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DOTTORATO DI RICERCA IN
AREA DEL FARMACO E TRATTAMENTI INNOVATIVI

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Histamine H_3 receptor ligands in progressive optic neuropathy

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“The real voyage of discovery consists not in seeking new landscapes but in having new eyes”

Marcel Proust

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ABSTRACT

Glaucoma is the second leading cause of blindness worldwide. It is characterized by progressive optic nerve atrophy and loss of retinal ganglion cells (RGCs) caused by ocular hypertension (OHT). Intra-ocular pressure (IOP) reduction is the only therapeutic approach demonstrated to preserve visual function in patients affected by glaucoma. Nitric oxide (NO) plays a pivotal role in IOP homeostasis in animals and glaucomatous patients. The first line of treatment consists of topical IOP-lowering medications. A significant number of patients requires more than one medication to achieve adequate baroprotection. The target IOP reduction of 25% is not easily reached/maintained, especially at night. Long-term IOP circadian fluctuation is associated with poor prognosis. The posterior hypothalamus is involved in circadian rhythm regulation and histamine is one of the neuro-hormones implicated in the arousal system. The histaminergic tone is reduced at night, nocturnal IOP is higher than diurnal one.

The aim of this thesis was to develop a reliable *in vivo* model of glaucoma and to investigate the role of the histaminergic system in IOP modulation; to study H_3 receptor (H_3R) antagonists' hypotensive action, alone and in combination with a NO donor (ciproxifan/molsidomine both 0.5%). Hence, the long-term baroprotective effects of these drugs on ocular hemodynamics was investigated as well as oxidative stress and inflammatory response in retinal glia and microglia. Furthermore, an *in vitro* model of glaucoma was set to study and possibly understand the mechanism of action of H_3R antagonists in reducing IOP.

Transient and stable OHT models were induced by injection of 50 µl of hypertonic saline (5% NaCl in sterile water) into the vitreous or 100 µl carbomer (0.1%) in the anterior chamber of New Zealand albino rabbits' eyes. IOP measurements were performed with a Tono-Pen prior to saline or carbomer injection (baseline), 60, 120 and 240 minutes after transient OHT induction and, every day for 12 days, in stable OHT model before drug dosing. Color Doppler ultrasound examination was performed to evaluate changes in retinal artery Resistivity Index (RI) before and after repeated dosing with the test items and their NO donor (molsidomine 1%) combination. Oxidative stress was assessed by 8-hydroxy-2-deoxyguanosine (8-OH*d*G) determination using an ELISA kit and fluorescent dye dihydroethidium (DHE) staining. Morphological changes in

retinal layers were assessed on histological sections by chemical (hematoxylin/eosin) and fluorescent staining (CD11B/IBA1 and GFAP/VEGFA double labeling). RT-PCR and Western blot analysis was performed to identify respectively mRNA and protein expression of histaminergic receptors, immunofluorescence staining from retinal and ciliary body biopsies to localize them. Finally, isolated cells from trabecular meshwork were characterized, by Immunofluorescence staining and dexamethasone-induced myocilin expression (Western Blot analysis), to study H_3R antagonist's effects on intracellular Ca^{2+} concentration with calcium imaging technique.

Selective histamine H_3R antagonists Ciproxifan and GSK189254 (0.5, 1%) and DL76 (1%), a novel H_3R antagonist, lowered IOP both in transient and stable OHT in a statistically significant manner. Chronic treatment with H_3R antagonists (1%), ciproxifan/molsidomine combination (both 0.5%) and timolol (1%) improved the vascular performance of central retinal artery, especially when the animals were treated with pharmacological ciproxifan/molsidomine. All histamine receptor subtypes were localized in mammalian retinal slides and, for the first time, in the ciliary body, trabecular meshwork. H_3R signal was present in trabecular meshwork cells as well. A clear correlation between IOP increase, vascular impairment of retinal artery and hypoxia-induced inflammation (VEGFA) was found, with the consequent increase of activated microglial cells and oxidative stress. These processes could be modulated by a baroprotective strategy involving H_3R antagonism. In conclusion, these results demonstrated that the histaminergic system participates in IOP regulation and that H_3R antagonists, alone or in association with NO donors, could represent a future promising therapy for glaucoma.

It would be very interesting, in the future perspective, to investigate the mechanism of action of H_3R antagonism. The connection that exists between microglia and H_4 receptor could represent an interesting target. H_4 receptors could also play a role in glaucoma regarding neuroprotection representing a completely novel strategy to contrast RGC loss and glaucomatous blindness.

LIST OF ABBREVIATIONS:

AH: aqueous humor

α-SMA: alfa smooth muscle actin

IBA1: Ionized calcium binding adaptor molecule 1

DHE: dihydroethidium

IOP: intraocular pressure

GFAP: glial fibrillary acid protein

H: histamine

Hr: hour

HR: histamine receptor

NO: nitric oxide

Min: minutes

OHT: ocular hypertension

PBS: phosphate buffered saline

RGC: retinal ganglionic cell

RT-PCR: retro transcriptase polymerase chain reaction

VEGFA: vascular endothelial growth factor A

WB: western blot

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1. INTRODUCTION

1.1 Definition

Glaucoma is a multifactorial, degenerative disorder of the optic nerve. Progressive optic neuropathy afflicts more than 60 million people worldwide (Quigley and Broman, 2006a) and it is mainly caused by glaucoma. Glaucoma is a group of eye diseases resulting in progressive ocular neuropathy, characterized by loss of retinal ganglionic cells (RGCs) and morphological alterations of optic nerve head. It is mainly caused by excessively high intraocular pressure (IOP). This increased pressure within the eye, if untreated, can lead to a reduction of local perfusion, chronic ischemia leading to the reduction of RGCs by autophagy and apoptosis (Wang *et al.*, 2015). When the number of RGCs is no longer suitable for neuronal transmission, the visual field becomes narrower. Optic nerve damage results in progressive, permanent vision loss, starting with unnoticeable blind spots at the edges of the field of vision, progressing to tunnel vision, and then to blindness. Patients with glaucoma present to eye health professionals with varying severity along a continuum (Varma *et al.*, 2011) (Figure 1.1).

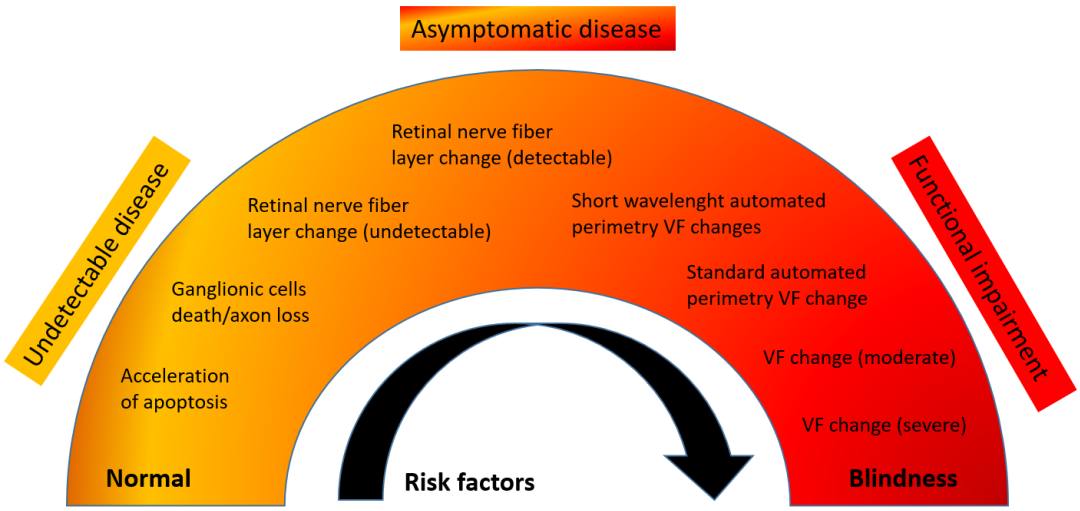


Figure 1.1. The glaucoma *continuum*. Modified from Varma *et al.*, 2011.

1.2 Classification of glaucoma

The types of glaucoma include the following:

- **Open-angle glaucoma (OAG)**
  - **Hypertensive OAG**

    The most common form of the disease. The drainage angle formed by the cornea and iris remains open, but the trabecular meshwork is partially blocked (Figure 1.2). Elevated intraocular pressure (IOP) is caused by an imbalance between production and outflow of aqueous humor (AH). The mechanical theory argues the importance of direct compression of the axonal fibers and support structures of the anterior optic nerve head by elevated IOP.

  - **Normotensive OAG**

    In normotensive glaucoma, the optic nerve head becomes damaged even though IOP is within the normal range. There is no consensus on the pathogenesis of this kind of glaucoma but the most reasonable explanation is the reduction of ocular perfusion with less oxygen and nutrients supplied to the optic nerve. This limited blood flow could be caused by atherosclerosis or other conditions that impair circulation. Recently, however, long course studies did show how IOP reduction pharmacologically or surgically obtained appears to be beneficial on visual field conservation also in normal-tension glaucoma (Oie *et al.*, 2017).

- **Angle-closure glaucoma ACG**

Also called closed-angle glaucoma, it occurs when the iris bulges forward to narrow or block the drainage angle formed by the cornea and iris. As a result, the fluid cannot circulate through the eye and pressure increases. Some people have narrow drainage angles, putting them at increased risk of angle-closure glaucoma.

Angle-closure glaucoma may occur suddenly (acute angle-closure glaucoma) or gradually (chronic angle-closure glaucoma). Acute angle glaucoma is a medical emergency. It can be triggered by sudden dilation of the pupils by mydriatic agents like atropine, tropicamide, and phenylephrine.

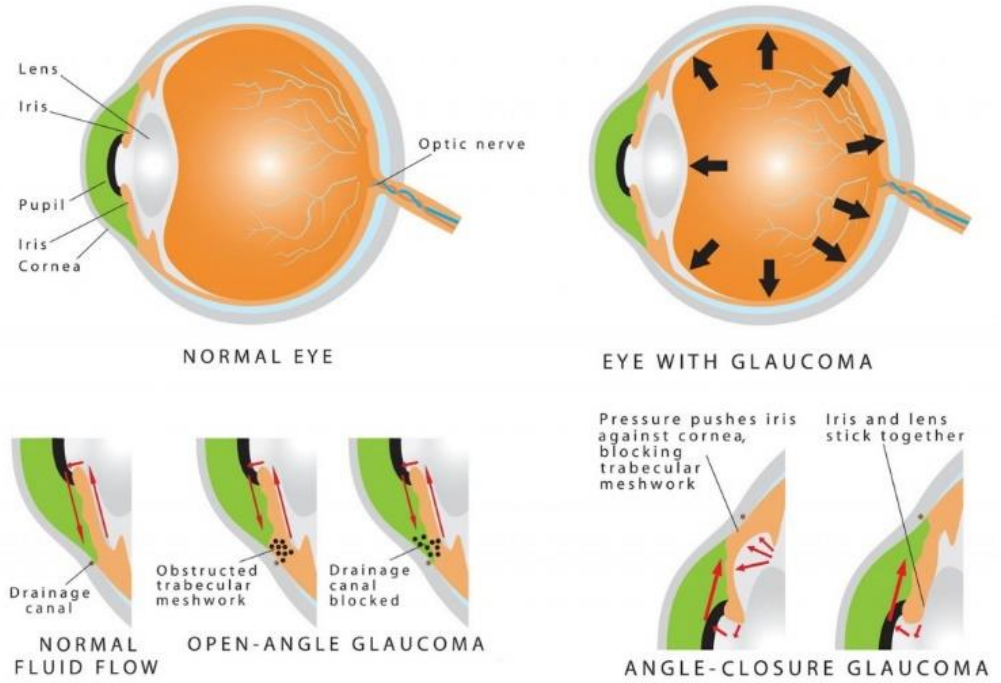


Figure 1.2. Normal eye *versus* hypertensive eye from "Glaucoma Is An Eye Disease And A Leading Cause Of Blindness", Royalty Free Cliparts, Vectors, And Stock Illustration (Image 43550697).

- **Glaucoma in children**

It is possible for infants and children to have glaucoma. It may be present from birth or develop in the first few years of life. The optic nerve damage may be caused by drainage blockages or an underlying medical condition. The most common form is due to congenital angle closure, but also a juvenile form of open angle glaucoma (JOAG) is described in the pediatric population. It is a rare condition associated with altered expression of the protein myocilin due to genetic alterations in the MYOC gene (Danford *et al.*, 2017)

- **Pigmentary glaucoma**

In pigmentary glaucoma (PG) is also a rare condition where pigment granules from the iris build up in the drainage channels, slowing AH outflow. Activities such as jogging sometimes stir up the pigment granules, depositing them on the trabecular meshwork and causing intermittent pressure elevations. Autopsy specimens of eyes with PG demonstrate disruption of the iris pigment epithelium cell membrane with extrusion of pigment granules. The trabecular meshwork in these eyes reveals collapse of trabecular sheets, extracellular free pigment granules and cellular debris clogging the inter-trabecular spaces, and macrophages and

degenerated trabecular endothelial cells filled with pigment (Jewelewicz *et al.*, 2009).

1.3 Epidemiology

Glaucoma represents a major public health problem. It is the second leading cause of blindness worldwide after cataracts. Globally, an estimated 60.5 million people (2.65% of the global population over 40) suffered from glaucoma in 2010. Of these, an estimated 44.7 million had primary open-angle glaucoma (POAG) and 15.7 million primary angle-closure glaucoma (PACG). More than 4.5 million were bilaterally blind from POAG in 2010, a number that is forecasted to rise to 5.9 million by 2020. The global prevalence of glaucoma for population aged 40 to 80 years is 3.54%. The prevalence of glaucoma is expected to reach 79.6 million in 2020, affecting all countries, although the largest increases are expected to be in China and India, which together will represent nearly 40% of cases worldwide (Varma *et al.*, 2011).

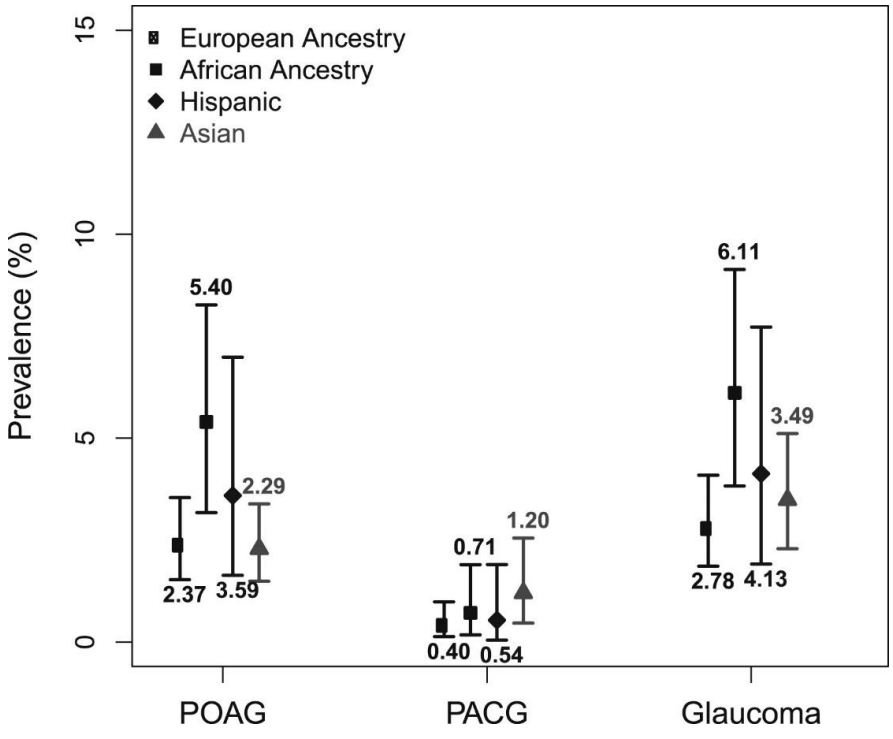


Figure 1.3. Pooled prevalence estimates of primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), and glaucoma by ethnic groups (Tham *et al.*, 2014).

The prevalence of POAG is highest in Africa, and the prevalence of PACG is highest in Asia. People of African ancestry appear to more likely to have POAG than

people of European ancestry (Figure 1.3) and people living in urban areas are more likely to have POAG than those in rural areas (Tham *et al.*, 2014).

In the United States of America, PAOG is the most common form of glaucoma and is the leading cause of irreversible blindness in African Americans, 19% of all blindness among African Americans compared to 6% in Caucasians (Quigley and Broman, 2006b). Other high-risk groups include people over 60, family members of those already diagnosed, diabetics, and people who are severely nearsighted (myopia).

The vision loss associated with glaucoma is largely irreversible. Those afflicted with the disease and living in developing countries are at a particular disadvantage; they have a higher risk of progressing to blindness, present with the more advanced disease, and often have a higher incidence of disease than developed nations (Omoti *et al.*, 2006; Vijaya *et al.*, 2014).

Everyone is at risk for glaucoma from children to senior citizens. Older people are at a higher risk for glaucoma but children can be born with glaucoma (approximately 1 out of every 10,000 babies born in the United States). Young adults can get glaucoma, too. African Americans, in particular, are susceptible at a younger age. Many risk factors are considered to be involved in the pathogenesis of glaucoma: ocular hypertension is commonly considered one of the major risk factors (Grieshaber and Flammer, 2007; Lee *et al.*, 2009).

1.4 Pathophysiology

A commonly held view is that glaucoma is a group of conditions with varying pathophysiological processes that share a common end-point of optic nerve head damage. There are multiple proposed mechanisms of damage, some of which are IOP-dependent, and others are IOP-independent. Elevation of intraocular pressure and axial shallowing of the anterior chamber from posterior pressure characterize aqueous humor misdirection. Graefe described the condition in 1869 using the phrase “malignant glaucoma” (Graefe, 1869). The precise mechanism of increased resistance to aqueous outflow remains unclear and is currently an active focus of research.

Many different abnormalities have been noted on histopathological examination of the drainage angle in patients with POAG. These include narrowed inter-trabecular spaces, thickened basement membranes, fused trabecular beams, reduction in

trabecular endothelial cell number, reduction in actin filaments, narrowing of collector channels, foreign material accumulation, scleral spur thickening, and closure of Schlemm's canal.

