration (right eye). ^{99m}Tc-dorzolamide that was cleared from the eye accumulated preferentially in kidneys, stomach and large intestine.

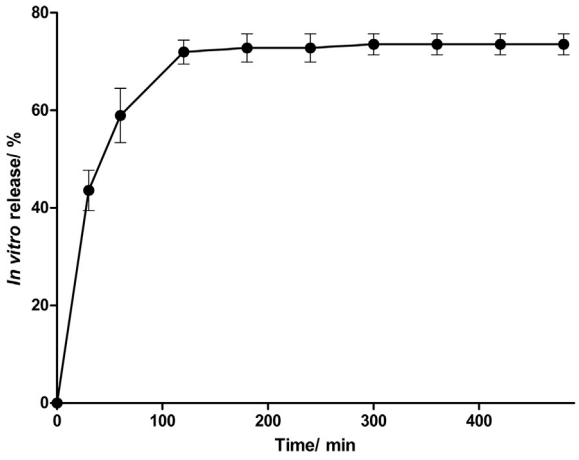

Fig. 4. Profile of *in vitro* release of dorzolamide from inserts. Data are represented as mean ± SD. Drug delivery was evaluated on Franz Cell, at 37.0 ± 0.5 °C. Receptor liquid: PBS (6.0 mL).

3.3. In vivo efficacy

The biological effectiveness of the inserts to controlled release dorzolamide was tested in an experimental model of glaucoma. Before the induction of glaucoma, the IOP of all groups was at normal levels. After the first week of glaucoma induction, it was observed a significant increase in IOP of all glaucomatous groups. No significant differences among the groups were observed ($p = 0.8848$). At this point, the treatments started. While the IOP of non-treated glaucomatous animals and of glaucomatous animals treated with PI remained high during the following four weeks, the IOP of glaucomatous animals treated with DI was significantly lowered during the following two weeks after a single insert administration. Commercial eye drops containing dorzolamide reduced the IOP during the daily treatment (two weeks) but, when the daily treatment was interrupted, the IOP increased again. No significant changes in the IOP were induced by the treatment of non-glaucomatous animals with DI or with eye drops containing dorzolamide (Fig. 7). The anti-glaucomatous effects of the DI did not affect the MAP (Fig. 8).

The effect of DI in lowering IOP resulted in preservation of the cells in the RGC layer. Fig. 9 **I** presents representative histological images of retina showing the effects of DI in number of cells in the RGC layer counting. As shown in Fig. 9 **II**, we noticed a large reduction in the number of cells in the RGC layer of all glaucomatous animals, except the ones treated with DI (glaucomatous non-treated group: 405 ± 11 cells; glaucomatous PI group: 420 ± 35 cells; glaucomatous dorzolamide eye drops group: 400 ± 34 cells, glaucomatous DI group: 490 ± 66 cells, control group: 485 ± 21 cells). Again, no significant changes were induced by DI in non-glaucomatous animals. These data indicate that controlled release of dorzolamide induced a neuroprotection of the cells that are present in the RGC layer.

During the *in vivo* tests, eventual eye irritation caused by the inserts was evaluated. In all experiments the inserts were well tolerated. No symptoms of ocular lesions, such as tearing, redness, edema and inflammation were noticed. Likewise, no anterior chamber inflammation or corneal changes were observed. Ocular surface structures and intraocular tissues were normal. Furthermore, fluorescein staining did not indicate corneal or conjunctival ulcerations (data not shown).

4. Discussion

Glaucoma is a chronic eye disease that requires long-term treatment in order to prevent progressive and irreversible blindness. In most glaucoma patients, medical therapy consists of topical eye drops or oral tablets (Nuzzi and Tridico, 2017). Controlled drug delivery systems offer manifold advantages over conventional systems particularly in glaucoma therapy as they increase the efficiency of drug delivery by improving the release profile and reducing drug toxicity (Liu et al., 2016). Ophthalmic inserts are sterile devices placed into cul-de-sac or conjuctival sac in order to contact the bulbar conjunctiva. Those systems allow controlled sustained release of drug, leading to reduced dosing frequency and increased contact time with ocular tissue (i.e., better bioavailability) (Gooch et al., 2012). Within this context, this study sought to develop and to evaluate a chitosan and hydroxyethyl cellulose-based ocular insert for sustained release of a hydrophilic drug (dorzolamide).

Inserts were produced as thin layer films and the interaction between the drug and the polymeric matrix was investigated by swelling studies, ATR-FTIR and DSC analyses. The decrease in the swelling index caused by the addition of dorzolamide is according to the findings of Panomsuk et al. (Panomsuk et al., 1996). These authors also reported that the addition of manitol (a hydrophilic molecule) to methylcellulose matrix reduces the swelling index of the membranes and suggested that this result can be caused by the formation of hydrogen bonding between the polymeric matrix and the drugs. Therefore, it was stated that the greater number and strength of hydrogen bonding, the lower the diffusion of water molecules in the hydrated polymeric matrix

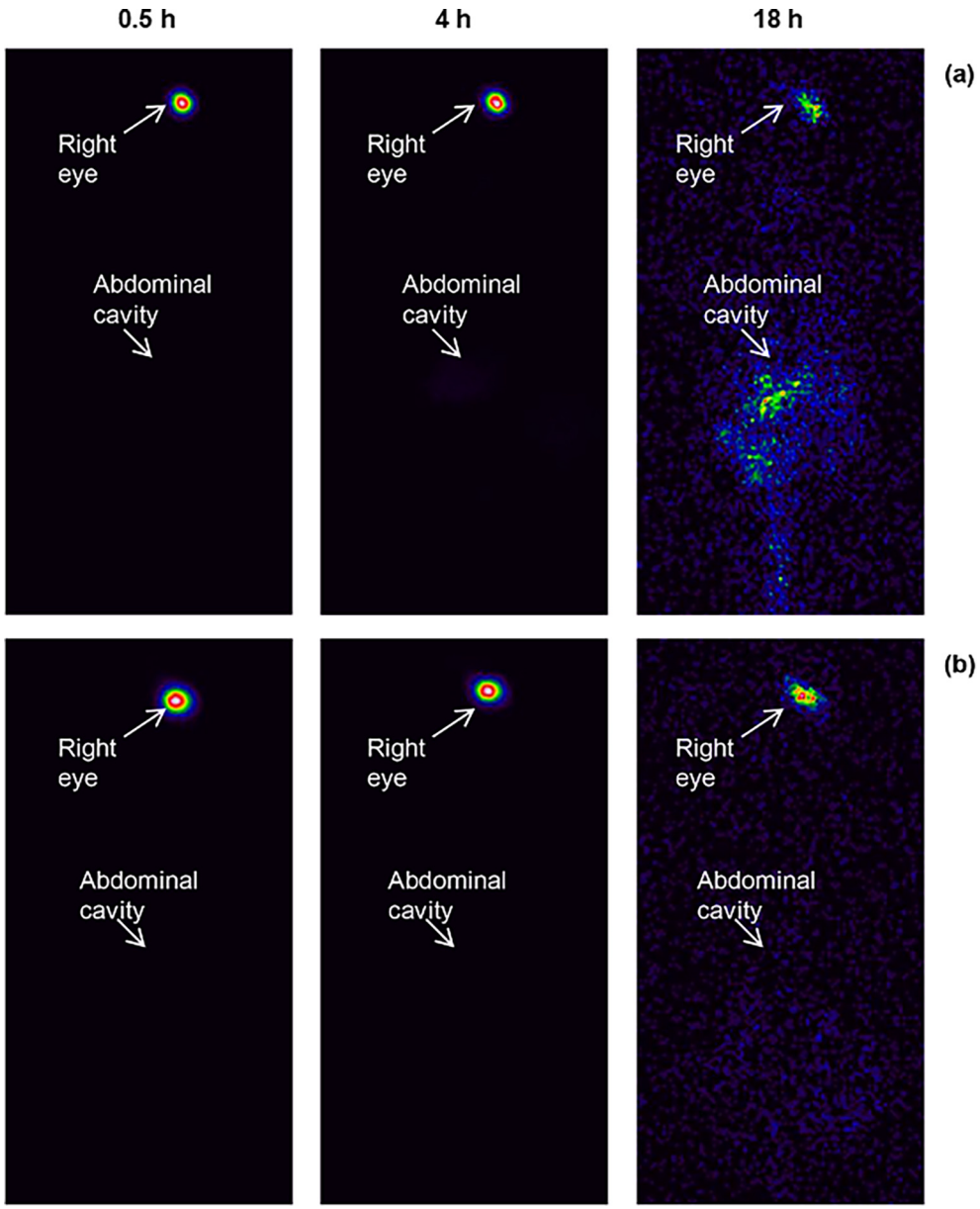

Fig. 5. Scintigraphic images of rats performed after ocular instillation of ^{99m}Tc-dorzolamide eye drops (a) and after administration of ^{99m}Tc-dorzolamide loaded inserts (b).

(Panomsuk et al., 1996), thus, lowering the speed of drug release.

In the DI spectrum, characteristic bands of N–H bonds of secondary amide (dorzolamide) shifted from 3372 cm^{-1} (Papadimitriou et al., 2008) to 3383 cm^{-1}, suggesting, again, a hydrogen bonding interaction between the drug and the matrix, thereby confirming the swelling tests. Moreover, characteristic dorzolamide bands, at 1589 cm^{-1} and 1534 cm^{-1} (C═C stretching) (Papadimitriou et al., 2008), were not identified in the DI spectrum, but it was observed that the band at 1546 cm^{-1} (N–H vibration) significantly increased, while chitosan band at 1634 cm^{-1} (C═O stretching) lowered. These findings suggest interaction between amino and carbonyl groups of chitosan and the C═C groups of dorzolamide, as described in the literature (Papadimitriou et al., 2008; Takahashi et al., 2005; Tandale and Wagh, 2011).