At the physiological level, cellular contraction/relaxation, permeability, cell stiffness, phagocytosis, extracellular matrix, cell survival and anti-oxidative activities are some of the cellular activities recognized to be important for maintaining homeostasis of AH outflow through the conventional pathway (Sabanay *et al.*, 2006; Stamer and Acott, 2012; Saccà *et al.*, 2016; Rao *et al.*, 2017)

1.4.1 Intraocular pressure related mechanisms of damage

Raised IOP is thought to damage the optic nerve head via induced mechanical changes at the *lamina cribrosa*, or via vascular dysfunction and resultant ischemia. There are several postulated mechanisms as to the cause of elevated IOP, the majority of which are related to reduced aqueous outflow. Structural changes include:

•Trabecular meshwork obstruction by foreign material (e.g. glycosaminoglycans, pigments, red blood cells)

• Trabecular cell loss of phagocytic activity and death resulting in trabecular beams fusing

• Loss of giant vacuoles from Schlemm's canal endothelium

• Reduced pore size and density in the wall of Schlemm's canal

These changes may be brought on by altered endogenous corticosteroid metabolism with secondary trabecular meshwork changes. IOP rise that can complicate the use of topical or systemic corticosteroid has been recognized for 50 years. Following isolation of the MYOC gene, it has been associated with genetic predisposition. This gene was previously known as the trabecular meshwork inducible glucocorticoid response or TIGR gene (Kersey and Broadway, 2006). Myocilin, the causal gene at the GLC1A locus (Online Mendelian Inheritance in Man [OMIM] 601652), was identified in pedigrees with juvenile open-angle glaucoma (JOAG), a term used to refer to POAG with an earlier age at onset, and an autosomal dominant mode of inheritance (Johnson *et al.*, 1993). Systemic corticosteroid treatment, especially dexamethasone, in childhood poses a risk for IOP elevation (Yamashita *et al.*, 2010).

Recently, the role of MYOC gene has been extensively studied. The sequence variations in the gene account for approximately 2% to 4% of glaucoma cases. One particular MYOC mutation, Gln368Stop (dbSNP accession number: rs74315329), is the most common genetic mutation causing glaucoma by increasing intraocular pressure (Nag *et al.*, 2017).

Lowering IOP (baroprotection) remains the only current therapeutic approach for preserving visual function in glaucoma patients.

1.4.2 Intraocular pressure independent mechanisms of damage

These include:

- Reduced ocular perfusion pressure (and hence the association with vascular diseases such as diabetes, hypertension, and migraine)
- Excitotoxicity, i.e. the damage from excessive glutamate
- Autoimmune-mediated nerve damage
- Loss of neurotrophic factors
- Failure of cellular repair mechanisms
- Abnormal autoregulation of retinal and choroidal vasculature

1.5 Available and future treatments

This disease is typically asymptomatic until advanced visual field loss occurs: therefore, it can be detected, at the right time for intervention, only with screening tests (IOP measurement with a tonometer) in prevention campaigns. Glaucoma prevention and treatment has been a major focus of international directives including the World Health Organization's Vision 2020 campaign.

At present, all treatment strategies are directed at lowering intraocular pressure. Initial treatment is usually started with topical or oral medications. However, with progressive damage, laser trabeculoplasty may be considered as an adjunctive therapy, followed by incisional glaucoma surgery, either with trabeculectomy or glaucoma drainage implant.

Glaucoma clinical trials over the past 20 years have provided critically important, evidence-based guidelines in the management of patients with glaucoma. Whether treatment is provided with medical therapy, laser, or surgery, these trials have shown

that glaucoma onset and progression can be controlled by lowering IOP, a well-established modifiable risk factor for glaucomatous optic neuropathy. IOP lowering has been found to be beneficial even in eyes with normal tension glaucoma. The Collaborative Normal-Tension Study Group found that a 30% IOP reduction dropped the rate of progression from 35% in the observation group to 12% in the treated group.

The Early Manifest Glaucoma Trial (EMGT) found that an IOP reduction by at least 25% reduced progression from 62% to 45% in the treated group compared to an untreated group (Leske *et al.*, 1999). Setting an initial target of 20-30% IOP reduction is recommended; however, it is very important to constantly reassess for optic nerve or visual field changes, and change target pressure, as needed.

The two possible strategies are to reduce aqueous humor production or to increase the outflow. The drainage of aqueous humor can be fulfilled via the conventional and unconventional pathway. The first pathway is through the trabecular meshwork, Schlemm's canal, collecting channels, and the episcleral venous system. In the second one, called the uveo-scleral pathway, aqueous humor drains through the ciliary muscle and exits through the supraciliary space and across the anterior or posterior sclera, into choroidal vessels.

The European Glaucoma Society in 2014 recommended initiating the treatment with a mono-therapy. Treatment is considered "effective" when the achieved IOP reduction on treatment is comparable to the published average range in similar population for that drug. According to a meta-analysis of randomized controlled trials, the highest reduction of IOP is obtained by prostaglandin analogs followed by non-selective β-blockers, α-adrenergic agonists, selective β-blockers and at last topical carbonic anhydrase inhibitors (van der Valk *et al.*, 2005). In figure 1.4 all the available pharmacological treatments in monotherapy or in combination are reported in a timeline (European Glaucoma Society, 2014).

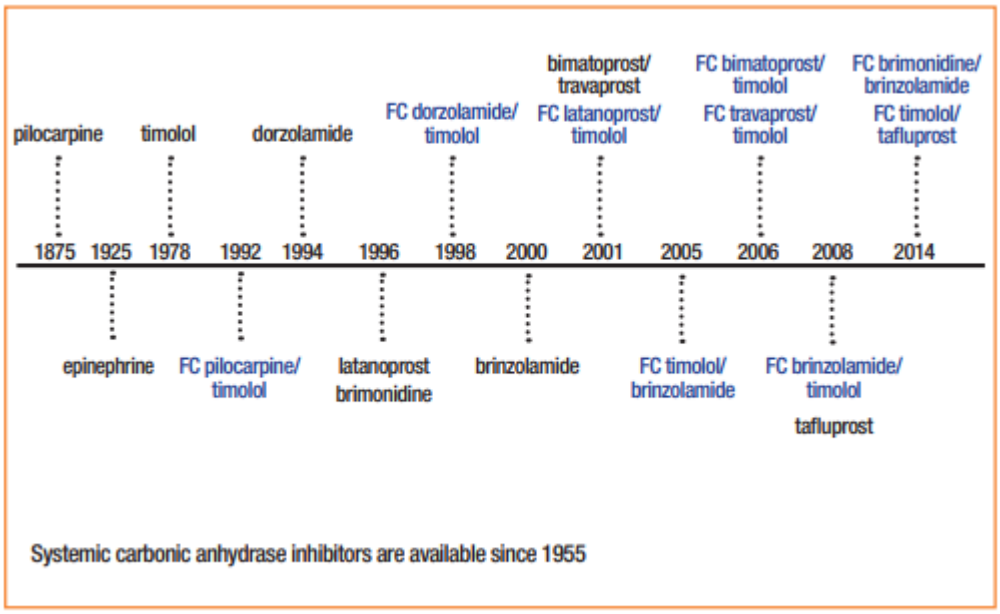


Figure 1.4. IOP lowering molecules and year of first clinical use. FC: fixed combination. In black monotherapy from European Glaucoma Society Guidelines 2017.

1.5.1 Medications that suppress aqueous humor production:

- **β-blockers** (timolol, betaxolol, carteolol, levobunolol, propranolol). They lower IOP by suppressing aqueous humor production by inhibition of synthesis of cyclic adenosine monophosphate (cAMP) in the ciliary epithelium. They decrease aqueous secretion and the most common side effect of topical β-blockers include burning and decreased corneal sensation (Arici *et al.*, 2000).

  Systemic side effects can be more severe. They include bradycardia; arrhythmia; heart failure; heart block; syncope; bronchospasm or airway obstruction (Diggory *et al.*, 1994); CNS effects (depression, weakness, fatigue, or hallucinations); impotence, and elevation of blood cholesterol levels. Diabetic patients may experience reduced glucose tolerance and hypoglycemic signs and symptoms can be masked. β-blockers may aggravate myasthenia gravis and abrupt withdrawal can exacerbate symptoms of hyperthyroidism. The β_1 selective antagonist, betaxolol, has fewer pulmonary side effects.

- **Adrenergic agonists** (epinephrine, dipivefrin) lower IOP through α_2 agonist-mediated aqueous humor production suppression and a secondary mechanism that increases aqueous outflow. Non-selective adrenergic agonists such as epinephrine lower IOP by several different mechanisms. Initially, a vasoconstrictive effect decreases aqueous production and c-AMP synthesis increases the outflow facility.

Ocular side effects include follicular conjunctivitis, burning, reactive hyperemia, adrenochrome deposits, mydriasis, and maculopathy in aphakic eyes, corneal endothelial damage, and ocular hypoxia. Systemic side effects include hypertension, tachycardia, and arrhythmia. Dipivefrin is a prodrug that is hydrolyzed to epinephrine as it traverses the cornea. It has significantly fewer systemic side effects than epinephrine. The potential side effects of nonselective adrenergic agonists have led to decline in their use.

- **Selective adrenergic agonists** (apraclonidine and brimonidine 0.1-0.2%) with the latter having much greater selectivity at the α_2 receptor. Brimonidine (0.1-0.2%) appears to increase uveoscleral outflow and lower IOP by about 26%. The side effects of selective adrenergic include contact dermatitis (40% with apraclonidine, < 15% for brimonidine, and <0.2% for brimonidine), follicular conjunctivitis, eyelid retraction, mydriasis, and conjunctival blanching.

  Systemically, they can cause a headache, dry mouth, fatigue, bradycardia, and hypotension. Long-term use of topical apraclonidine is frequently associated with allergy and tachyphylaxis. Generally, brimonidine seems to produce fewer ocular side effects than apraclonidine. Brimonidine is generally the preferred first-line drug in the first, second and early third trimester. Late in the third trimester, brimonidine should be discontinued because it can induce central nervous system depression in newborns wherein topical carbonic anhydrase inhibitors may be the optimal choice (Sethi *et al.*, 2016).
- **Carbonic Anhydrase Inhibitors** (CAIs) lower IOP by decreasing aqueous production by direct antagonist activity on the ciliary epithelial carbonic anhydrase. Over 90% of ciliary epithelial enzyme activity needs to be abolished to decrease aqueous production and lower IOP. Common side effects of topical CAIs include bitter taste, blurred vision, punctate keratopathy, and lethargy.

  Systemic CAIs include acetazolamide and methazolamide. Topical CAIs include brinzolamide 1% and dorzolamide 2%. A reduction in IOP of 14-17% is expected with these agents. Systemic CAIs are associated with numerous side effects, including transient myopia; paresthesia of the fingers, toes, and perioral area; urinary frequency; metabolic acidosis; malaise; fatigue; weight loss; depression; potassium depletion; gastrointestinal symptoms; renal calculi formation; and rarely, blood

dyscrasia. Due to the side effects of the systemic CAIs, they are most useful in acute situations or as a temporizing measure before surgical intervention.

1.5.2 Medications that increase aqueous outflow

- **Prostaglandin analogs** like $PGF_2\alpha$ synthetic analog latanoprost, bimatoprost, and travoprost lower IOP by increasing aqueous outflow through the unconventional outflow pathway or uveoscleral outflow. The exact mechanism by which prostaglandins improve uveoscleral outflow is not fully understood but may involve relaxation of the ciliary muscle and remodeling of the extracellular matrix elements of the ciliary muscle. These agents have been shown to increase the outflow by as much as 50%. Latanoprost and travoprost, and bimatoprost (prostamide) represent the newest, the most effective, and the most commonly used class of medications. Latanoprost 0.005% and travoprost 0.004% are pro-drugs that penetrate the cornea and become biologically active after being hydrolyzed by corneal esterases. Bimatoprost 0.03% decreases IOP by increasing uveoscleral outflow by 50% and increasing trabecular outflow by approximately 25-30%. Both latanoprost and travoprost reduce IOP by approximately 25-30% (Medeiros *et al.*, 2016).

  Ocular and systemic side effects such as conjunctival injection, hypertrichosis, trichiasis, hyperpigmentation of the periocular skin and hair growth around the eyes are generally being well-tolerated. These tend to be reversible with cessation of the drug. A unique side effect is increased iris pigmentation which is thought to be secondary to increased melanin content in the iris stromal melanocytes without the proliferation of cells. This tends to occur in 10-20% of blue iris within 18-24 months of initiating therapy, and 60% eyes with mixed green-brown or blue-brown iris. This side effect is much less evident with bimatoprost (Zaleski-Larsen *et al.*, 2017). Use of prostaglandin analogs and prostamides have also been associated with exacerbations of herpes keratitis, anterior uveitis, and cystoid macular edema in susceptible individuals.
- **Parasympathomimetic agonists** (pilocarpine, carbachol) lower IOP by increasing aqueous outflow related to contraction of the ciliary muscle in eyes with open angles and pupillary constriction in cases of pupillary block glaucoma. Topical cholinergic agonists such as pilocarpine cause contraction of the longitudinal ciliary muscle,

which pulls the scleral spur to tighten the trabecular meshwork, increasing the outflow of aqueous humor. This results in a 15-25% reduction in IOP. The direct agents -(pilocarpine) are cholinergic receptor agonists; the indirect agents (echothiophate iodide) inhibit cholinesterase and prolong the action of native acetylcholine. Carbachol is a mixed direct agonist/acetylcholine releasing agent.

Systemic side effects of pilocarpine are rare; however, ocular side effects are common. Ocular side effects include brow ache, induced myopia, miosis, leading to decreased dark vision, shallowing of the anterior chamber, retinal detachment, corneal endothelial toxicity, the breakdown of the blood-brain barrier, hypersensitivity or toxic reaction, cicatricial pemphigoid of the conjunctiva, and atypical band keratopathy. The indirect agents have ocular side effects that are generally more intense than those of the direct agents. In addition, indirect agents can cause iris cysts in children and cataract in adults. Finally, prolonged respiratory paralysis may occur during general anesthesia in patients who are on cholinesterase inhibitors because of their inability to metabolize paralytic agents such as succinylcholine. The use of cholinergic agents has declined in recent years with the availability of newer medications that have comparable efficacy and fewer side effects.

1.5.3 Combination medications

Fixed combination medications offer the potential advantage of increased convenience, compliance, efficacy, and cost. In the USA there are currently 2 fixed-combination medications on the market, dorzolamide hydrochloride 2% and timolol maleate ophthalmic solution 0.5% and brimonidine tartrate 0.2%, timolol maleate ophthalmic solution 0.5% and brimonidine tartrate 0.2% and brinzolamide 1%.

In Europe fixed combinations are also available, after a first line monotherapy treatment, fixed combination and non-fixed combination can be used according to the specific characteristics of each patient towards a tailored therapy. This trend is encouraged in the last guidelines published by the European Glaucoma Society (European Glaucoma Society, 2017).

The efficacy and ocular side effects for both fixed-combination medications are similar to their individual components. The efficacy and tolerability of both dorzolamide

hydrochloride-timolol maleate 0.5%/2% and brimonidine tartrate-timolol maleate 0.2%/0.5% appear to be similar to each other.

1.5.4 Hyperosmotic agents

Hyperosmotic agents such as oral glycerine and intravenous mannitol can rapidly lower IOP by decreasing vitreous volume. They do not cross the blood-ocular barrier and therefore exert oncotic pressure that dehydrates the vitreous. Side effects associated with the hyperosmotic agents can be severe and include a headache, back pain, diuresis, circulatory overload with angina, pulmonary edema, and heart failure, and CNS effects such as obtundation, seizure, and cerebral hemorrhage. Because of these potentially serious side effects, they are not used as long-term agents. They are typically used in acute situations to temporarily reduce high IOP until more definitive treatments can be rendered.

1.5.5 Hypotensive agents in the future

All these drugs are not enough, given the elevated number of therapeutic failure in IOP control. A significant number of patients are “nonresponders” to these treatments even when they are administered in combination. For these reasons and for the presence of side effects an increasing number of patients request surgical interventions (Bettin and Di Matteo, 2013).

The therapeutic options available for lowering IOP, especially as it relates to enhancement of AH outflow through the trabecular pathway, are limited. Towards addressing this challenge, bench and bedside research conducted over the course of the last decade and a half has identified the significance of inhibiting Rho kinase for lowering IOP. Rho kinase is a downstream effector of Rho GTPase signaling that regulates act myosin dynamics in numerous cell types. Studies from several laboratories have demonstrated that inhibition of Rho kinase lowers IOP via relaxation of the trabecular meshwork which enhances AH outflow. By contrast, activation of Rho GTPase/Rho kinase signaling in the trabecular outflow pathway increases IOP by altering the contractile, cell adhesive and permeability barrier characteristics of the trabecular

meshwork and Schlemm's canal tissues, and by influencing extracellular matrix production and fibrotic activity (Rao *et al.*, 2017).

Another approach is to push nitric oxide (NO) availability in the eye. NO is an endogenous messenger almost ubiquitous in the human body. It is produced, together with l-citrulline, by oxidation of l-arginine under the action of an enzyme called NO synthase (NOS). At present, three different isoforms of this enzyme are known: the endothelial NOS (eNOS, NOS-III) and neuronal NOS (*n*NOS, NOS-I) are constitutively expressed, while the third is an inducible isoform (*i*NOS, NOS-II) known to increase dramatically in certain pathological conditions and following pro-inflammatory stimuli (Moncada *et al.*, 1991).

At low concentrations (<nM) NO triggers a variety of regulatory and cytoprotective effects. By contrast, at high concentrations (>µM) it induces cellular toxicity consequent on the generation of reactive nitrogen oxide species (RNOS) or on the direct inhibition of functional biological molecules (Blangetti *et al.*, 2017).

Several classes of NO-donors have been studied as potential drugs to treat glaucoma (Fabrizi *et al.*, 2012; Impagnatiello *et al.*, 2012; Cavet *et al.*, 2014).

Recently the LUNAR study (Clinicaltrials.gov identifier: NCT01749930), a randomized, multicenter, double-masked, parallel-group, clinical trial conducted at 46 investigational sites in the United States (40), the United Kingdom (3), Germany (2), and Italy (1) between January 2013 and November 2014, highlighted the efficacy of Latanoprostene Bunod (LBN 0.024%). This drug is a novel NO-donating prostaglandin F2α analog. The compound is rapidly metabolized into latanoprost acid, a prostaglandin analog, and butanediol mononitrate, a NO-donating moiety. Latanoprostene bunod, given topically once a day in the evening, was noninferior to timolol 0.5% and, over 3 months of treatment, achieved significantly greater IOP lowering at all time points evaluated. It significantly increased the percentage of subjects with a change from baseline in IOP ≥25% in 387 subjects with open-angle glaucoma and demonstrated a good safety profile (Medeiros *et al.*, 2016).

1.6 Histaminergic system

1.6.1 Biological effects of histamine.

Histamine exerts its effects through the engagement of four G-protein coupled receptors H_1R, H_2R, H_3R, H_4R (Figure 1.5).