From DSC curves, it is possible to notice one endothermic peak in the first run (Fig. 3**a**) and another exothermic peak in the second run (Fig. 3**b**), both irregular. The first peak (112.44 °C for PI and 95.61 °C for DI) is attributed to the evaporation of residual water and the second one (283.38 °C for PI and 226.38/249.13 °C for DI) is a result of the degradation of the main polymeric chains (Rodrigues et al., 2009). The degradation peak of the polymeric chains was dismembered and a decrease in the degradation temperature was observed when dorzolamide was added to the inserts. These data corroborate with those findings from both ATR-FTIR and swelling studies, which indicated an interaction between the polymeric matrix and the drug. Moreover, melting point of dorzolamide (endothermic peak at 279 °C) (Katiyar et al., 2014) was not identified, suggesting that dorzolamide was molecularly dispersed in the polymeric matrix.

The ability of the formulation to sustained release dorzolamide was evaluated by *in vitro* drug release, scintigraphic images, *ex vivo* biodistribution studies and *in vivo* effectiveness studies. From *in vitro* sustained release profile, it was noticed that about 75% of dorzolamide was released at the first 3 h. Nevertheless, scintigraphic images showed that, at 0.5 and 4 h post-administration, ^{99m}Tc-dorzolamide remained in the eye even after eye drops instillation. Thus, late images were taken (at 18 h post-administration) and it was observed that most of

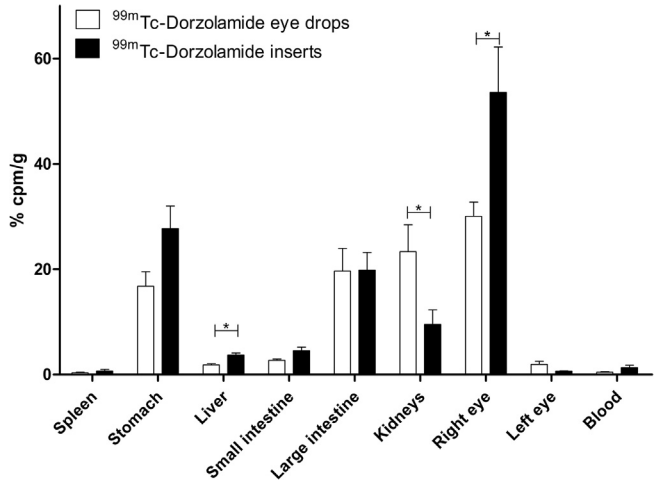

Fig. 6. Biodistribution profile in rats obtained at 18 h after ocular instillation of ^{99m}Tc-dorzolamide eye drops and after ocular administration of ^{99m}Tc-dorzolamide loaded inserts. Data are expressed as the percentage of radioactivity per gram of tissue (%cpm/g) and are represented as mean ± SD (*$p < 0.05$).

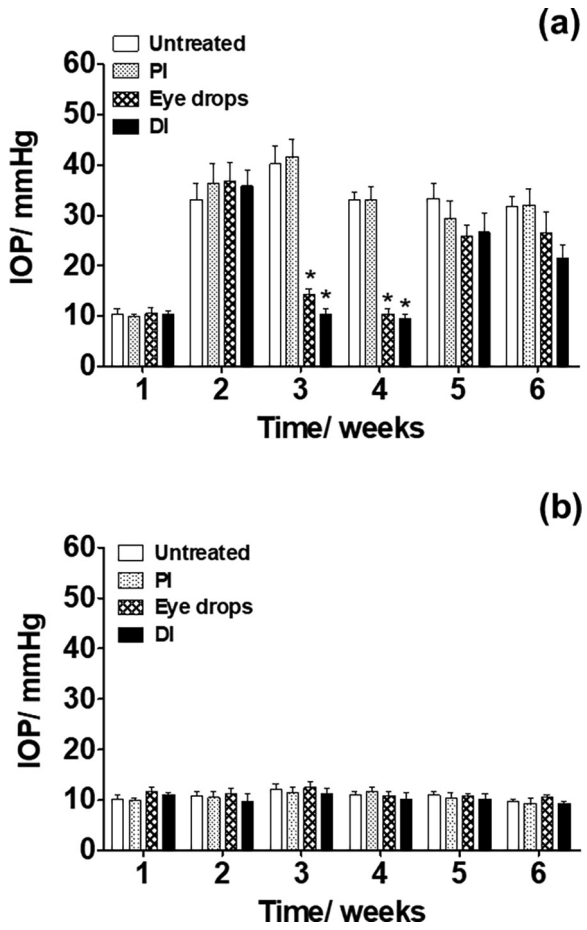

Fig. 7. Effects of dorzolamide inserts (DI) administration on intraocular pressure (IOP) of glaucomatous (a) and non-glaucomatous (b) groups. Treatments were initiated after confirmation of IOP elevation, i.e. after the second week. Data are represented as mean ± SD (*$p < 0.01$ vs. untreated). PI: placebo inserts.

radiolabeled dorzolamide from eye drops was cleared from the eye, while polymeric inserts enhanced precorneal residence time of dorzolamide. *Ex vivo* biodistribution studies were in agreement with scintigraphic images since the results showed that more than 50% of ^{99m}Tc-

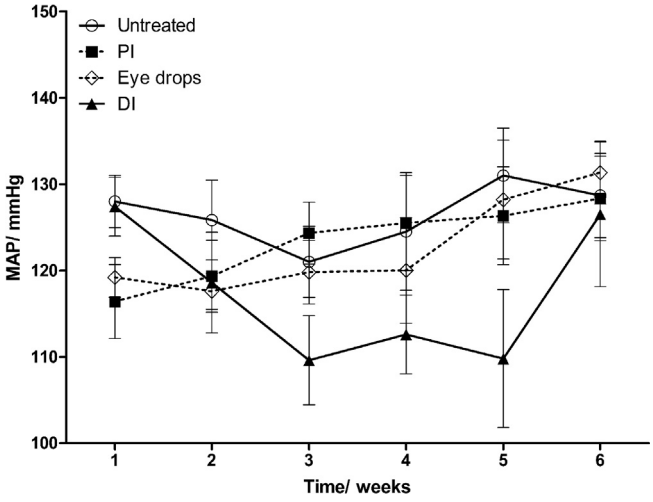

Fig. 8. Effects of dorzolamide inserts (DI) administration on mean arterial pressure (MAP). Data are represented as mean ± SD. PI: placebo inserts.

dorzolamide loaded in the inserts remained in the eye after 18 h. Likewise, when the DI was used to treat animals with glaucoma, it was able to reduce the IOP for up two weeks after a single administration, therefore increasing the time of action of the drug. These different profiles, obtained by *in vitro* and *in vivo* assays, are in accordance with our previous findings (Foureaux et al., 2015; Franca et al., 2014) and can be justified by an experimental limitation. In Franz cell system, inserts are constantly hydrated, and this hydration promotes fast drug release, by swelling. In the eye, as we have a limited volume of liquid (tears), the hydration is lowered and, then, the drug is released more slowly than *in vitro*.

The enhancement of the precorneal retention time of dorzolamide can be attributed to mucoadhesive properties of chitosan and, also, to the good interaction between the drug and the polymeric matrix. It has already been proved that chitosan, when delivered in the eye, shows a prolonged precorneal residence time (Kuntner et al., 2011; Yuan et al., 2006). In the present study, we demonstrated that, due to physico-chemical interaction between the drug and the polymeric matrix, the matrix was able to enhance precorneal residence time of dorzolamide. The ability of polymeric matrixes in enhancing pre-corneal residence time was also similarly reported by Gupta et al., when they evaluated PLGA nanoparticles entrapping ^{99m}Tc-sparfloxacin (Gupta et al., 2010) or ^{99m}Tc-levofloxacin (Gupta et al., 2011). Similar results were achieved by Wilson et al., when evaluating PVA inserts entrapping ^{99m}Tc-DTPA (Wilson et al., 1998). The preferable site of ^{99m}Tc-dorzolamide accumulation, released from eye drops, was the kidneys, which are the most important way of elimination of dorzolamide since it is a high hydrophilic drug.

In keeping with our findings, Papadimitriou et al. (2008) also obtained *in vitro* sustained release of the dorzolamide, when the drug was loaded in chitosan nanoparticles, but this formulation was not tested *in vivo*. Wadhwa et al. (2009) added hyaluronic acid to chitosan polymeric nanoparticles and only 20% of dorzolamide was released *in vitro* during 24 h. However, when this formulation was tested in non glaucomatous rabbits, it was able to decrease the IOP for only 72 h. Here, we added hydroxyethyl cellulose to the polymeric matrix, in order to lower the speed of drug delivery and we showed that these inserts were more efficient than those ones, since the IOP was lowered for a longer period (2 weeks). Also, it is important to notice that the procedure used to prepare the inserts was easier than that one used to produce nanoparticles.

Natu et al. developed polymeric inserts of poly (ε-caprolactone) and poly (ethylene oxide)-b-poly-(propylene oxide)-b-poly-(ethylene oxide) as dorzolamide carriers, which were able to sustainable release of the