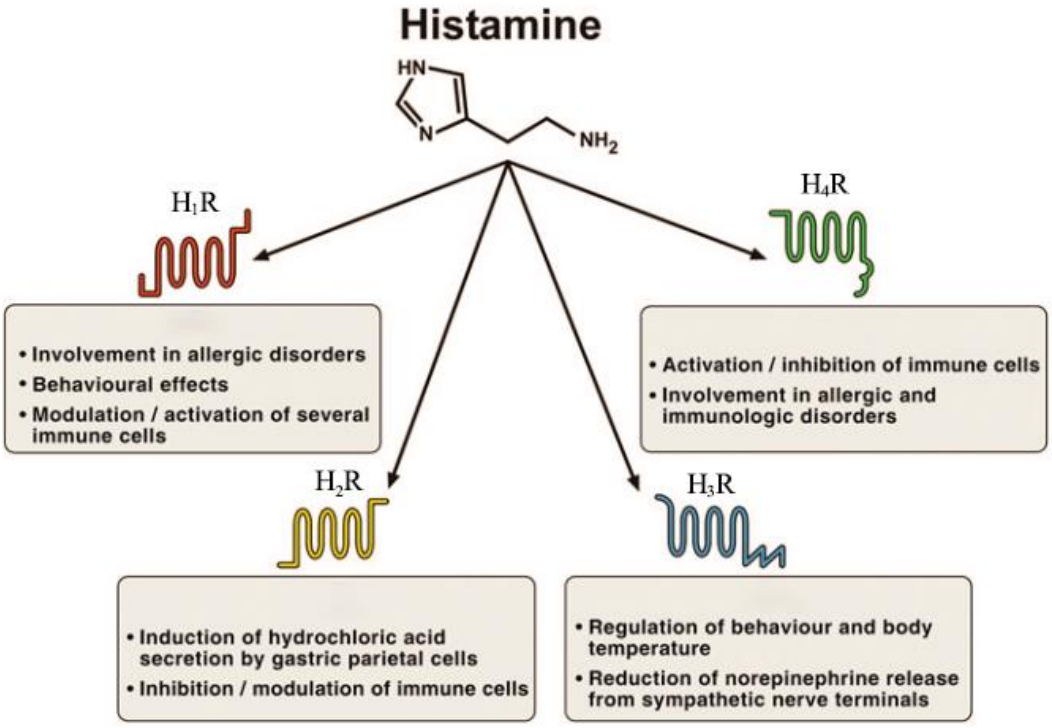


Figure 1.5: Histamine and the four receptors of the histaminergic system. Modified from Hattori and Seifert, 2017.

H_1R is expressed on endothelial cells and bronchial smooth muscle cells and plays a major role in allergic disorders. The presence of H_1R in the central nervous system mediates several behavioral effects as well as pro-inflammatory and immune-modulatory activities due to its expression on several immune cells. H_2R activation induces the secretion of hydrochloric acid from gastric parietal cells and modulates/inhibits a variety of immune cells. H_3R regulates various aspects of behavior and body temperature at the level of the central nervous system. In addition, H_3R inhibits norepinephrine release from sympathetic nerve terminals in the heart. H_4R modulates the migration and activation of a wide spectrum of immune cells and is thereby involved in allergic and immune-mediated disorders.

1.6.2 Histamine H_1 receptor

The H_1R is ubiquitously expressed, specifically in lungs, CNS, and blood vessels It preferentially couples to Gαq/11 proteins, causing phospholipase C (PLC) and protein kinase C (PKC) activation as well as inositol-1,4-5-trisphosphate (IP_3) formation and intracellular Ca2+ release (Panula *et al.*, 2015). The typical signs of type I allergic reaction like pruritus increased vascular permeability and edema is caused by H_1R activation. Therefore, administration of H_1R antagonists (so-called antihistamines) belongs to the most important anti-allergic therapeutic interventions (Simons and Simons, 2011).

The H_1R is expressed on various types of immune cells, specifically on T cell subsets and dendritic cells and influences T cell polarization (Neumann et al. 2014). Moreover, as indicated by results from H_1R-deficient mice, the H_1R plays a role in various models of inflammatory diseases, e.g. nasal allergy, Th2-driven allergic asthma, atopic dermatitis or experimental autoimmune encephalitis (EAE) (Neumann *et al.*, 2013).

In the CNS, H_1R is involved in the regulation of locomotor activity, emotions, cognitive functions, arousal, sleep and circadian rhythm or pain perception (Schneider *et al.*, 2014). Sedation, the most important side effect of brain-penetrating first-generation antihistamines, is caused by antagonism at H_1R in the CNS (Simons and Simons, 2011; Neumann *et al.*, 2013).

1.6.3 Histamine H_2 receptor

The H_2 receptor antagonist cimetidine was launched by Sir James Black's team in 1977 as the first effective pharmacological treatment for dyspepsia, gastric or duodenal ulcers, or gastroesophageal reflux disease.

The H_2R is ubiquitously expressed, most importantly in stomach, heart, and CNS (Schneider *et al.*, 2014; Panula *et al.*, 2015). Agonist binding to this receptor results in activation of Gαs-proteins that stimulate the adenylyl-cyclase- mediated production of the second messenger cAMP. The central role of the H_2R in the regulation of gastric acid production is the basis for the therapeutic use of H_2R antagonists to treat gastroesophageal reflux disease (Schubert and Peura, 2008).

The function of the H_2R in the brain is less well documented as for H_1R but includes modulation of cognitive processes and of circadian rhythm. Moreover, H_2R

influences glucose metabolism and food intake (Schneider *et al.*, 2014). Experiments with knockout mice have revealed that the histamine H_2R is involved in the regulation of immune responses, specifically in the modulation of Th1- or Th2-cell polarization.

The human histamine H_2R (*h*H_2R) has been pharmacologically characterized in both human cells and in the Sf9 cell expression system (Seifert, 2013). Neutrophils are specifically well suited for the analysis of hH_2R pharmacology because they are primary cells that can be easily isolated from human blood in large numbers. The *h*H_2R inhibits superoxide anion production induced by chemotactic peptides in neutrophils and eosinophils (Reher *et al.*, 2012). Furthermore, it is discussed that decreased *h*H2R function may contribute to inflammation in bronchial asthma (Seifert, 2013).

1.6.4 Histamine H_3 receptor

The Gαi/o-coupled histamine H_3R is mainly expressed in neurons and acts as a presynaptic auto- and hetero-receptor. It inhibits the release of histamine (Arrang *et al.*, 1983, 1985) but also of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, or glutamate (Haas *et al.*, 2008).

Additionally, there is increasing evidence that H_3R is expressed post-synaptically (Ellenbroek and Ghiabi, 2014)**,** where it regulates, e.g. dopamine D_1R signaling**.** H_3R deficiency reduces addictive behavior in mouse models of ethanol consumption, which is probably due to the reward-inhibiting function of an increased histamine release (Vanhanen *et al.*, 2013)**.** This renders the H_3R an interesting target for the treatment of alcohol addiction (Nuutinen *et al.*, 2012). Despite the decade-long research on H_3R pharmacology, only the inverse H_3R agonist pitolisant is currently used as an orphan drug to treat narcoleptic patients (Dauvilliers *et al.*, 2013). Mouse models suggest that, in contrast to the other three histamine receptor subtypes, the H_3R does not seem to play a major role in immunological processes and inflammation (Neumann *et al.*, 2013).

Histamine H_3Rs are one of several classes of prejunctional hetero-inhibitory receptors. In the hearts of mammals, including human, sympathetic nerve endings express H_3-receptors (Levi and Smith, 2000). H_3R can inhibit sympathetic neurotransmission in the heart (Figure 1.6), which has been demonstrated by the findings that selective H_3-receptor agonists such as (R)α-methylhistamine and imetit decrease the inotropic and chronotropic responses to cardiac sympathetic nerve

stimulation (Endou *et al.*, 1994). Histamine appears to be locally released from mast cells in myocardial ischemia (Hatta *et al.*, 1997) and H_3Rs are plausibly fully activated under ischemic conditions. Indeed, the selective H_3R antagonist thioperamide could double the overflow of noradrenaline at reperfusion in the early phase of myocardial ischemia where noradrenaline exocytosis is enhanced (Imamura *et al.*, 1994).

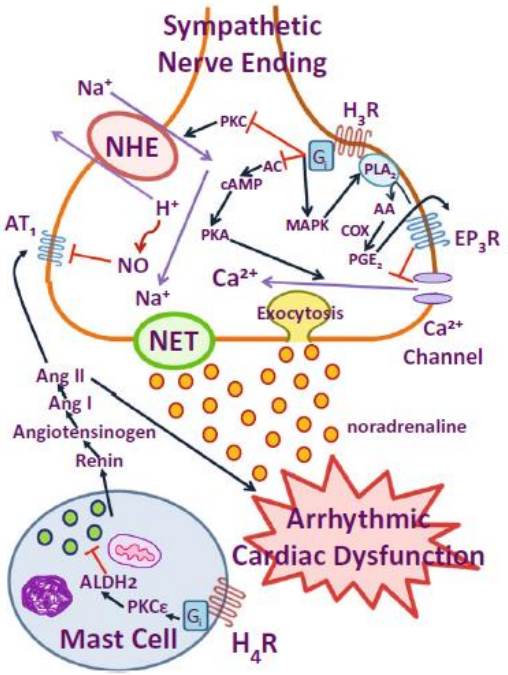


Figure 1.6: Scheme illustrating the H_3R and H_4R-mediated inhibitions of the events that trigger noradrenaline release from sympathetic nerve terminals, mast cell renin release, and, consequently, the development of arrhythmic cardiac dysfunction in myocardial ischemia. From (Hattori and Seifert, 2017).

1.6.5 Histamine H_4 receptor

The H_4 receptor is the most recently discovered histamine receptor. The cloning of the H_3 receptor provided a template for the search of other histamine receptors. This culminated with six independent groups reporting the cloning of the H_4 receptor in 2000 (Panula *et al.*, 2015). Since that time much research has focused on characterizing the receptor's role in the inflammatory process because it originally appeared to be predominantly expressed in hematopoietic cells.

H_4R modulates the migration and activation of a wide spectrum of immune cells (e.g., mast cells, basophils, eosinophils, monocytes, dendritic cells, NK, iNK T and γδ cells, CD8+ T cells, Treg, and Th2 cells) and is thereby involved in allergic and immune-mediated disorders. H_4R antagonism prevents histamine-induced $[Ca^{2+}]i$ increase, mast cell chemotaxis, and submucosal mast cell accumulation in the trachea of mice after

histamine inhalation (Thurmond, 2014). H_4R antagonism prevents bleomycin-induced pulmonary fibrosis alone and in association with naproxen (Arianna Carolina Rosa *et al.*, 2014; Lucarini *et al.*, 2016).

There is evidence that the H_4 receptor is expressed in the nervous system. The expression has been reported in the murine hippocampus, granule cell layer of the cerebellum, the thalamus, and in the spinal cord (Connelly *et al.*, 2009).

Recently the first series of observations have been conducted on behavioral phenotype of histamine H_4 receptor knockout mice, focusing on central neuronal functions (Sanna *et al.*, 2017). According to the authors, H_4R modulates various neurophysiological functions such as locomotor activity, anxiety, nociception and feeding behavior, confirming the importance of the integrity and functionality of neuronal H_4R in the histaminergic regulation of neuronal functions. Furthermore, H_4 receptor agonism seems to be implicated in the modulation of neuropathic pain (Sanna *et al.*, 2015). The functional expression of H_4R on human and rodent neurons highlights their implication in neuronal functions, but the difficulty to generate H_4R antibodies with high specificity (Beermann *et al.*, 2012) generates results that should be interpreted with some caution.

An interesting point of view is the role of microglia in neuroinflammation. Dysregulation of brain histamine is increasingly appreciated as a potential contributor to neuropsychiatric disease (Haas *et al.*, 2008). For example, dysregulation of histamine biosynthesis has been implicated as a rare genetic cause of Tourette syndrome (TS) (Baldan *et al.*, 2014). Abnormalities in histaminergic signaling have also been hypothesized to contribute to Parkinson's disease (Shan *et al.*, 2012). Histamine regulates microglia *in vivo*, via the H_4 receptor. Chronic histamine deficiency in histidine-decarboxylase knockout mice reduces ramifications of microglia in the striatum and in the hypothalamus, but not elsewhere in the brain. Depletion of histaminergic neurons in the hypothalamus has a similar effect (Frick *et al.*, 2016).

1.6.6 Histamine receptors in the eye

Histamine and adrenaline, proposed for the treatment of glaucoma, was the so-called "*Glaucosans"* in the late 20s of the past 20th century (Duke-Elder and Law, 1929). Several ocular hypertensive effects have been reported in chronic glaucoma patients following the use of histamine H_2 receptor antagonists for the treatment of peptic ulcer, however, some relatively recent studies have failed to demonstrate the significant action of topical administered H_2 blockers on IOP in humans (Razeghinejad *et al.*, 2011).

The H_1 and H_2 receptor antagonists have an anticholinergic activity that may induce glaucoma. Promethazine has been shown to produce an idiopathic swelling of the lens that could increase the risk of pupillary block angle-closure glaucoma. Cimetidine and ranitidine increased the intraocular pressure in a patient with glaucoma being treated for duodenal ulcer (Lachkar and Bouassida, 2007).

Retina, optic nerve and various brain structure of both albino and pigmented rabbits contain histamine in the range of 40-400 ng/g tissue, while choroid tissue of both animal strains was characterized by several times higher amine content. The synthesis and release of histamine (Arrang *et al.*, 1983, 1985) are controlled by presynaptic histamine H_3 auto-receptors that are located in the CNS. The heterogeneous distribution of the isoforms suggests that isoform-specific H_3 receptor regulation is important in several brain functions (Drutel et al., 2001).

In macaque retinas stimulation of H_3R increases the delayed rectifier component of the voltage-dependent potassium conductance in ON bipolar cells, hyperpolarizing the cells by 5 mV, on average (Yu *et al.*, 2009). In dark-adapted baboon retinas, histamine also decreases the rate of maintained firing and the amplitude of the light responses of ON ganglion cells (Akimov *et al.*, 2010).

The primates' retina receives input from histaminergic neurons that are active during the day in the posterior hypothalamus. H_1 receptors are localized on horizontal cells and in a small number of amacrine cells (Figure 1.7).

H_2 receptors appear to be closely associated with synaptic ribbons inside cone pedicles (Vila *et al.*, 2012).

Retinae of mammals, including humans, receive input from the brain via axons emerging from the optic nerve. One set of retinopetal axons arises from perikarya in the posterior hypothalamus and uses histamine, and the other arises from perikarya in the

dorsal raphe and uses serotonin. These serotonergic and histaminergic neurons are not specialized to supply the retina; rather, they are a subset of the neurons that project via collaterals to many other targets in the CNS as well. They are components of the ascending arousal system, firing most rapidly when the animal is awake and active. Many of the effects of histamine and serotonin on light responses suggest that retinopetal axons optimize retinal function at the ambient light intensity during the animal's waking period. Histamine reduces the amplitude of light responses in a subset of monkey retinal ganglion cells, a finding consistent with a role for retinopetal axons in light adaptation in these diurnal animals (Gastinger, Tian, *et al.*, 2006). There are only a small number of these retinopetal axons, but their branches in the inner retina are very extensive. H_3R was previously localized on the tips of ON bipolar cell dendrites in both rod and cone terminals (Gastinger *et al.*, 1999; 2006).

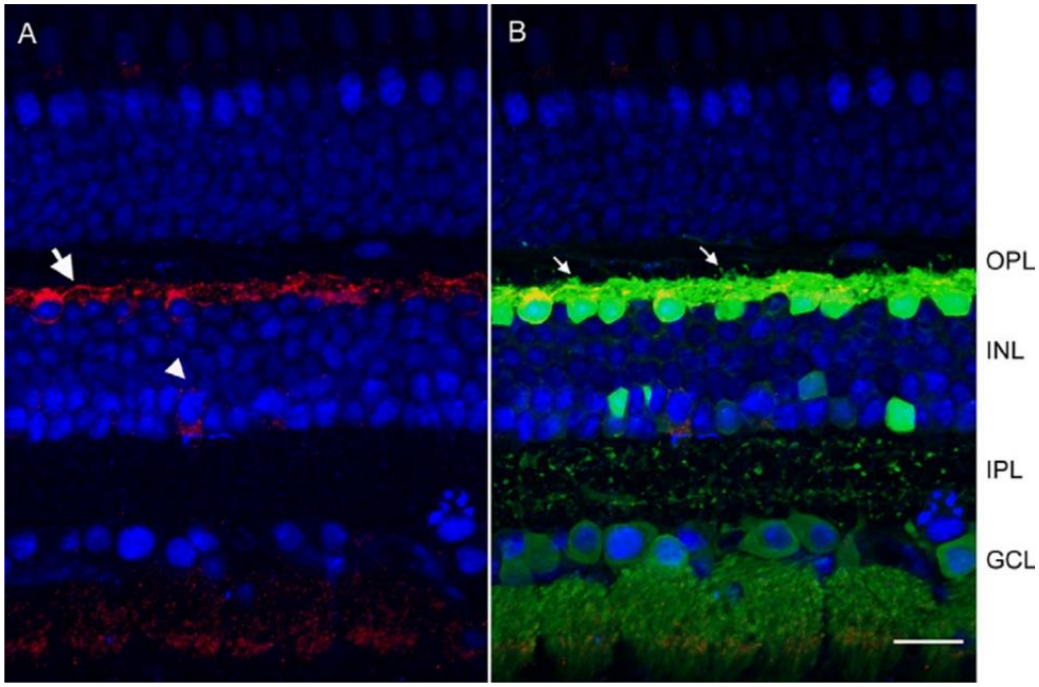


Figure1.7. Localization of H_1R (red) and parvalbumin (green) in a vertical section of the retina. OPL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL retinal ganglionic cell layer (from Gastinger *et al.*, 2006).

All three major targets of histamine are in the outer plexiform layer, but the retinopetal axons containing histamine terminate in the inner plexiform layer.

Taken together, these findings suggest that histamine acts primarily via volume transmission in primate retina contributing to the increase of the operating range of cones and conserving ATP in bright, ambient light.

2. AIM OF THE RESEARCH

The aim of my research project was to establish a possible role of the histaminergic system in retinal neuroprotection. The project was focused:

1. to evaluate the presence and the different distribution of the histamine receptor subtypes in the retina, ciliary bodies, trabecular meshwork (TM) and TM derived cells.
2. to investigate the role of H_3 receptor antagonism in the modulation of IOP
3. to study the potentiation of the hypotensive effect of H_3 receptor antagonism with a nitric oxide donor.

Intraocular pressure is the result of a balance between secretion and outflow of aqueous humor. This balance is perfectly kept in healthy subjects (Nau *et al.*, 2013). Three variables are of interest in this balance: the rate of aqueous humor formation, the resistance to outflow, and the episcleral venous pressure. These three variables change at night.

IOP has a circadian rhythm, aqueous humor production decreases during the night in diurnal mammals like rabbits, drainage facility decreases as well and as a result, IOP increases at night (Smith and Gregory, 1989).The nocturnal increase in IOP in rabbits is reported to be attenuated in animals with cervical ganglionectomy (Gregory *et al.*, 1985). This demonstrates that intact sympathetic innervation to the eye is required for maintenance of the normal circadian rhythm of IOP and establish a role for the pineal hormones in the regulation of the rhythm of IOP (Braslow and Gregory, 1987).

The histaminergic system is deeply implicated in circadian rhythm and fulfills a major role in the maintenance of waking (Lin *et al.*, 2011). During the day the histaminergic tone could play a role in maintaining the IOP balance. The histaminergic neurons are located exclusively in the posterior hypothalamus from where they project to most areas of the central nervous system and to the retina via retinopetal axons as well (Gastinger, Tian, *et al.*, 2006). Furthermore, histamine is reported to be responsible for ciliary muscle contraction in human eyes and therefore in IOP reduction (Markwardt *et al.*, 1997).