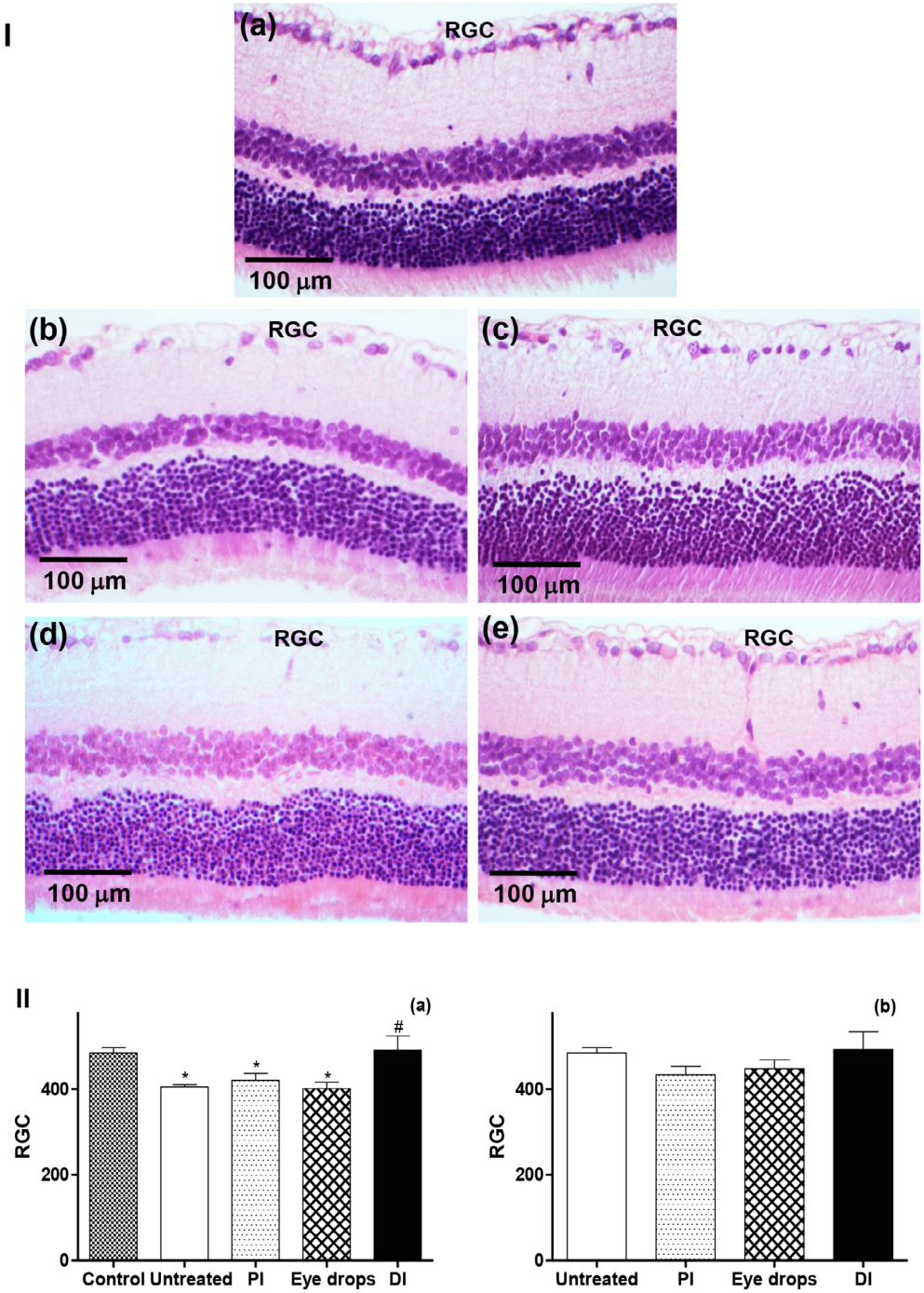

Fig. 9. Effects of dorzolamide inserts (DI) administration on retinal ganglion cells (RGC) counting evaluated by histological analysis. **(I)** Representative photomicrographs of retinas showing the higher number of RGC in non-glaucomatous animals (a) compared to non-treated glaucomatous rats (b), placebo insert (PI) treated glaucomatous rats (c) and dorzolamide eye drops treated glaucomatous rats (d), and the beneficial effects of DI in this parameter (e). **(II)** Quantification of RGC in retinas of glaucomatous (a) and non-glaucomatous (b) groups. Data are represented as mean ± SD (*$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. untreated glaucomatous rats).

drug for 20 days (Natu et al., 2011a,b) and to decrease the IOP of normal and glaucomatous rabbits during 3 months (Natu et al., 2011a,b). However, those inserts are surgically implanted in the ocular conjunctiva and this procedure caused conjunctivitis in approximately 9% of the treated animals (Natu et al., 2011a,b). On the other hand, the inserts developed by us were able to *in vivo* sustainable release of dorzolamide lowering the IOP for a relatively long period even being applied topically and without causing serious damages to the eyes.

It is important to notice that DI not only was able to reduce the IOP of glaucomatous rats, but also showed a neuroprotective effect, which was demonstrated by the reduction of the death of the cells present in the RGC layer. In previous study we have demonstrated that apoptosis participate in this process (Foureaux et al., 2013). Although, both eye drops and DI groups had the IOP lowered by the same period (two weeks), reduction in the cells of the RGC layer was observed only in rats treated with eye drops. This result could be explained by the difference between the drug release systems evaluated. Since eye drops released the drug immediately, the drug concentration in the eye lowered during the day, which likely resulted in transient increments of the IOP, leading to cell death in the RGC layer. In a sustained release formulation, the amount of the drug released in the eye is constant. Thus, transient increments in the IOP during the day do not happen and the cells of the RGC layer are protected. It is not well established if the elevation or fluctuation of the IOP is the most important risk factor for development and progression of glaucoma or if both factors are crucial for the development of the disease (Boland and Quigley, 2007), but it seems that both factors are important for cell death in the RGC layer.

In summary, considering all our current results, it can be concluded

that chitosan and hydroxyethyl cellulose-based ocular inserts are promising formulations to be used as sustained-release drug delivery systems of dorzolamide for glaucoma treatment. Therefore, this formulation can be considered as a novel strategy in the field of glaucoma management.

Funding sources

This work was supported by the National Council for Scientific and Technological Development (CNPq, Brazil) [grant numbers 470193/2012-3, 310347/2014-9] and the Foundation for Research Supporting of the State of Minas Gerais (FAPEMIG, Brazil) [grant number APQ-01728-14]. Coordination and Improvement of Higher-Level Personnel (CAPES, Brazil) provided fellowship for JRF.