Taken together, the reported data strongly support a potential role of histamine modulation via H_3R antagonism in regulating IOP. Aqueous humor production or the

outflow rate can possibly be influenced by a neuro-hormone like histamine as histamine is a transmitter of the ascending arousal system, firing most rapidly when the animal is awake and active. The contributions of these retinopetal axons to vision may be predicted from the known effects of serotonin and histamine on retinal neurons (Gastinger, Tian, *et al.*, 2006). On the other hand, a nitric oxide donor can boost the effect by reducing the episcleral vein pressure acting on the third variable of interest in IOP balance.

The H_3R antagonists chosen for the study were ciproxifan, DL76, GSK 189245. All ciproxifan properties are very well described. The compound is lipophilic and has a long duration of action, characteristics very useful for the treatment of a chronic disease and a very useful laboratory tool. In addition, I wanted to evaluate the possible enhancement of ciproxifan IOP lowering activity by a pharmacological combination of this compound with a NO donor molsidomine in a chronic glaucoma animal model.

In order to investigate the pathophysiologic role of the histaminergic system during ocular hypertension I decided to evaluate the changes, quantitative variations, in histaminergic receptors expression in naïve *versus* hypertensive eyes and as well as in hypertensive treated eyes (ciliary bodies and retinae).

The main responsible for outflow impairments are the modifications in the trabecular meshwork. There is a growing interest in increasing trabecular meshwork outflow by extracellular matrix remodeling and/or by modulation of contractility/TM cytoskeleton disruption, that is why a primary TM cell culture, derived from rabbits' TM, was started. Furthermore, I took into serious account the principle of the reduction of the number of animals to use in experiments (Article 47 of Directive 2010/63/EU on the protection of animals used for the scientific purpose). TM cells are the target of plenty of investigations. It seemed therefore reasonable to focus the attention on this part of the eye and especially on cells derived from this unique structure

The final part of the project was addressed in the validating a primary trabecular meshwork cell culture as a reliable model to study the H_3R antagonist action mechanism in reducing ocular hypertension. The validation protocol was carried out by cytocharacterizing the cell culture and reproducing the experiment of Markwardt *et al*.,1997.

3. SUMMARY OF RESEARCH PROJECT

1. Histaminergic receptors characterization in the eye of rabbits
    - Histaminergic receptor subtypes expression in retina, ciliary body and optic nerve (protein and mRNA levels)
    - Histaminergic receptor subtypes localization on TM cell cultures
2. Validation of animal models of glaucoma
    - Transient IOP elevation with hypertonic saline injection in posterior chamber
    - Chronic ocular hypertension with carbomer injection in anterior chamber
3. Pharmacological evaluation of H_3R antagonists (ciproxifan, DL76, GSK198245) on IOP reduction in transient IOP elevation model
    - Effects on transient ocular hypertension, the definition of the best dose.
    - Evaluation of specificity of H_3R antagonism action with the H_3R agonist imetit
    - Evaluation of the role of H_2R agonist amthamine and /H_1R antagonist pyrilamine
    - Evaluation of the potential source of histamine by choroidal mast cells depletion
4. Pharmacological evaluation of H_3R antagonists (ciproxifan, DL76, GSK198245) alone and in association with a NO-donor in carbomer model of glaucoma
    - Evaluation of IOP in chronic treatment
    - Analysis of vascular performance of retinic artery
    - Evaluation of oxidative stress in retina
    - Evaluation of retinal ganglionic cells conservation
    - Evaluation of retinal hypoxia modulation
    - Analysis of retinal microglial modulation
    - Histaminergic receptor subtypes expression in retina and TM in chronic ocular hypertension
5. Investigation on the mechanism of action of H_3R antagonists on TM cells
    - TM cells isolation and characterization
    - Effects of H_3R antagonism on intracellular calcium concentration in TM cells

4. MATERIALS AND METHODS

4.1 Animal models of glaucoma

The experimental procedures were carried out in New Zealand albino rabbits. All procedures on animals conformed to those of the Association for Research in Vision and Ophthalmology Resolution, in agreement with the Good Laboratory Practice for the use of animals, with the European Union Regulations (Directive 2010/63/EU), upon authorization of Italian Ministry of Health (number 1179/2015-PR).

Male albino rabbits (body weight 2-2.5 kg) were kept in individual cages, food and water were provided *ad libitum*. The animals were maintained on a 12-12h light/dark cycle in a temperature controlled room (22°-23°C). Animals were identified with a tattoo in the ear, numbered consecutively. All selected animals underwent ophthalmic and general examinations before the beginning of the study.

A total of 10 rabbits were used for the parallel study of each of the 4 compounds and for the vehicle. The probability to reach a statistically significant difference between treatment groups and control group was set to 80% with a significance level of 0.05 if the mean difference between treatments is 8 mmHg. This is based on assuming that the standard deviation of the response variable is 3. In detail, to calculate the sample size the power analysis was performed taking into account the 2013 guidelines by Charan and Kantharia, considering that:

1- the difference between the average of the two groups

2- the standard deviation taken from previously published studies.

3- the level of significance, which is usually set at the level of 5% (p = 0.05).

3 - the power: the probability of finding the effect that the study aims to achieve. This can be maintained between 80% and 99% depending on the type of research but is usually maintained at 80%.

4- the direction of the hypothesis (one or two tails).

The software used for this calculation was a free software called G* power 3 (Faul *et al.*, 2007).

The animal models of glaucoma used were:

1. Transient IOP elevation model obtained by injection of 0.1 ml of sterile hypertonic saline (5%) into the vitreous bilaterally in locally anesthetized rabbits with one drop of 0.2 % oxybuprocaine hydrochloride (Orihashi *et al.*, 2005). In this model ocular hypertension is maintained for 4 hours.

2. Chronic IOP elevation model obtained by injection of 0.1 ml carbomer 0.25% (Siccafluid, Farmila THEA Pharmaceutical) bilaterally into the anterior chamber of New Zealand albino rabbits pre-anesthetized with tiletamine plus zolazepam (Zoletil 100, 0.05 mg/kg) plus xylazine (Xilor 2%, 0.05 ml/kg) i.m. (Xu *et al.*, 2002; Fabrizi *et al.*, 2012). In this model ocular hypertension is maintained for 2 weeks.

4.2. IOP measurements

All the IOP measurements were performed by applanation tonometry using a tonopen Avia (Reichert Technologies, Depew, NY, USA). This method has proven to be as reliable as Goldmann applanation tonometry or GAT, the most accurate way of measuring IOP (Gupta *et al.*, 2016). GAT is the gold standard for IOP measurement in clinical practice (Goldmann and Schmidt, 1957). Tonopen Avia tonometry was used both in transient and chronic IOP elevation model. One drop 0.2% oxybuprocaine hydrochloride diluted 1:1 with saline, was instilled in each eye immediately before each set of pressure measurement.

4.2 Validation of the models of glaucoma

Both models proposed were validated using gold standard drugs for the treatment of glaucoma i.e timolol maleate 1%, travoprost 0.004% and dorzolamide 2% (Sigma Aldrich, Milan, Italy) dissolved in sterile physiologic solution and 0.1% DMSO *versus* vehicle.

Eye irritancy of various compounds was always measured after repeated administration using the Draize Eye Test (Wilhelmus, 2001).

In the transient IOP elevation model (hypertonic saline model), treatments and IOP measurements were performed according to the scheme in figure 4.1 with the compounds (treatments) reported in table 4.1 on a total of 15 rabbits.

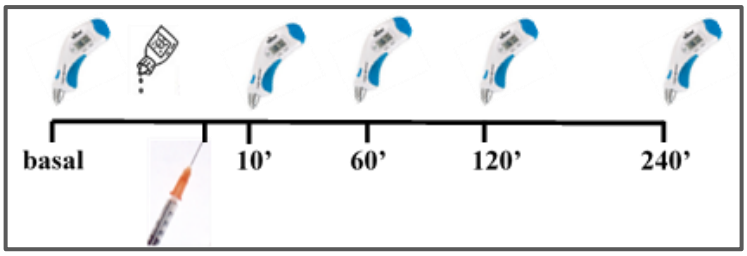


Figure 4.1: Hypertonic saline experimental model with IOP measurements time point

5 left eyes	Timolol 1%
5 right eyes	Vehicle
5 left eyes	Travoprost 0.004%
5 right eyes	Vehicle
5 left eyes	Dorzolamide 2%
5 right eyes	Vehicle

Table 4.1. Treatment groups: timolol n= 5; travoprost n=5; dorzolamide n=5 and vehicle n=15

In the chronic IOP elevation model (carbomer model), treatments and IOP measurements were performed according to the scheme in figure 4.2 with the compounds (treatments) reported in table 4.2 on 10 rabbits. IOP was measured each morning before drug instillation.

The carbomer model is 2 weeks long period of "once a day" eye drop instillation and tonometry evaluation.

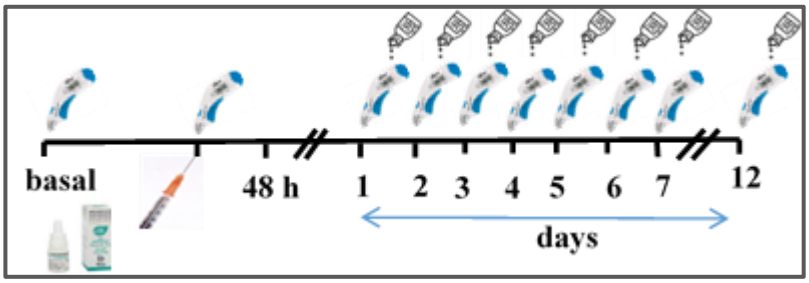


Figure 4.2: Carbomer experimental model with treatment dosing and IOP measurements time point

5 left eyes	Timolol 1%
5 right eyes	Dorzolamide 2%
5 left eyes	Travoprost 0.004%
5 right eyes	Vehicle

Table 4.2: treatment groups: timolol n= 5; travoprost n=5; dorzolamide n=5 and vehicle n=5

The experimental carbomer model of glaucoma is established as soon as the ocular hypertension is stable. This happens 1 or 2 days after carbomer injection. The increase in IOP after carbomer injection was evaluated after 30 minutes, one hour and then three times a day (at 8 am, 2 and 8 pm) until stabilization for the first 48 hours (Galassi *et al.*, 2006).

4.3 Histaminergic ligands used in the experiments on both glaucoma models

All compounds were purchased by Sigma Aldrich (Milan, Italy) with the exception of ciproxifan, provided by Professor Holger Stark (Heinrich Heine Institute, Dusseldorf University, Germany) and DL76 provided by Doctor Dorota Lazewska (Department of Technology and Biotechnology of Medicinal Drugs, Jagiellonian University, Kraków, Poland).

Eye irritancy of various compounds was always evaluated after repeated administration using the Draize Eye Test (Wilhelmus, 2001). Histaminergic ligands are reported with their relative receptor affinities in table 4.3.

Histaminergic ligands	***h*H$_1$R p*K*i**	***h*H$_2$R p*K*i**	***h*H$_3$R p*K*i**	***h*H$_4$R p*K*i**
Pyrilamine/mepyramine	**8.8**	**4.63**	**<5**	**<5**
Amthamine	**-**	**6.82**	**-**	**5.30**
Ciproxifan	**<5**	**<5**	**7.1**	**5.73**
DL-76	**-**	**-**	**22 (CHO cells)**	**-**
GSK189245	**-**	**-**	**9.59**	**-**

Table 4.3: Histaminergic ligands modified by (Panula *et al.*, 2015; Szafarz *et al.*, 2015).

4.4 Experimental design for H_3R antagonism

The experimental design to find the best dose was carried out in transient IOP elevation model as showed in figure 4.1 with the instillation of 30 μl of H_3 receptor antagonists at 3 different concentrations (0.1, 0.5, 1%). The pharmacological evaluation of the specificity H_3R antagonism was studied by pre-treating the animals with 20 μl of H_3R agonist imetit at an equimolar concentration with the H_3R antagonist. The same procedure was carried out with H_1R antagonist pyrilamine/mepyramine and H_2R agonist amthamine.

To evaluate the contribution of histamine from mast cells the animals were pretreated with compound 48/80 (C48/80) 15μg/kg instilled in the eye. The compound was administered topically once a day for two days before IOP evaluation in 4 animals in order to deplete choroidal mast cells from histamine, following the scheme reported in figure 4.3. The control group was composed of 4 animals as well.

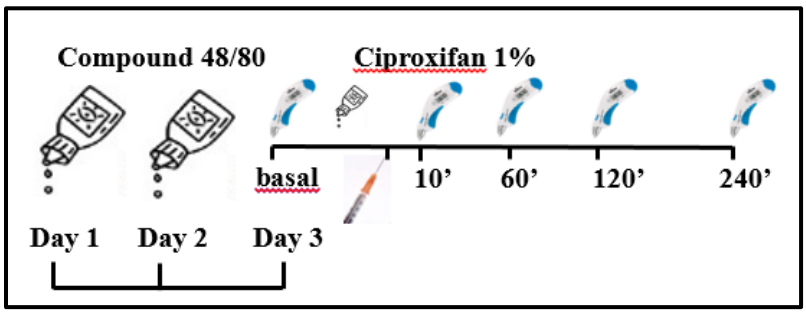


Figure 4.3 Hypertonic saline model in C48/80 preconditioning

4.4.1 Evaluation of mast cells' granule release

This assay was carried out on 5 μm thick histological choroid sections. The sections were cut from the paraffin-embedded posterior eye samples and stained with Astra blue (Fluka, Buchs, Switzerland). This cationic dye binds specifically to heparin containing mast cell granules and can be used as a probe to evaluate the cells' content of secretion granules by optical density measurements. Digital images of optical fields containing mast cells were randomly taken from the 2 different experimental groups using 40x objective. The images were analyzed with the ImageJ 1.33 software. In each animal, 10 different optical fields were analyzed by 2 different researchers (me and Doctor Alessandro Pini), the mean optical density value (± SEM) was then calculated for the entire experimental group.

4.4 Experimental design in chronic model of glaucoma

The experimental design for the chronic treatments was carried out in carbomer induced ocular hypertension reproducing the validated model shown in figure 4.4. The protocol was carried out for 4 groups of animals (n=10 per group) in two different sets.

In the first set, I tested the effects of ciproxifan, DL 76 and GSK189254 and timolol maleate *versus* vehicle in reducing IOP and inducing retinal baroprotection.

In the second set I tested the effects of ciproxifan 0.5%, ciproxifan 0.5% plus molsidomine 0.5%, timolol maleate 1% *versus* vehicle (Figure4.4).

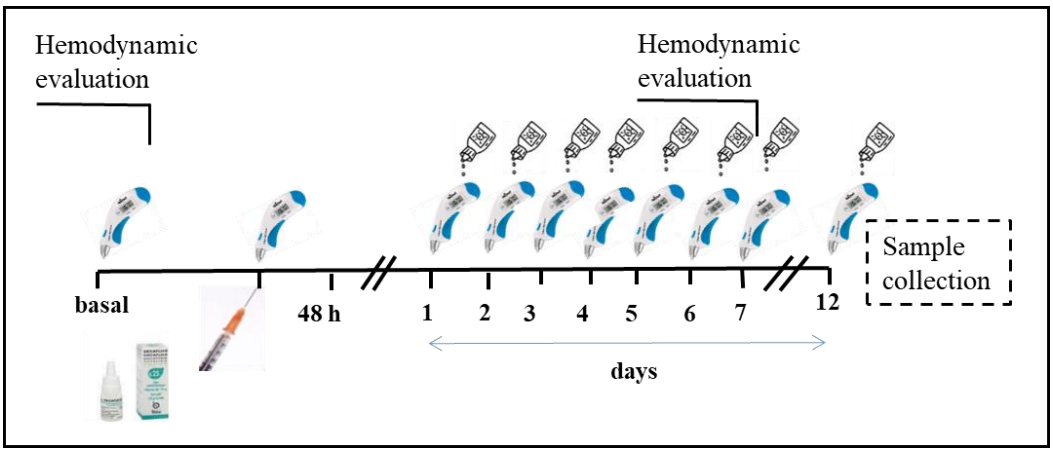


Figure 4.4: Carbomer experimental protocol with treatment dosing schedule and IOP measurements, hemodynamic evaluation and sample collection time points.

4.5 Hemodynamic evaluation

The hemodynamic evaluation was performed using an eco-color-doppler Philips Ultrasound HD7 XE (Brothel, WA, USA). All animals of the chronic glaucoma model underwent Color Doppler Imaging (CDI) investigation at the beginning of the experimental setting and end of drug treatments. Special attention was devoted to the evaluation of retinic artery circulation. Blood flow velocities are measured and the Pourcelot Resistivity Index (RI) calculated (Impagnatiello *et al.*, 2012)**.** Resistivity index or Pourcelot Index is the ratio given by the difference between systolic and diastolic velocity over-systolic velocity and gives a dimension of local vascular impairment According to the experimental protocol hemodynamic evaluation was performed in basal conditions and at the end pharmacological treatments (figure 4.4).

4.6 Sample collection

Sample collection was also performed in basal conditions and at the end of the experiments. Animals were killed with a lethal dose of anesthetics (zolazepam/tiletamine and penthotal sodium 0.05g/ kg). For each treatment, group one eye was excised and fixed with 4% paraformaldehyde in phosphate-buffered saline for histological analysis and the contralateral one was dissected to collect retina and ciliary

body, that were quickly frozen in liquid nitrogen and stored at −80°C. Samples of stomach, spleen, and hippocampus were also collected to use them as positive controls for H_2, H_4, and H_1/H_3 receptors, respectively.

4.7 Western blot analysis of histaminergic receptors

Ciliary bodies, retinae, and stomach were homogenized in ice-cold lysis buffer (NaCl 0.9%, tris-HCl 20 mmol/L pH 7.6, Triton X-100 0.1%, phenylmethylsulfonyl fluoride 1 mmol/L, leupeptin 0.01%) plus protease inhibitor cocktail (Cell Signaling, Milan, Italy) sonicated, and centrifuged at 4°C for 10 minutes at 10,000*g*. Total protein levels were quantified using Pierce Protein Assay (Rockford, IL, USA). Tissue samples (30 µg protein per lane) were subjected to 8% SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight (4°C) with antibodies for H_1, H_2, H_3 and H_4 receptors (dilution are reported in table 4.5). Primary antibodies were diluted in T-PBS (20 mM Tris-HCl buffer, 150 mM NaCl and 0.05% Tween 20) containing 5% non-fat dry milk. After 3 rinses with T-PBS, membranes were incubated with anti-rabbit IgG conjugated with peroxidase, diluted 1:5000 in T-PBS at RT for 1 hr. β-actin protein was used as the internal control for normalization. The immunoreaction was revealed by enhanced chemiluminescence (Luminata Crescendo Western HRP substrate, Merck Millipore Darmstadt, Germany), and quantified by densitometric analysis with the Image J software (NIH, USA). The same procedure was carried out at end of the chronic experimental protocol to evaluate the effects of ocular hypertension and all the different treatments on the expression of histaminergic receptors.