CRediT authorship contribution statement

Juçara R. Franca: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Giselle Foureaux:** Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Leonardo L. Fuscaldi:** Investigation, Writing - original draft, Writing - review & editing. **Tatiana G. Ribeiro:** Validation, Writing - review. **Rachel O. Castilho:** Funding acquisition, Methodology, Resources, Writing - review & editing. **Maria I. Yoshida:** Investigation, Methodology, Writing - review & editing. **Valbert N. Cardoso:** Methodology, Resources, Writing - review & editing. **Simone O.A. Fernandes:** Resources, Validation, Writing - review & editing. **Sebastiao Cronemberger:** Validation, Writing - review & editing. **José Carlos Nogueira:** Methodology, Validation, Writing - review & editing. **Anderson J. Ferreira:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **André A.G. Faraco:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: J.R. Franca, A.A.G. Faraco, R.O. Castilho, S. Cronemberger, M.I. Yoshida and T. G. Ribeiro would like to inform that we submitted a patent application to the National Institute of Industrial Property (INPI/Brazil) under the number 014119992358 (29/07/2011). The application is related to several drug delivery systems developed by our research group, including the one described in this particular submission.

References

Angadi, S.C., Manjeshwar, L.S., Aminabhavi, T.M., 2010. Interpenetrating polymer network blend microspheres of chitosan and hydroxyethyl cellulose for controlled release of isoniazid. Int. J. Biol. Macromol. 47, 171–179. https://doi.org/10.1016/j.ijbiomac.2010.05.003.

Boland, M.V., Quigley, H.A., 2007. Risk factors and open-angle glaucoma: classification and application. J. Glaucoma 16, 406–418. https://doi.org/10.1097/IJG.0b013e31806540a1.

Bourges, J.L., Bloquel, C., Thomas, A., Froussart, F., Bochot, A., Azan, F., Gurny, R., BenEzra, D., Behar-Cohen, F., 2006. Intraocular implants for extended drug delivery: therapeutic applications. Adv. Drug Deliv. Rev. 58, 1182–1202. https://doi.org/10.1016/j.addr.2006.07.026.

Caramella, C., Ferrari, F., Bonferoni, M.C., Rossi, S., Sandri, G., 2010. Chitosan and its derivatives as drug penetration enhancers. J. Drug Deliv. Sci. Technol. 20, 5–13. https://doi.org/10.1016/S1773-2247(10)50001-7.

Davis, B.M., Crawley, L., Pahlitzsch, M., Javaid, F., Cordeiro, M.F., 2016. Glaucoma: the retina and beyond. Acta Neuropathol. 132, 807–826. https://doi.org/10.1007/s00401-016-1609-2.

Dobaria, N.B., Badhan, A.C., Mashru, R.C., 2009. A novel itraconazole bioadhesive film for vaginal delivery: design, optimization, and physicodynamic characterization. AAPS PharmSciTech 10, 951. https://doi.org/10.1208/s12249-009-9288-0.

Eroğlu, H., Sargon, M.F., Öner, L., 2007. Chitosan formulations for steroid delivery: effect of formulation variables on in vitro characteristics. Drug Dev. Ind. Pharm. 33, 265–271. https://doi.org/10.1080/03639040600713134.

Foureaux, G., Franca, J.R., Nogueira, J.C., Fulgêncio, G. de O., Ribeiro, T.G., Castilho, R.O., Yoshida, M.I., Fuscaldi, L.L., Fernandes, S.O.A., Cardoso, V.N., Cronemberger, S., Faraco, A.A.G., Ferreira, A.J., 2015. Ocular inserts for sustained release of the angiotensin-converting enzyme 2 activator, diminazene aceturate, to treat glaucoma in rats. PLoS One 10, e0133149. https://doi.org/10.1371/journal.pone.0133149.

Foureaux, G., Nogueira, J.C., Nogueira, B.S., Fulgêncio, G.O., Menezes, G.B., Fernandes, S.O.A., Cardoso, V.N., Fernandes, R.S., Oliveira, G.P., Franca, J.R., Faraco, A.A.G., Raizada, M.K., Ferreira, A.J., 2013. Antiglaucomatous effects of the activation of intrinsic angiotensin-converting enzyme 2. Investig. Opthalmol. Vis. Sci. 54, 4296. https://doi.org/10.1167/iovs.12-11427.

Franca, J.R., Foureaux, G., Fuscaldi, L.L., Ribeiro, T.G., Rodrigues, L.B., Bravo, R., Castilho, R.O., Yoshida, M.I., Cardoso, V.N., Fernandes, S.O., Cronemberger, S., Ferreira, A.J., Faraco, A.A.G., 2014. Bimatoprost-loaded ocular inserts as sustained release drug delivery systems for glaucoma treatment: in vitro and in vivo evaluation. PLoS One 9, e95461. https://doi.org/10.1371/journal.pone.0095461.

Fuscaldi, L.L., de Barros, A.L.B., Santos, C.R. de P., de Oliveira, M.C., Fernandes, S.O.A., Cardoso, V.N., 2015. Feasibility of the 99mTc-HYNIC-βAla-Bombesin(7–14) for detection of LNCaP prostate tumour in experimental model. J. Radioanal. Nucl. Chem. 305, 379–386. https://doi.org/10.1007/s10967-015-4030-8.