4.8 Semi-quantitative RT-PCR of histaminergic receptors

Total RNA was isolated from a tissue according to the manufacturer's protocol (NucleoSpin RNA II; Machery-Nagel, Bethlehem, PA). Concentration and purity were assessed by spectrophotometric analysis (Genequant-Pro, GE Healthcare, Milano, Italia). One µg of total RNA was reverse-transcribed to cDNA with M-MLV Reverse Transcriptase (Omniscript; QIAGEN, Milan, Italy) using random primers in a 20 µL reaction incubated at 25°C for 5 min followed by 60 min at 42°C and 5 min at 70°C. Two hundred ng of cDNA was amplified as follows: 15 min at 95°C, 1 min of denaturation at

94°C, 30 s of annealing, and 30 s of extension at 72°C for 38 cycles. Primer sequences are summarized in table 4.4. Amplification products were highlighted with ethidium bromide on 1.5% agarose gel. The intensities of the bands were quantified by densitometric analysis. Doctor Laura Lucarini helped me designing the primers.

Name	Primer sequence (5'-3')	TM °C	Name	Primer sequence (5'-3')	TM °C	Product length (bp)
[a]r H1R F	AGCATGGAACGTCCAGTAGT	58.73	r H1R R	TTGCCCTCACACATCCTGTC	59.96	109
[b]r H2R F	GGATACCGCGACTACGAACC	60.32	r H2R R	ATCGTGGGAAAGCTGACACG	60.67	94
[c]r H3R F	TCACTGGAGAAGCGCATGAA	59.68	r H3R R	GAGCCCAAAGATGCTCACGA	60.39	114
[d]r H4R F	GTAGGAAACGCGGTGGTCAT	60.39	r H4R R	TGAGCCAAAACAGGCAGACT	59.82	184

[a]XM_002713049.2 PREDICTED: Oryctolagus cuniculus histamine receptor H_1 (*h*H_1R), transcript variant X1, mRNA

[b]XM_008255497.1 PREDICTED: Oryctolagus cuniculus histamine H_2 receptor-like (LOC103348006), mRNA

[c]NM_001082226.1 Oryctolagus cuniculus histamine receptor H_3 (*h*H_3R), mRNA

[d]XM_002713460.2 PREDICTED: Oryctolagus cuniculus histamine receptor H_4 (*h*H_4R), mRNA

Table 4.4. Histaminergic receptors gene sequences used

4.9 Immunofluorescence of ciliary bodies and retinae

Histological sections, 5 µm thick, were cut from the paraffin-embedded posterior and anterior eye samples. The sections were deparaffinized and boiled for 10 min. in sodium citrate buffer (10 mM, pH 6.0; Bio-Optica, Milan, Italy) for antigen retrieval. After a pre-incubation in 1.5% bovine serum albumin (BSA) in PBS, pH 7.4 for 20 min at RT to minimize the aspecific binding, the sections were incubated overnight at 4 °C with primary antibodies directed against H_{1-4} receptors as reported in table 4.6.

A double labeling was performed with glial fibrillary acid protein (GFAP) and vascular endothelial growth factor A (VEGFA) to evaluate retinal hypoxia modulation. A second double labeling was made to evaluate retinal microglial modulation with IBA1, a marker for resting microglia, and CD11B, a marker of activated microglia. The immunoreactions were revealed by incubation with the corresponding (Table 46) anti-Fluor 488 and 594-conjugated IgG for 2 hours at RT. The sections were mounted in an aqueous medium (Fluoremount, Sigma, Milan, Italy) with 4',6-diamidino-2-phenylindole (DAPI). All antibodies' dilutions are reported in table 4.6.

Representative images were acquired by an Olympus BX63 microscope coupled to CellSens Dimension Imaging Software version 1.6 (Olympus, Milan, Italy).

4.10 Determination of 8-hydroxy-2′-deoxyguanosine (8-OhdG)

Frozen ciliary bodies and retinae samples were thawed at room temperature, and cell DNA isolation was performed as previously described with minor modifications. Briefly eye samples were homogenized in 1 ml of 10 mM PBS, pH 7.4, sonicated on ice for 1 min, added with 1 ml of 10 mmol/l Tris-HCl buffer, pH 8, containing 10 mmol/l EDTA, 10 mmol/l NaCl, and 0.5% SDS, incubated for 1 hr at 37°C with 20 µg/ml RNase 1 (Sigma-Aldrich, Saint Louis, MO, USA) and overnight at 37°C under argon in the presence of 100 µg/ml proteinase K (Sigma-Aldrich). The mixture was extracted with chloroform/isoamyl alcohol (10/2 v/v). DNA was precipitated from the aqueous phase with 0.2 volumes of 10 mmol/l ammonium acetate, solubilized in 200 µl of 20 mmol/l acetate buffer, pH 5.3, and denatured at 90°C for 3 min. The extract was then supplemented with 10 IU of P1 nuclease (Sigma-Aldrich) in 10 µl and incubated for 1 hr at 37°C with 5 IU of alkaline phosphatase (Sigma-Aldrich) in 0.4 mol/l phosphate buffer,

pH 8.8. All of the procedures were performed in the dark under argon. The mixture was filtered by an Amicon Micropure-EZ filter (Merck-Millipore), and 50 µl of each sample was used for 8-hydroxy-2-deoxyguanosine (8-OH*d*G) determination using an ELISA kit (JalCA, Shizuoka, Japan), following the instructions provided by the manufacturer. The absorbance of the chromogenic product was measured at 450 nm and expressed as ng/mg of DNA. The results were calculated from a standard curve based on an 8-OH*d*G solution. The values are expressed as ng 8-OH*d*G/ng total DNA (Lodovici *et al.*, 2000).

4.11 Fluorescent dye dihydroethidium (DHE) staining

Histological sections, 5 µm thick, were cut from the paraffin-embedded posterior and anterior eye samples, deparaffinized and incubated with fluorescent dye DHE 2 µM (Abcam, Cambridge, Uk) in a humidity chamber for 30 mins at 37°C. Coverslips were then placed on the slides and kept in the dark. The sections were mounted in an aqueous medium (Fluoremount, Sigma, Milan, Italy). Fluorescence was detected (absorbance: 518 nm, emission: 605 nm) using Olympus BX61 microscope with Olympus BX63 microscope coupled to CellSens Dimension Imaging Software version 1.6 (Olympus, Milan, Italy).

4.12 Histology and assessment of retinal ganglionic cell (RGC)

Histological sections, 5 µm thick, were cut from the paraffin-embedded posterior and anterior eye samples. All sections were stained in a single session to minimize artefactual differences in the staining. Photomicrographs of the histological slides were randomly taken with a digital camera connected to a light microscope. Quantitative assessment of the stained sections was performed by computer-aided count on the optical field using image analysis program ImageJ (NIH, Bethesda, MD). Values are means ± SEM of individual rabbit (five images each) from the different experimental groups (tested blindly by two different researchers i. e. me and Dr. Laura Lucarini).

4.13 TM cells isolation and culture

Trabecular meshwork was isolated under the sterile condition and incubated in a phosphate buffer solution with 1 mg/ml type 1 collagenase and 0.4 mg/ml of BSA for

2 hours at 37°C. The enzymatic digestion was blocked with HAM F-12 culture medium and the sample was centrifuged at 500 *g* for 10' at room temperature. After supernatant removal, the pellet was suspended in 1 ml of complete medium and seeded in an F24 flask with HAM F-12 culture medium added with 10% fetal calf serum, 1% penicillin and streptomycin. The cellular culture was stabilized with four passages before cytological characterization with immunofluorescence, WB, and functional analysis. All passages were ruled as follow: cells were cultured with HAM F12 culture medium (containing 10% fetal calf serum and 1% penicillin and streptomycin) until subconfluence, which was usually reached upon 4 days of culture. At this point, cells were washed three times with PBS. Adherent cells were detached from the surface of the culture vessel both by a mechanic (rocking of culture vessel) and were enzymatic (3 ml of trypsin for 15 min). The supernatant was recovered and centrifuged at 500 *g* for 10 min at RT. The pelleted cells were resuspended in growth medium (HAM F12 medium, 10% fetal calf serum, 1% penicillin and streptomycin).

4.14 TM cells characterization with myocilin expression induction

Cells were grown in 6 multiwell plates at a starting concentration of 0.25 x 10^4 in 0.5 ml of complete HAM F12 culture medium as previously described. The day after the cells were incubated with 100nM and 1µM dexamethasone added to the culture medium for 5 days in order to obtain corticosteroid induced stress. Cells were washed twice with PBS. One ml of lysis buffer ((NaCl 0.9%, tris-HCl 20 mmol/L pH 7.6, Triton X-100 0.1%, phenylmethylsulfonyl fluoride 1 mmol/L, leupeptin 0.01%) plus 100µl of protease inhibitor cocktail (Cell Signaling, Milan, Italy) was added at each well. Pellet was collected after manual scraping. Protein dosage and western blot analysis were performed as described in paragraph 4.7. The antibody used for myocilin expression revelation was anti-myocilin and the dilution is reported in reported in table 4.5. Myocilin is a functional marker of dexamethasone conditioned TM cells and a marker of glaucoma (Kwon *et al*., 2011).

4.15 TM cells characterization with immuno-fluorescence staining

Cells were grown in 8 multiwell chamber slide (Sigma Aldrich, Milan, Italy) at a starting concentration of $0{,}25x10^4$ in 0.5 ml of complete culture medium, for 4-5 days until 80% confluence. Slides were washed twice with cold PBS and then fixed with 4% paraformaldehyde in PBS at RT 15 minutes. Then, cells were permeabilized with 0.1% Triton-X100 in PBS for 15 minutes. After washing 3 times with PBS, the slides were blocked for 30 minutes in PBS containing 2% BSA at room temperature and then incubated for 1 hour with α smooth muscle actin (SMA), aquaporin, myosin heavy chain (MYHC) and vimentin antibody in 2% BSA/PBS. After 3 washings with 0.1% T-PBS, bound antibody was detected with the corresponding, FITC conjugate, secondary polyclonal antibody. Nuclei were counterstained with a 1:5000 dilution of DAPI for 15 minutes. Representative images were acquired by an Olympus BX63 microscope coupled with a Cell Sens Dimension Control immunostaining was performed omitting the primary or secondary antibodies to verify the specificity of the immunostaining.

4.16 Calcium imaging on TM cells

Cells were grown in a 6 multiwell plate on a polylisinated removable glass at a starting concentration of $0.25x10^4$ in 0.5 ml of complete HAM F12 culture medium till 80% confluence. Intracellular Ca^{2+} levels was evaluated loading the cells with 1-5 μl/L fluo-3-acetoxymethyl ester and 1.0 μl/mL Pluronic F127 solved in extracellular solution containing (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 3 KCl, 10 Hepes, 10 D(+) glucose, adjusted to pH 7.3 with NaOH, osmolarity 300–310 mosmol l^{-1}. Cells were incubated for 40 min at 37°C. Fluo-3 fluorescence levels were detected at 540±15 nm with a fluorescence microscope (Olympus, Milan, Italy). Fluorescence acquisition and analysis were performed using Imaging Workbench software (INDEC BioSystems, Los Altos, Ca, USA). The data were reported as a mean fractional variation (±S.E.M) relative to the initial values measured for individual cells.

4.17 Antibodies used for western blot analysis

Target	Antigen	Supplier	Catalog#	Antibody	Host	usage	Conc
TM cells	Myocilin	Santa Cruz Biotechnology	sc-137233	Monoclonal	Mouse	primary	1:1500
Retina TM/ ciliary bodies TM cells	H_1R	Santa Cruz Biotechnolgy	sc-374621	Monoclonal	Mouse	primary	1:1000
Retina TM/ ciliary bodies TM cells	H_2R	Santa Cruz Biotechnolgy	sc-33974	Monoclonal	Goat	primary	1:1000
Retina TM/ ciliary bodies TM cells	H_3R	Santa Cruz Biotechnolgy	sc-390140	Monoclonal	Mouse	primary	1:1000
Retina TM/ ciliary bodies TM cells	H_4R	Santa Cruz Biotechnolgy	sc-33967	Monoclonal	Goat	primary	1:1000
Actin	β-actin	Sigma	A2228	Monoclonal	Mouse	primary	1:10000
Mouse FC	Mouse FC	Cell signaling	6402-05	Polyclonal	Rabbit anti-mouse IgG HRP	secondary	1:2000
Goat FC	Goat FC	Santa Cruz Biotechnology	Sc-2020	Polyclonal	Donkey anti-goat IgG-HRP	secondary	1:2000

Table 4.5: List of primary and secondary antibodies

4.18 Antibodies used for immunofluorescence staining

Immunofluorescence staining							
Target	**Antigen**	**Supplier**	**Catalog#**	**Antibody**	**Host**	**usage**	**Conc**
TM cells TM	Aquaporin 1	Santa Cruz Biotechnolgy	sc-32737	Monoclonal	Mouse	primary	1:250
TM cells	Alfa - SMA	Abcam	ab7817	Monoclonal	Mouse	primary	1:125
TM cells	Vimentine	Abcam	ab11256	Polyclonal	Goat	primary	1:100
TM cells	MYHC 11	Abcam	ab82541	Polyclonal	Rabbit	primary	1:125
TM cells	H_1R	Santa Cruz Biotechnolgy	sc-374621	Monoclonal	Mouse	primary	1:100
TM cells	H_2R	Santa Cruz Biotechnology	sc-33974	Monoclonal	Goat	primary	1:100
TM cells	H_3R	Santa Cruz Biotechnolgy	sc-390140	Monoclonal	Mouse	primary	1:100
TM cells	H_4R	Santa Cruz Biotechnology	sc-33967	Monoclonal	Goat	primary	1:100
Retina (resting microglia)	IBA 1	Abcam	ab5076	Polyclonal	Goat	primary	1:350
Retina (microglia)	CD11B	Abcam	ab8878	Monoclonal	Rat	primary	1:50
Retina (glia)	GFAP	Abcam	ab4674	Polyclonal	Chicken	primary	1:500
Retinal hypoxia	VEGF	Abcam	Ab46154	Polyclonal	Rabbit	primary	1:75
Rabbit FC	Rabbit FC	Jackson IR	711-545-152	Polyclonal	Donkey	secondary Alexa Fluor^R 488	1:500
Rabbit FC	Rabbit FC	Jackson IR	711-585-152	Polyclonal	Donkey	secondary Alexa Fluor^R 594	1:500
Rat FC	Rat FC	Jackson IR	711-545-152	Polyclonal	Donkey	secondary Alexa Fluor^R 488	1:500
Chicken FC	Chicken FC	Jackson IR	703-545-155	Polyclonal	Donkey	secondary Alexa Fluor^R 488	1:500
Mouse FC	Mouse FC	Jackson IR	515-585-062	Polyclonal	Sheep	secondary Alexa Fluor^R 594	1:500
Goat FC	Goat FC	Jackson IR	805-585-180	Polyclonal	Bovine	secondary Alexa Fluor^R 594	1:500

Table 4.6: List of primary and secondary antibodies

4.19 Statistical analysis

For each assay, data were reported as mean values (± S.E.M.) of individual average measures of the different animals per group. The significance of differences among the groups was assessed by one-way ANOVA or two way ANOVA for multiple comparisons followed by Bonferroni post-test and unpaired t-test with Welch's correction. Calculations were made with Prism 6.1 statistical software (GraphPad Software Inc., San Diego, CA, USA). A probability value (p) of <0.05 was considered significant.

5. RESULTS

The results are presented in one chapter divided into six subchapters (5.1 to 5.6) organized chronologically from the validation of the animal experimental model, to the first preliminary results obtained on cells isolated from the main effector of IOP modulation i.e. the trabecular meshwork.

5.1 Validation of glaucoma models

This part of the work was fundamental as it was propaedeutic to all the functional experiments regarding baroprotection. These part of the work provided two valid experimental animal models of glaucoma. The transient model was very useful to screen the IOP lowering properties of H_3 receptor antagonists and to range the best dose of the different compounds. The stable IOP elevation model was subsequently validated to reproduce a model of chronic ocular pathology, i.e glaucoma, much more similar to naturally occurring one and to have a chronic dosing setting for experiments.

5.1.1 Transient IOP elevation with hypertonic saline injection

IOP rose from 15.28 mmHg to 38.3±4.2 mmHg after half an hour and remained stable for 3 hours, starting to reduce spontaneously in vehicle-treated animals after 3 hours. The treatments started to reduce IOP from 1 hour. The best time points to understand the ability of a compound to reduce IOP are the one at 60 and 120 min from the saline injection. Travoprost and timolol 1% treatments reduced IOP > 25 % at these time points.

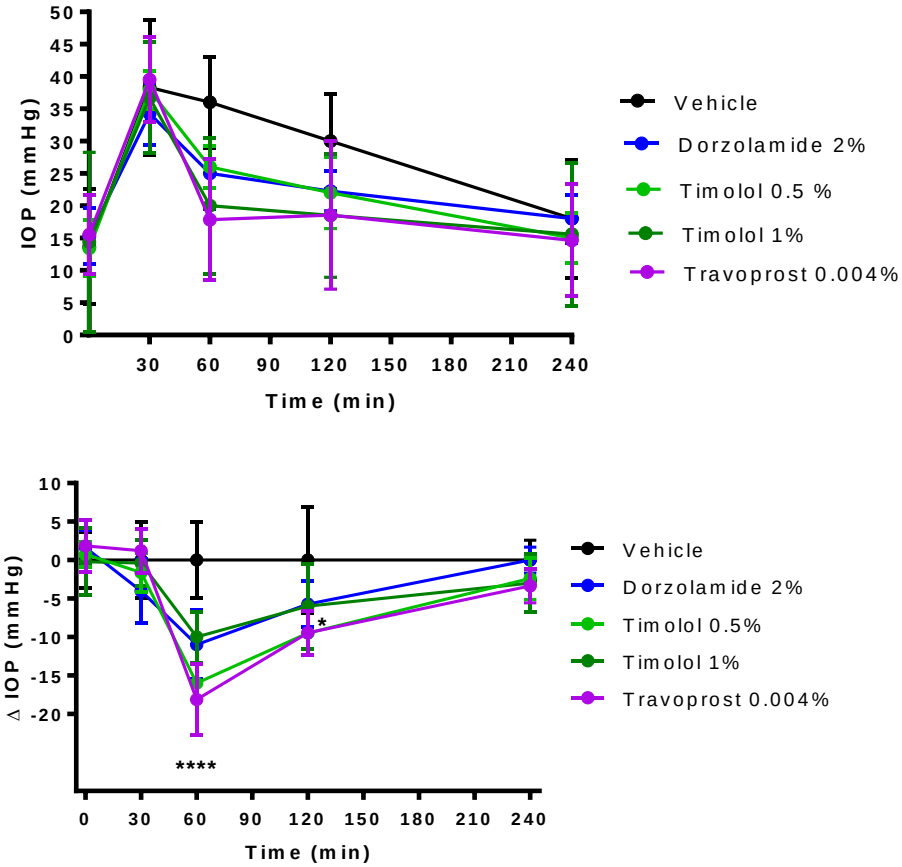


Figure 5.1. IOP time course. Data are expressed as mean±SEM (n=5) ****p<0.0001 timolol 0.5, 1%, travoprost 0.004% at 60 and 120 min, dorzolamide 2% at 60 min; *p<0.05 timolol 0.5% and dorzolamide 2% at 120 min *versus* vehicle. Two way ANOVA followed by Bonferroni *post hoc* test.