Fuscaldi, L.L., de Barros, A.L.B., Santos, C.R. de P., de Souza, C.M., Cassali, G.D., de Oliveira, M.C., Fernandes, S.O.A., Cardoso, V.N., 2014. Evaluation of the optimal LNCaP prostate tumour developmental stage to be assessed by 99mTc-HYNIC-βAla-Bombesin(7–14) in an experimental model. J. Radioanal. Nucl. Chem. 300, 801–807. https://doi.org/10.1007/s10967-014-3040-2.

George, M., Abraham, T.E., 2006. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan — a review. J. Control. Release 114, 1–14. https://doi.org/10.1016/J.JCONREL.2006.04.017.

Gooch, N., Molokhia, S.A., Condie, R., Burr, R.M., Archer, B., Ambati, B.K., Wirostko, B., 2012. Ocular drug delivery for glaucoma management. Pharmaceutics 4, 197–211. https://doi.org/10.3390/pharmaceutics4010197.

Gulsen, D., Chauhan, A., 2005. Dispersion of microemulsion drops in HEMA hydrogel: a potential ophthalmic drug delivery vehicle. Int. J. Pharm. 292, 95–117. https://doi.org/10.1016/j.ijpharm.2004.11.033.

Gupta, H., Aqil, M., Khar, R.K., Ali, A., Bhatnagar, A., Mittal, G., 2011. Biodegradable levofloxacin nanoparticles for sustained ocular drug delivery. J. Drug Target. 19, 409–417. https://doi.org/10.3109/1061186X.2010.504268.

Gupta, H., Aqil, M., Khar, R.K., Ali, A., Bhatnagar, A., Mittal, G., 2010. Sparfloxacin-loaded PLGA nanoparticles for sustained ocular drug delivery. Nanomed. Nanotechn. Biol. Med. 6, 324–333. https://doi.org/10.1016/j.nano.2009.10.004.

Han, H.D., Nam, D.E., Seo, D.H., Kim, T.W., Shin, B.C., Choi, H.S., 2004. Preparation and biodegradation of thermosensitive chitosan hydrogel as a function of pH and temperature. Macromol. Res. 12, 507–511. https://doi.org/10.1007/BF03218435.

Hartman, R.E., Kamper, J.E., Goyal, R., Stewart, J.M., Longo, L.D., 2012. Motor and cognitive deficits in mice bred to have low or high blood pressure. Physiol. Behav. 105, 1092–1097. https://doi.org/10.1016/j.physbeh.2011.11.022.

ICH Harmonized Tripartite Guideline, 2005. Validation of analytical procedures: text and methodology Q2(R1).

Irimia, T., Dinu-Pîrvu, C.-E., Ghica, M.V., Lupuleasa, D., Muntean, D.-L., Udeanu, D.I., Popa, L., 2018a. Chitosan-based in situ gels for ocular delivery of therapeutics: a state-of-the-art review. Mar. Drugs 16. https://doi.org/10.3390/md16100373.

Irimia, T., Ghica, M.V., Popa, L., Anuța, V., Arsene, A.-L., Dinu-Pîrvu, C.-E., 2018b. Strategies for improving ocular drug bioavailability and corneal wound healing with chitosan-based delivery systems. Polymers (Basel) 10. https://doi.org/10.3390/polym10111221.

Katiyar, S., Pandit, J., Mondal, R.S., Mishra, A.K., Chuttani, K., Aqil, M., Ali, A., Sultana, Y., 2014. In situ gelling dorzolamide loaded chitosan nanoparticles for the treatment of glaucoma. Carbohydr. Polym. 102, 117–124. https://doi.org/10.1016/j.carbpol.2013.10.079.

Kulkarni, S.V., Damji, K.F., Buys, Y.M., 2008. Medical management of primary open-angle glaucoma: Best practices associated with enhanced patient compliance and persistency. Patient Prefer. Adherence 2, 303–314.

Kuntner, C., Wanek, T., Hoffer, M., Dangl, D., Hornof, M., Kvaternik, H., Langer, O., 2011. Radiosynthesis and assessment of ocular pharmacokinetics of 124i-labeled chitosan in rabbits using small-animal PET. Mol. Imaging Biol. 13, 222–226. https://doi.org/10.1007/s11307-010-0352-7.

Li, J., Xu, Z., 2002. Physical characterization of a chitosan-based hydrogel delivery system. J. Pharm. Sci. 91, 1669–1677. https://doi.org/10.1002/jps.10157.

Liu, D., Yang, F., Xiong, F., Gu, N., 2016. The smart drug delivery system and its clinical potential. Theranostics 6, 1306–1323. https://doi.org/10.7150/thno.14858.

Ludwig, A., 2005. The use of mucoadhesive polymers in ocular drug delivery. Adv. Drug Deliv. Rev. 57, 1595–1639. https://doi.org/10.1016/j.addr.2005.07.005.

Mincione, F., Scozzafava, A., Supuran, C.T., 2008. The development of topically acting carbonic anhydrase inhibitors as antiglaucoma agents. Curr. Pharm. Des. 14, 649–654.