5.1.2 Stable IOP elevation with carbomer injection in anterior chamber

IOP rose from 15.85±2.77 mmHg to 34.75±3.2 mmHg after 48 hours and remained stable for 2 weeks. The treatments started to reduce IOP from day 4. Travoprost and timolol treatments reduced IOP >25% starting from day 4. This hypotensive effect was evident at days 7, 8 10 and 11. IOP reduction with reference anti-glaucoma drugs was effective and stable in comparison to the vehicle and therefore this stable model of OHT could be a valid experimental animal model to study a new class of baroprotective drugs.

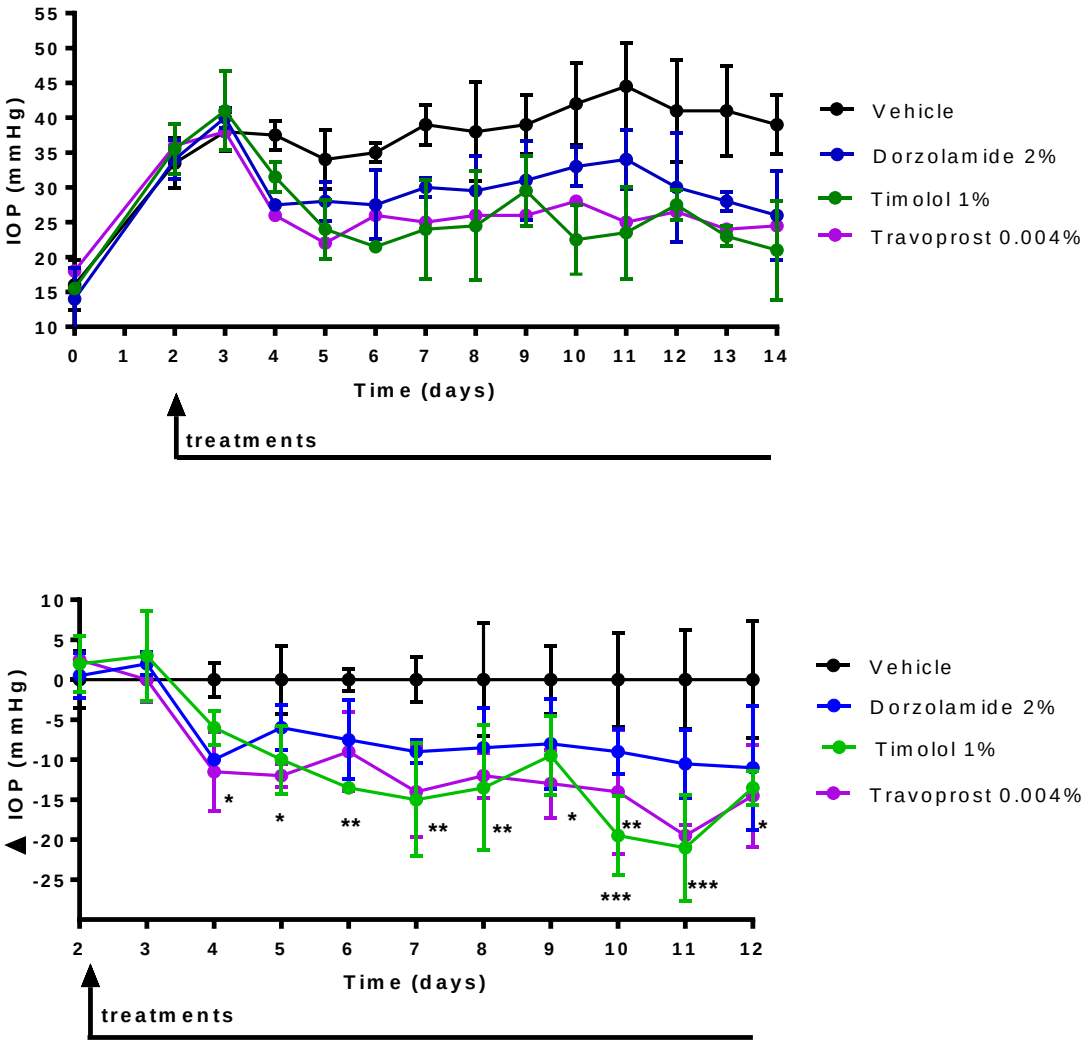


Figure 5.2. IOP time course. Data are expressed as mean±SEM (n=5). ***p<0.001 timolol and travoprost at day 11, travoprost 0.004 at day 10; **p<0.01 timolol and travoprost at day 7,8 timolol at day 6, travoprost 0.004 at day 10; *<0.05 dorzolamide and travoprost at day 4, timolol and travoprost at day 5,12 and 8 travoprost 0.004% at day 9 *versus* vehicle. Two way ANOVA followed by Bonferroni *post hoc* test.

5.2 Histaminergic receptors characterization in New Zealand white rabbits

The presence of histamine H_3 receptor subtype both as protein and mRNA expression in rabbits' eyes opened a possibility for investigations on the role H_3R blockade by three different H_3R antagonists (GSK189254, ciproxifan and DL 76) on IOP reduction in the models of ocular pathology previously validated.

5.2.1 Histaminergic $H_{1\text{-}4}$ receptor protein expression

H_1R and H_4R were found in the retina and optic nerve at a higher concentration compared to those revealed in trabecular meshwork and stomach, which was used as a positive control. High levels of H_3R expression was found in the retina, optic nerve and trabecular meshwork and its presence were confirmed also in the stomach. H_2R was found only in the stomach while it resulted to be undetectable, as a protein, in ciliary bodies and retinae.

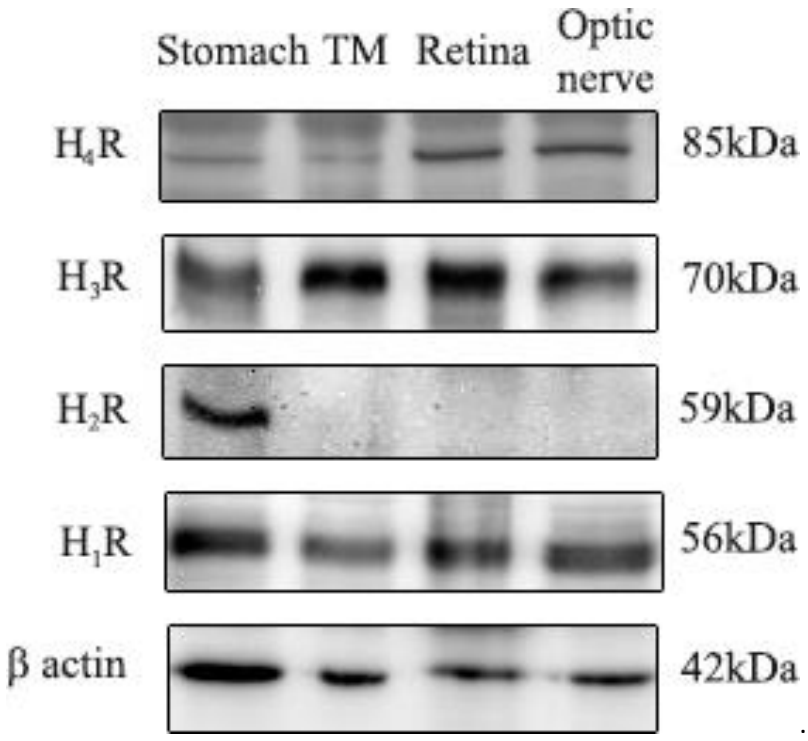


Figure 5.5. Western blot analysis of histaminergic receptors in ocular tissues and stomach

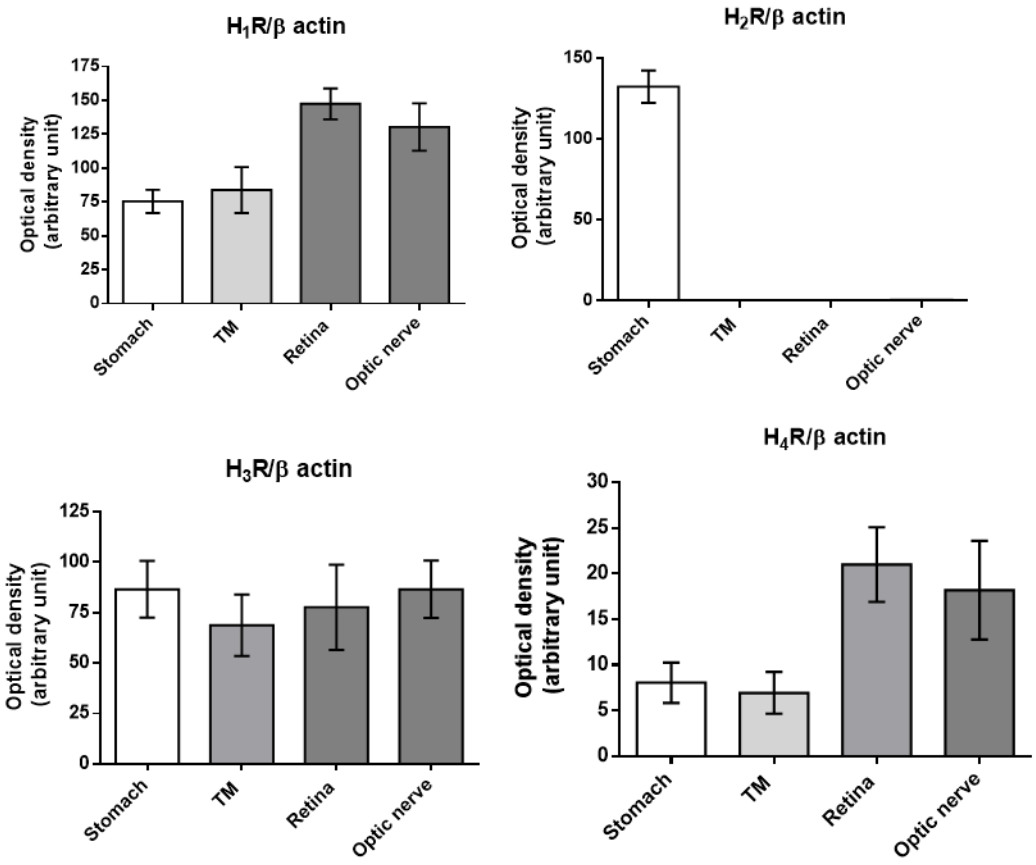


Figure 5.6. Western blot analysis of histamine receptor subtypes in the trabecular meshwork, retina, optic nerve, and stomach. Optical densitometry (n=3).

5.2.2 Histaminergic receptors mRNA expression

The mRNA presence of histaminergic receptors was evaluated using RT-PCR. Hippocampus was used as positive control for H_1 and H_3 receptors, stomach for H_2 receptor and spleen for the H_4 receptor, respectively. The mRNA of the H_2 receptor was found in every tissue studied. H_1 and H_3 receptors' mRNA was found in the retina and ciliary body and in the hippocampus as expected. H_4 receptor's mRNA was found in the retina, ciliary body and in the spleen. The mRNA of H_2R was then present in the same ocular structure where protein expression was not detected by western blot analysis.

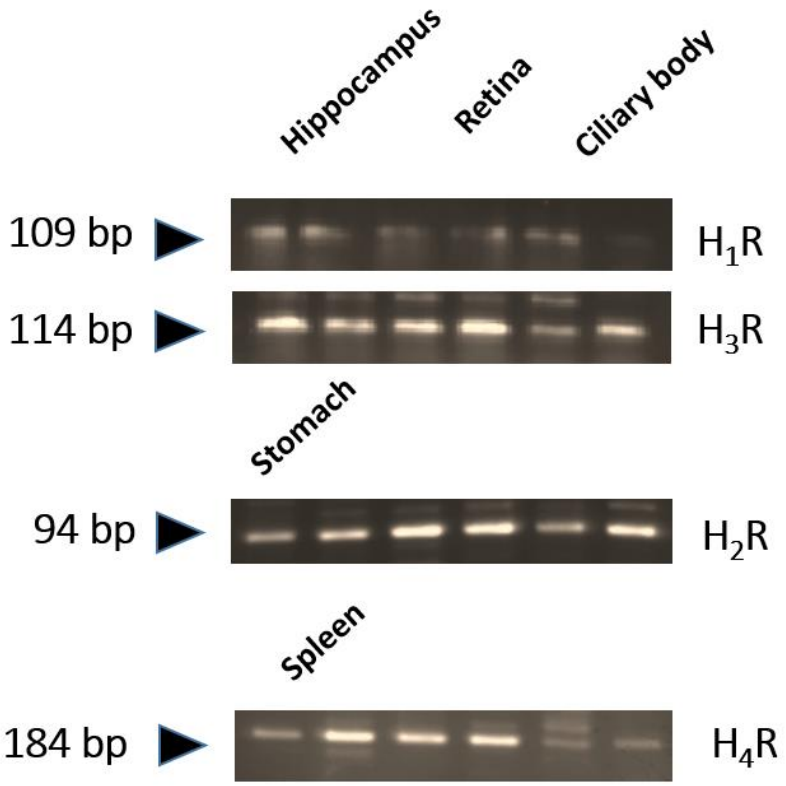


Figure 5.7: Histaminergic receptors mRNA bands

5.1.3 Histaminergic receptors localization in ocular tissue

The immunofluorescence analysis revealed the presence of histamine H_3 and H_4 receptors in the retina, and H_1, H_3 and H_4 in the ciliary body (figure 5.8). H_2 receptor signal was not found confirming the data obtained with WB analysis. In ciliary bodies, H_1 receptor expression was localized mainly in the proximity of vessels (Fig 5.8).

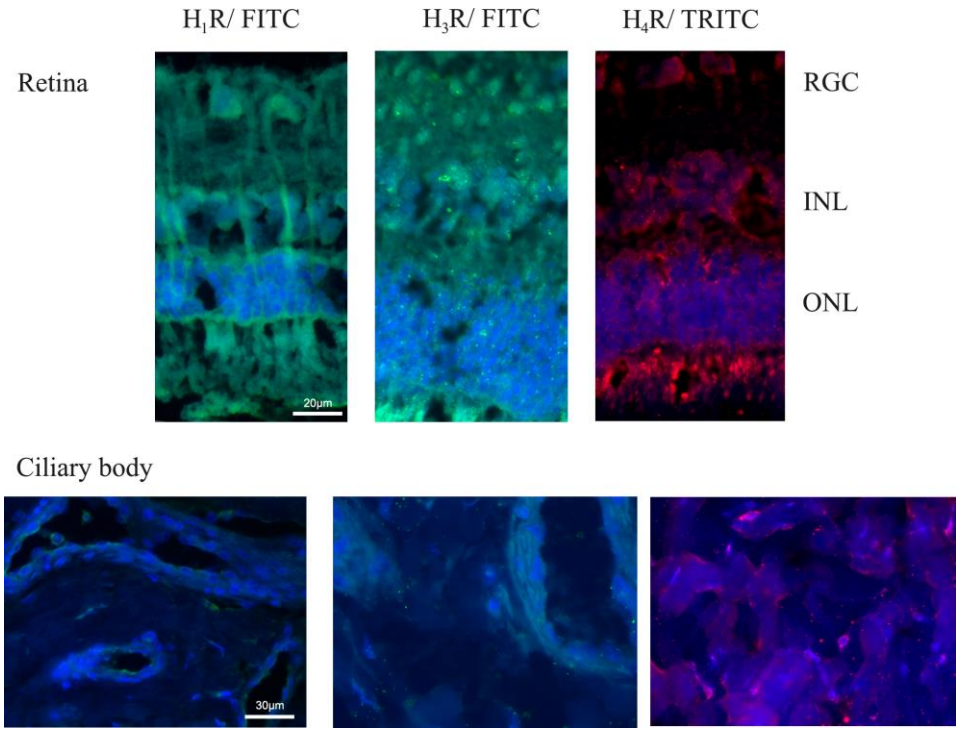


Figure 5.8. Representative images of retinae and ciliary bodies labeled with H_1, H_3, and H_4R antibodies.

At 100x magnification, it was possible to localize H_3 and H_4R at the retinal ganglionic cells (RGC) layer (figure 5.9).

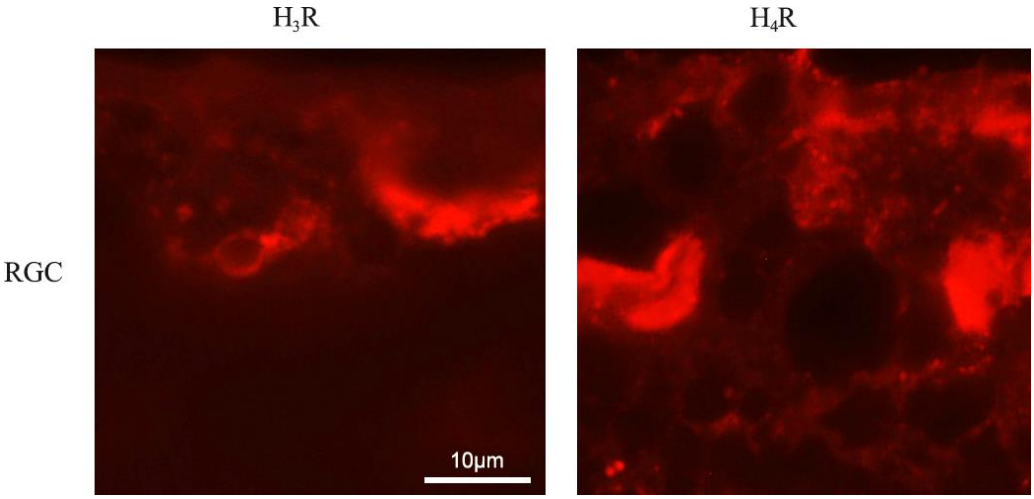


Figure 5.9. Representative images of retinal ganglion cell (RGC) layer

5.3 Pharmacological studies of H_3R antagonists in transient ocular hypertension model

At this point, the establishment of the best dose of H_3R antagonists was carried out in transient ocular hypertension model. This model also provided useful information on the selectivity of H_3R antagonism as the hypotensive effect was blocked both by pretreating the animals with H_3R agonist imetit and H_1R antagonist pyrilamine, suggesting a role for H_1R as well in IOP regulation. The source of histamine was found in choroidal mast cells. Animals preconditioned with C 48/80 did not respond to ciproxifan treatment (no IOP reduction) suggesting that mast cells, in an acute IOP elevation experimental setting, can act as histamine provider cells.

5.3.1 Establishing of the best dose.

In this experimental set, IOP rose from 16.8±5.6 mmHg at baseline to 39.63±4.85 mmHg after hypertonic saline injection. Ciproxifan dose-dependently reduced IOP at 60' after saline injection in a statistically significant manner with $p<0.0001$ with ciproxifan 1% at 60' and 120; $p<0.01$ with ciproxifan 0.5% at 120'; $p<0.05$ with ciproxifan 0.5% at 120' *versus* vehicle) (Figure 5.10). I did not observe any side effects and the drug did not cause any change in pupil diameter.

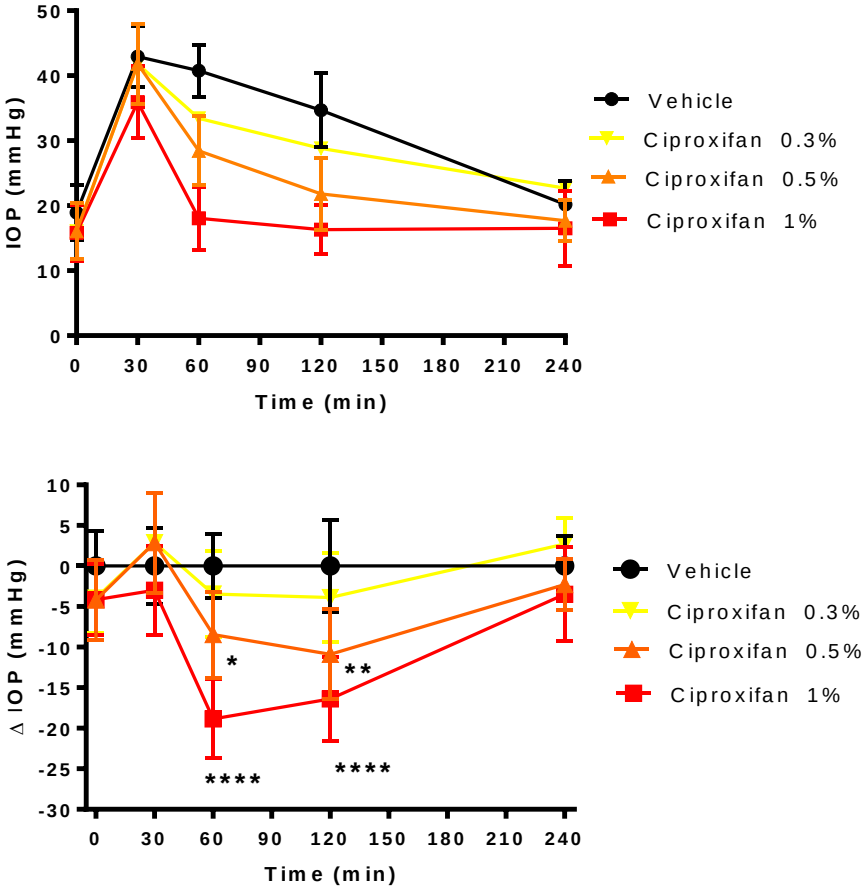


Figure 5.10. IOP time course. Data are expressed as mean±SEM (n=5). ****p<0.0001 ciproxifan 1% at 60' and 120; **p<0.01 ciproxifan 0.5% at 120'; *p<0.05 ciproxifan 0.5% at 60' *versus* vehicle Two-way ANOVA followed by Bonferroni *post hoc* test. .

In this experimental set, IOP rose from 16.8±3.8 mmHg at baseline to 39.5±5.2 mmHg after hypertonic saline injection. DL76 dose-dependently reduced IOP with p<0.0001 for DL76 1% at 60' and 120 and p<0.001 for DL76 0.5% at 60'and at 120 *versus* vehicle (Figure 5.10). No side effects were observed and the drug did not cause any change in pupil diameter.

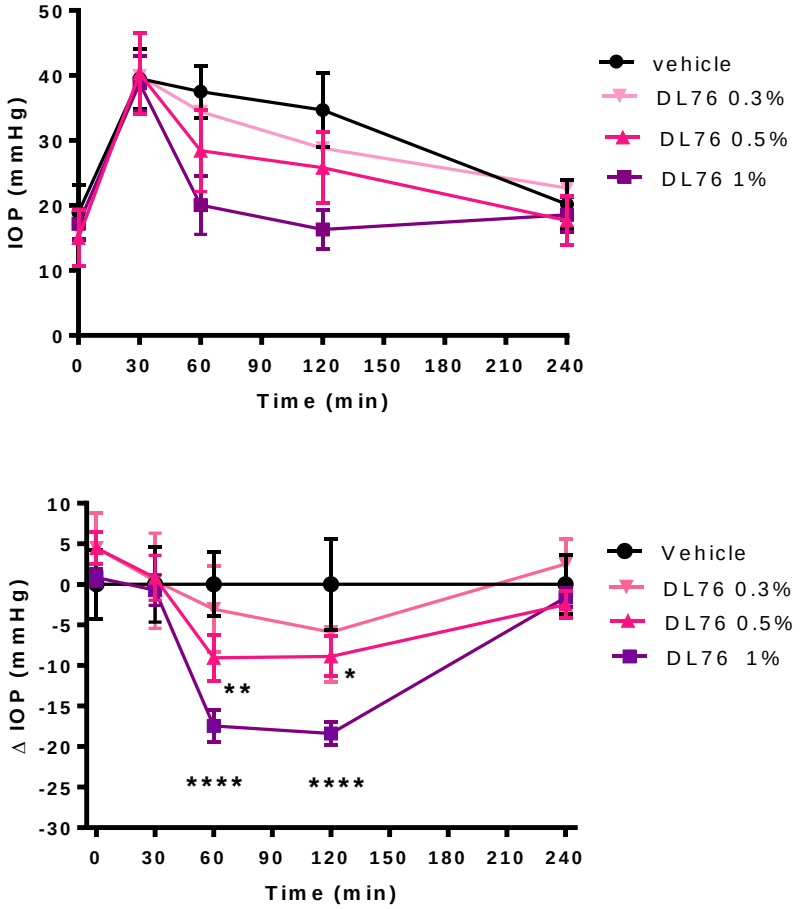


Figure 5.11. IOP time course. Data are expressed as mean±SEM (n=5). ****p<0.0001 DL76 1% at 60' and 120, **p<0.001 DL76 0.5% at 60';*p<0.05 DL76 0.5% at 120' *versus* vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test.

In this experimental set, IOP rose from 14.9±4.2 mmHg at baseline to 40.2±4 mmHg after hypertonic saline injection. GSK189254 0.3 and 0.5% was not effective in reducing IOP. GSK189254 1% (Figure 5.10) reduced IOP in a statistically significant manner with p<0.01 GSK189254 1% at 120' and p<0.05 GSK189254 1% at 60' *versus* vehicle.

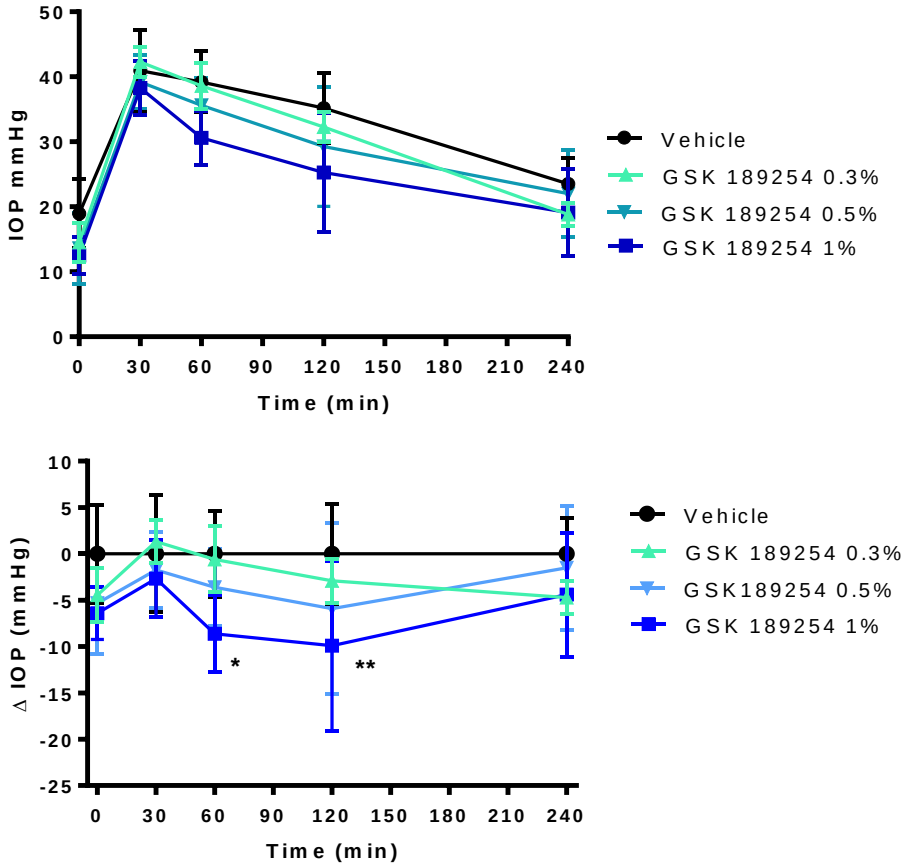


Figure 5.12. IOP time course. Data are expressed as mean±SEM (n=5). **p<0.01 GSK189254 1% at 120', *p<0.05 GSK189254 1% at 60' *versus* vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test.

5.3.2 Comparison between the best H_3R antagonists' doses and gold standard treatment timolol 1%

In this experimental set, IOP rose from 15.7±3.4 mmHg at baseline to 37.7±4.2 mmHg after hypertonic saline injection. Both ciproxifan and DL76 showed an IOP lowering profile very similar to timolol ($p<0.01$ ciproxifan 1% at 60', $p<0.05$ D76 and timolol 1% at 60 and 120' *versus* vehicle). GSK189254 1% in contrast with the previous data was not effective in reducing IOP either at 60 and 120'(Figure 5.13).

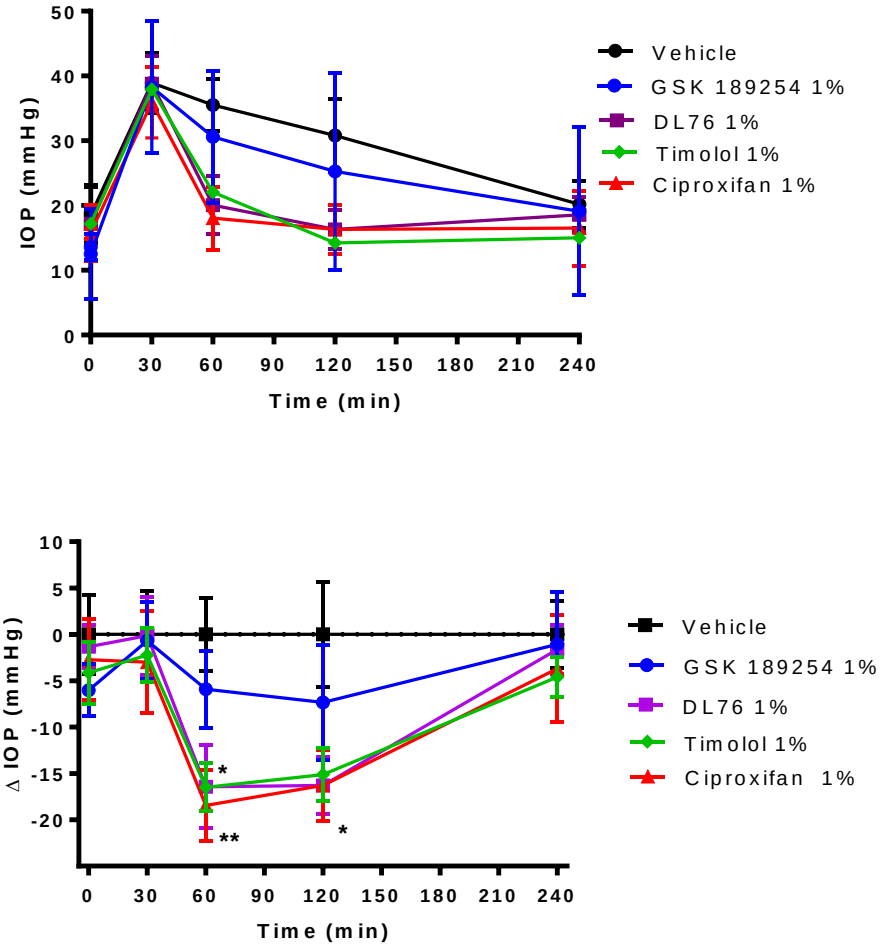


Figure 5.13. IOP time course. Data are expressed as mean±SEM (n=5). **p<0.01 ciproxifan 1% at 60', *p<0.05 D76 and timolol 1% at 60 and 120' *versus* vehicle.Two-way ANOVA followed by Bonferroni *post hoc* test.

5.3.3 Evaluation of specificity of H_3R antagonism action with H_3R agonist Imetit

In this experimental set, IOP rose from 16.2±3.5 mmHg at baseline to 40.2±5.3 mmHg after hypertonic saline injection. The IOP-lowering activity of ciproxifan and DL76 was suppressed by imetit 1%, proving that H_3R antagonism can be specifically counteracted by pre-treatment with an H_3 agonist (Figure 5.14).

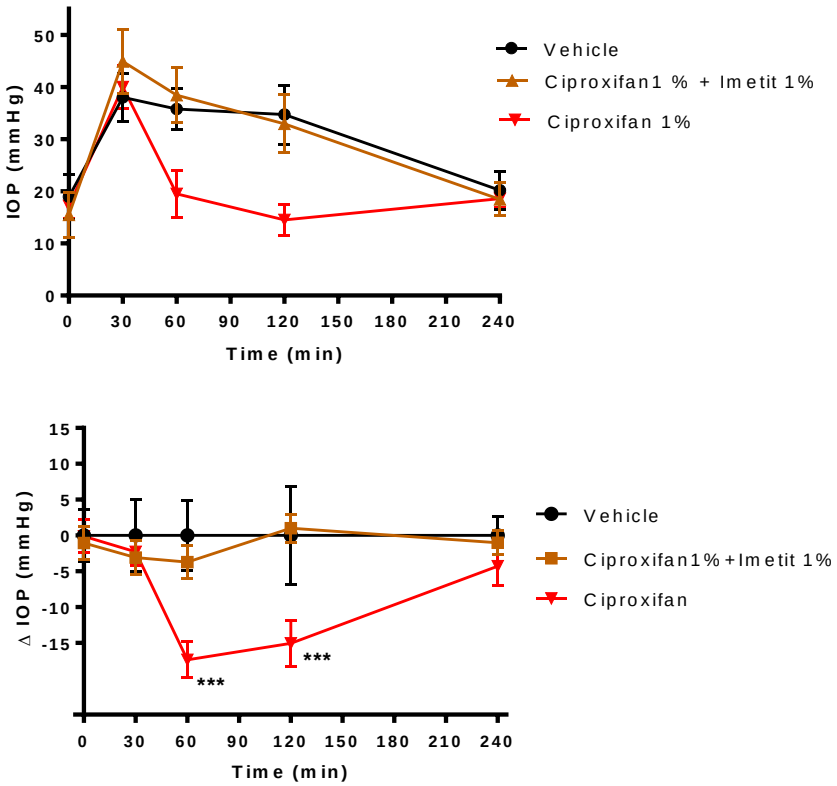


Figure 5.14. IOP time course. Data are expressed as mean±SEM (n=5). ***p<0.0001 ciproxifan 1% *versus* vehicle and *versus* ciproxifan + imetit 1% at 60' and 120'. Two-way ANOVA followed by Bonferroni *post hoc* test.

5.3.4 Role of H_2R agonist amthamine and /H_1R antagonist pyrilamine

IOP rose from 17.8±4.2 mmHg at baseline to 38.5 ±4.4 mmHg 10' after hypertonic saline injection. Ciproxifan % IOP lowering activity was suppressed by imetit 1%, proving that H_3R antagonism can be specifically counteracted by pre-treatment with an H_3 receptor agonist (Figure 5.15).

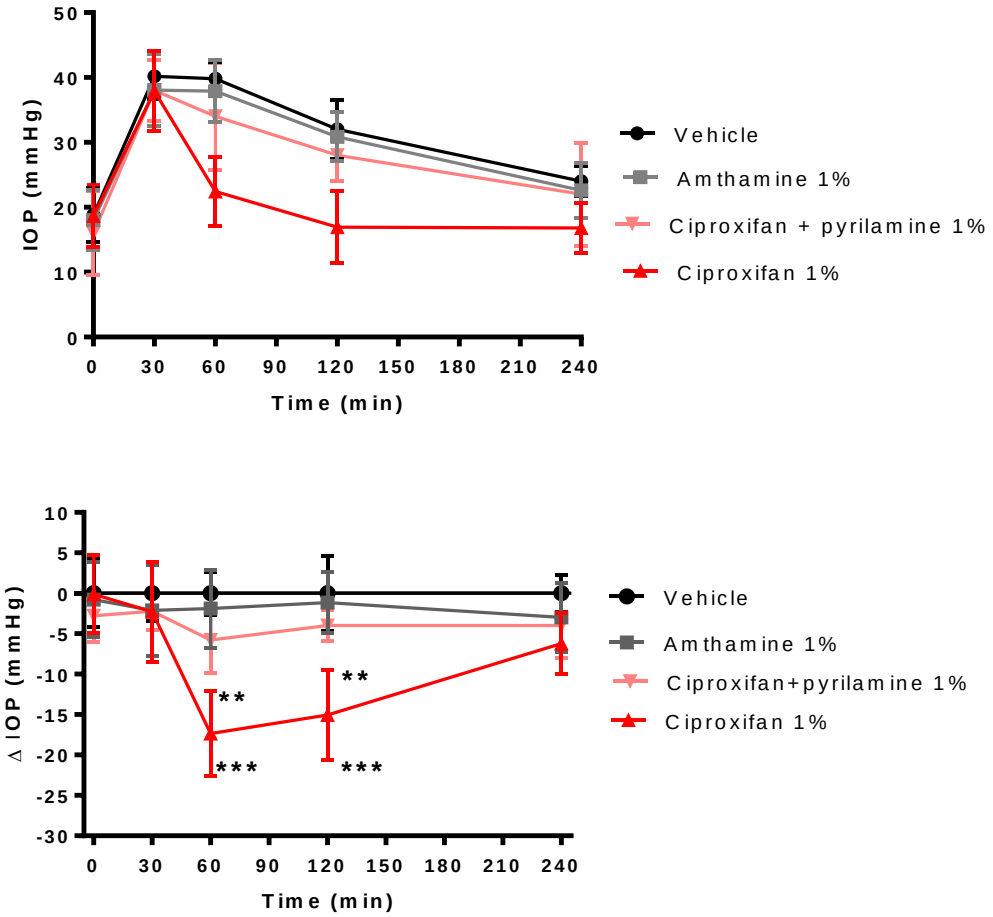


Figure 5.15. IOP time course. Data are expressed as mean±SEM (n=5). ***p<0.001 ciproxifan 1% at 60' and 120 *versus* amthamine and vehicle. **p<0.01 ciproxifan *versus* ciproxifan+pyrilamine.Two-way ANOVA followed by Bonferroni *post hoc* test.