Moreno, M.C., Marcos, H.J.A., Oscar Croxatto, J., Sande, P.H., Campanelli, J., Jaliffa, C.O., Benozzi, J., Rosenstein, R.E., 2005. A new experimental model of glaucoma in rats through intracameral injections of hyaluronic acid. Exp. Eye Res. 81, 71–80. https://doi.org/10.1016/j.exer.2005.01.008.

Natu, M.V., Gaspar, M.N., Fontes Ribeiro, C.A., Cabrita, A.M., de Sousa, H.C., Gil, M.H., 2011a. In vitro and in vivo evaluation of an intraocular implant for glaucoma treatment. Int. J. Pharm. 415, 73–82. https://doi.org/10.1016/J.IJPHARM.2011.05.047.

Natu, M.V., Gaspar, M.N., Ribeiro, C.A.F., Correia, I.J., Silva, D., de Sousa, H.C., Gil, M.H., 2011b. A poly(ε-caprolactone) device for sustained release of an anti-glaucoma drug. Biomed. Mater. 6, 025003. https://doi.org/10.1088/1748-6041/6/2/025003.

Nuzzi, R., Tridico, F., 2017. Glaucoma: biological trabecular and neuroretinal pathology with perspectives of therapy innovation and preventive diagnosis. Front. Neurosci. 11, 494. https://doi.org/10.3389/fnins.2017.00494.

Panomsuk, S.P., Hatamaka, T., Aiba, T., Katayama, K., Koizumi, T., 1996. A study of the hydrophilic cellulose matrix: effect of drugs on swelling properties. Chem. Pharm. Bull. (Tokyo) 44, 1039–1042. https://doi.org/10.1248/cpb.44.1039.

Papadimitriou, S., Bikiaris, D., Avgoustakis, K., Karavas, E., Georgarakis, M., 2008. Chitosan nanoparticles loaded with dorzolamide and pramipexole. Carbohydr. Polym. 73, 44–54. https://doi.org/10.1016/J.CARBPOL.2007.11.007.

Perumal, V.A., Lutchman, D., Mackraj, I., Govender, T., 2008. Formulation of monolayered films with drug and polymers of opposing solubilities. Int. J. Pharm. 358, 184–191. https://doi.org/10.1016/J.IJPHARM.2008.03.005.

Rodrigues, L.B., Leite, H.F., Yoshida, M.I., Saliba, J.B., Junior, A.S.C., Faraco, A.A.G., 2009. In vitro release and characterization of chitosan films as dexamethasone carrier. Int. J. Pharm. 368, 1–6. https://doi.org/10.1016/j.ijpharm.2008.09.047.

Sigurdsson, H.H., Stefánsson, E., Gudmundsdóttir, E., Eysteinsson, T., Thorsteinsdóttir, M., Loftsson, T., 2005. Cyclodextrin formulation of dorzolamide and its distribution in the eye after topical administration. J. Control. Release 102, 255–262. https://doi.org/10.1016/j.jconrel.2004.10.004.

Song, R., Murphy, M., Li, C., Ting, K., Soo, C., Zheng, Z., 2018. Current development of biodegradable polymeric materials for biomedical applications. Drug Des. Devel. Ther. 12, 3117–3145. https://doi.org/10.2147/DDDT.S165440.

Takahashi, H., Chen, R., Okamoto, H., Danjo, K., 2005. Acetaminophen particle design using chitosan and a spray-drying technique. Chem. Pharm. Bull. (Tokyo) 53, 37–41.

Tandale, Y.N., Wagh, V.D., 2011. Formulation and evaluation of dorzolamidehydrochloride polymeric film. Int. J. PharmTech Res. 3, 1211–1218.

Valenta, C., 2005. The use of mucoadhesive polymers in vaginal delivery. Adv. Drug Deliv. Rev. 57, 1692–1712. https://doi.org/10.1016/j.addr.2005.07.004.

Wadhwa, S., Paliwal, R., Paliwal, S.R., Vyas, S.P., 2009. Chitosan and its role in ocular therapeutics. Mini Rev. Med. Chem. 9, 1639–1647.

Ways, T.M.M., Lau, W.M., Khutoryanskiy, V.V., 2018. Chitosan and its derivatives for application in mucoadhesive drug delivery systems. Polymers (Basel) 10. https://doi.org/10.3390/polym10030267.

Wilson, C.G., Zhu, Y.P., Frier, M., Rao, L.S., Gilchrist, P., Perkins, A.C., 1998. Ocular contact time of a carbomer gel (GelTears) in humans. Br. J. Ophthalmol. 82, 1131–1134. https://doi.org/10.1136/BJO.82.10.1131.

Yuan, X., Li, H., Yuan, Y., 2006. Preparation of cholesterol-modified chitosan self-aggregated nanoparticles for delivery of drugs to ocular surface. Carbohydr. Polym. 65, 337–345. https://doi.org/10.1016/J.CARBPOL.2006.01.020.

Zambito, Y., Di Colo, G., 2010. Chitosan and its derivatives as intraocular penetration enhancers. J. Drug Deliv. Sci. Technol. 20, 45–52. https://doi.org/10.1016/S1773-2247(10)50005-4.