5.3.5 Evaluation of the potential source of histamine with a model of choroidal mast cell granules depletion by compound 48/80 pre-treatment

In this experimental set, IOP rose from 17.7±3.1 mmHg at baseline to 38.8 ±3.7 mmHg 10' after hypertonic saline injection. Ciproxifan 1% IOP lowering activity was suppressed by mast cells' granules depletion (Figure5.16).

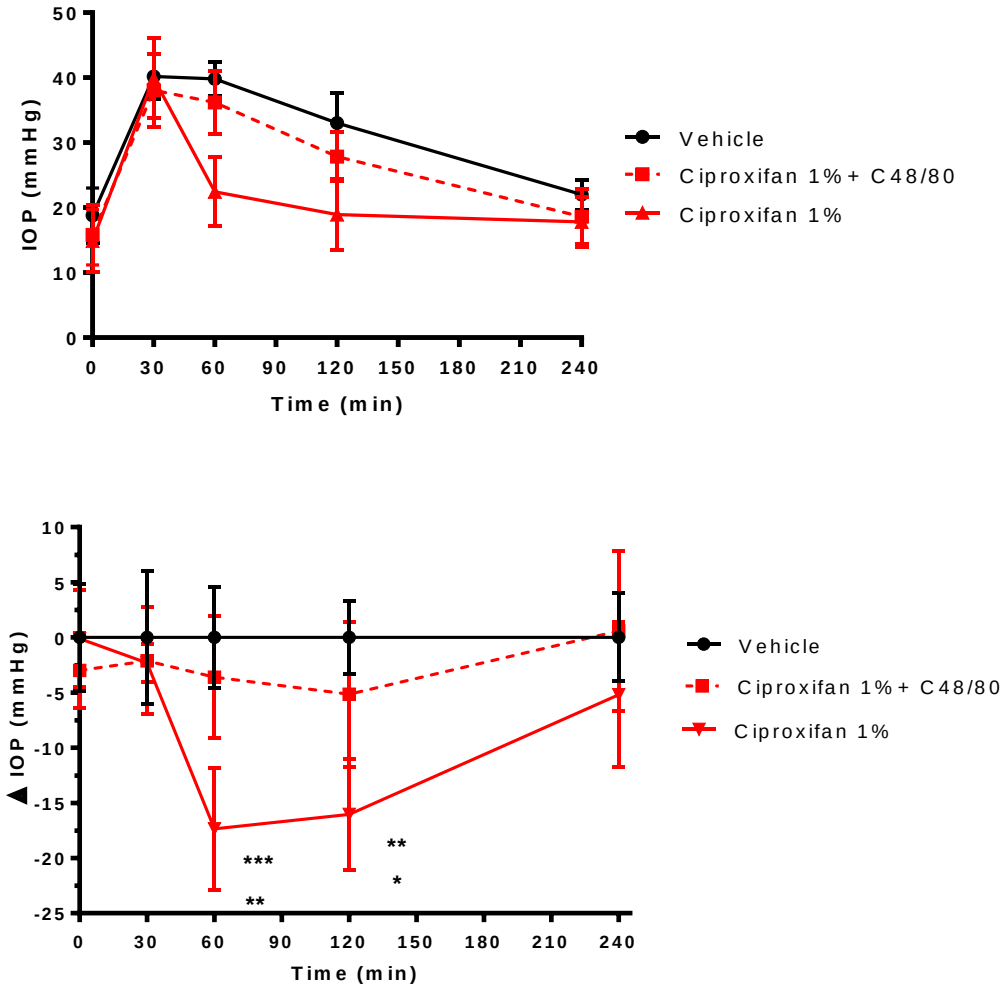


Figure 5.16. IOP time course. Data are expressed as mean±SEM (n=4). ***p<0.001 ciproxifan 1% at 60' *versus* vehicle, **p<0.01 ciproxifan 1% at 60' *versus* ciproxifan + C48/80, ciproxifan 1% *versus* vehicle at 120' *p<0.05 ciproxifan 1% at 60' *versus* ciproxifan+C48/80. Two-way ANOVA followed by Bonferroni *post hoc* test.

The choroids of animals treated 48 hours prior to the IOP profile experiments were characterized by mast cells depleted in histamine granules. The difference in a number of degranulated mast cells between the two groups of animals was statistically significant (Figure 5.17).

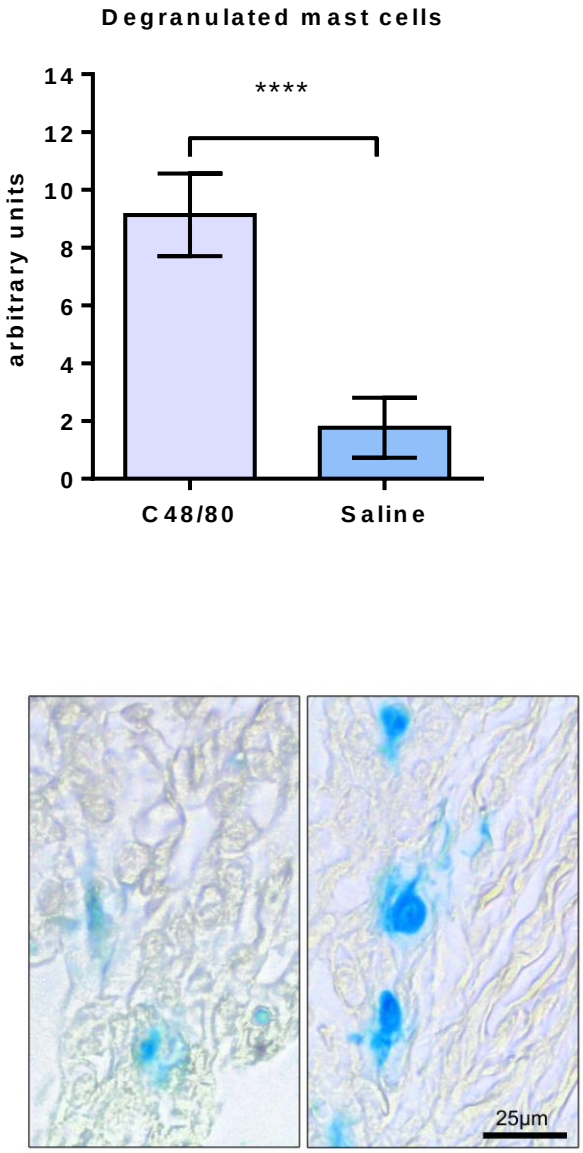


Figure 5.17. ****p<0.0001 C48/80 treated eyes (degranulated mast cells) *versus* saline-treated eyes with *t-test* with Welch's correction with corresponding representative ASTRA blue stained images below.

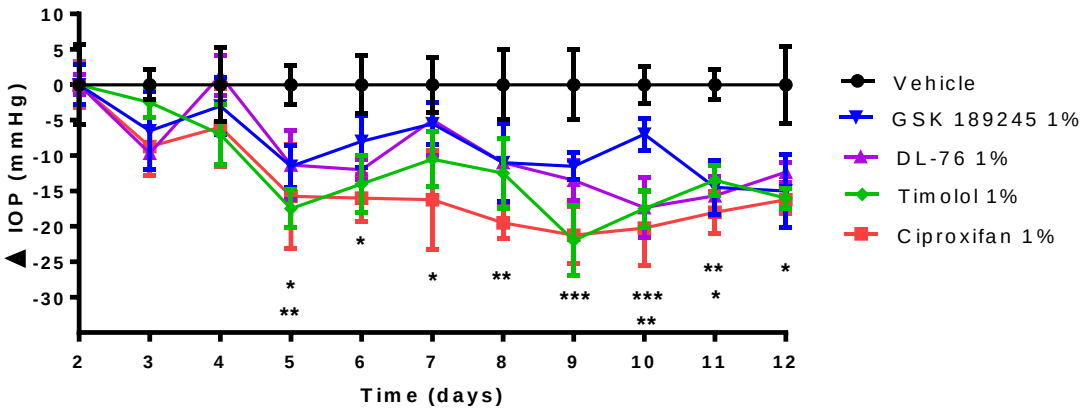
5.4 H_3R antagonists treatment alone and in association with a nitric oxide donor in chronic IOP elevation model.

Chronic treatments with H_3R antagonist was able to reduce IOP in the stable model of OHT showing features similar to the reference drug timolol, and improving the vascular performance of retinic artery. This positive effects i.e, baroprotection and better vascularization contributed to reducing oxidative stress and slowed down the death of RGCs and their substitution by gliosis phenomena. Chronic treatment with H_3 receptor antagonists reduced the hypoxia-induced expression of VEGFA and a favorable modulation of microglia.

A different pattern of protein expression (WB analysis) was found in OHT versus and naïve animals. In the retinae of animals with OHT, the expression in the TM of H_1R was decreased while H_3R was increased. Interestingly in H_3R antagonists treated eyes, the receptor expression was close to naïve/basal level. The H_4R expression was barely detectable in the TM of all experimental groups without significative differences.

5.4.1 IOP reduction with chronic treatment using H_3R antagonist

IOP rose from 13.5±3.8 to 4.1 at 2 days after carbomer injection and remained stable for 2 weeks in vehicle-treated animals**.** The treatment with ciproxifan and DL-76, both 1%, showed a statistically significant reduction of IOP starting from day 5 versus vehicle. The effect was stable throughout the whole experimental session (Figure 5.18).



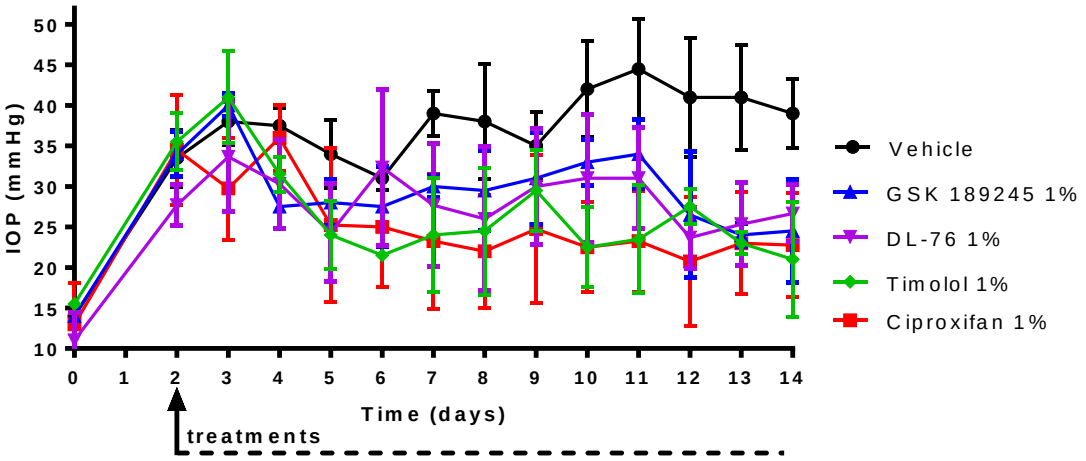


Figure 5.18. IOP time course. Data are expressed as mean±SEM (n=10). ***p<0.001 ciproxifan at day 9 and 10; **p<0.01 ciproxifan day 8, 11 timolol and D76 t day 10.*p<0.05 ciproxifan at day 5, 6, 7, 12, DL76 and GSK 189254 at day 11, 12 and timolol at day 12 *versus* vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test.

The Pourcelot or Resistivity Index (RI) significantly increased in glaucomatous animals compared to naïve ones (animals undergoing the test at the beginning of the experimental set prior to carbomer injection). The treatment with H_3R antagonists ciproxifan and DL76 at the highest dose was able to reduce the RI. The same effect was obtained by chronic timolol treatment (Figure 5.20).

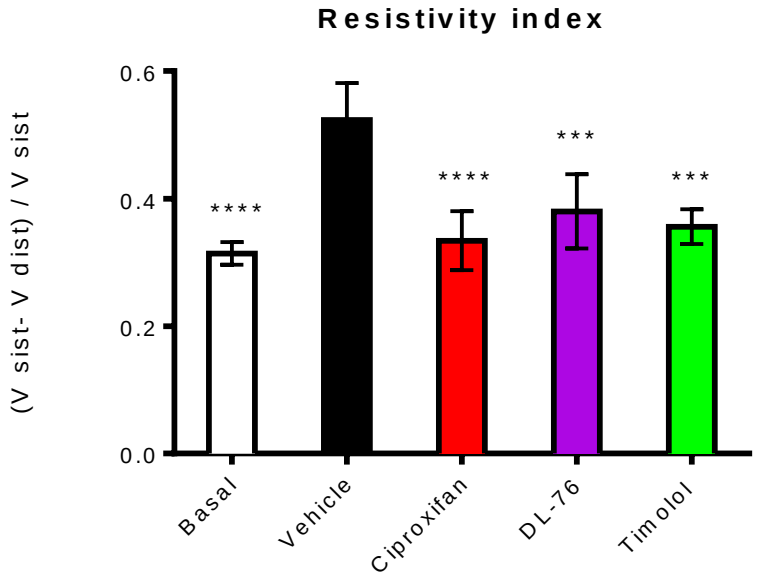


Figure 5.19. Pourcelot or resistivity index. Data are expressed as mean±SEM (n=10). ****p<0.0001 basal (naïve animal) *versus* vehicle (animal with OHT). ***p<0.001 DL76 and timolol 1% *versus* vehicle. One-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

5.4.2 Evaluation of retinal ganglionic cells conservation

The death of retinal ganglionic cells (RGCs) is the main cause of visual impairment in glaucoma. The chronic treatment with H_3 receptor antagonists significantly prevented the cell death of neurons in the RGC layer. The morphological evaluation of timolol treated animal retinae showed a slightly better conservation of ganglionic cells, RGCs (in the upper part of each image with fewer nuclei) appeared to be preserved and therefore visible also in the sections of animals treated with ciproxifan and DL76 (Figures 5.20, 5.21).

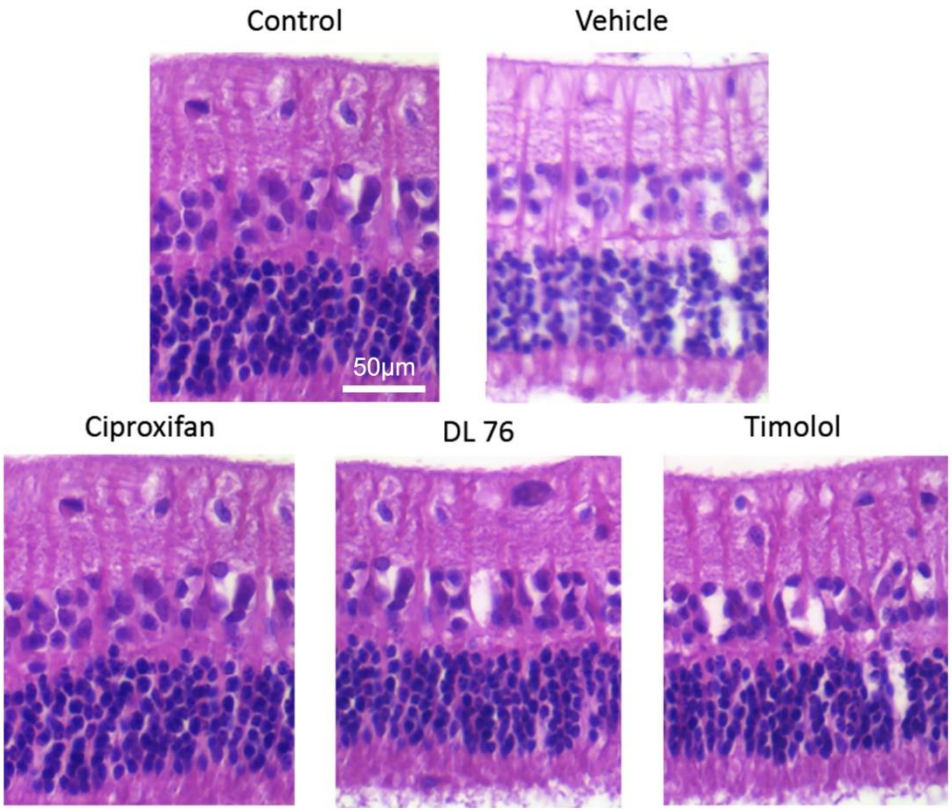


Figure 5.20. Representative images of hematoxylin/eosin-stained histological sections of retinae from different treated groups. RGCs are visible in the upper layer

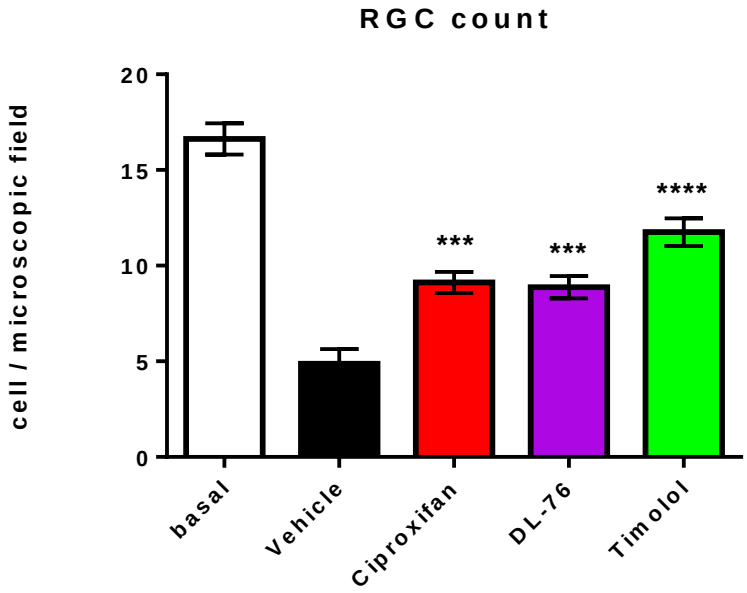


Figure 5.21. Ganglionic cells count. Data are expressed as mean±SEM (n=5). ****p<0.0001 Timolol *versus* vehicle (glaucomatous animal). ***p<0.001 ciproxifan and DL76 a 1% *versus* vehicle. One-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

5.4.3 Evaluation of oxidative stress in retina

Oxidative stress was evaluated qualitatively with dihydroethidium (DHE) staining and quantitatively with 8-hydroxydeoxyguanosine (8-OHdG) level measurement respectively. The morphological evaluation of DHE staining revealed a higher oxidative damage in nuclei of retinal layers of vehicle group animals in comparison with those of control one. The more intense the red the most damage on the DNA caused by oxidative stress. The intensity of red was very strong in the retina of vehicle-treated hypertensive eyes. H_3R antagonists and timolol treated eyes resulted in retinal images with less intense fluorescent red signal meaningless DNA damage. These treatments were able to reduce the oxidative damage (Figure 5.22).

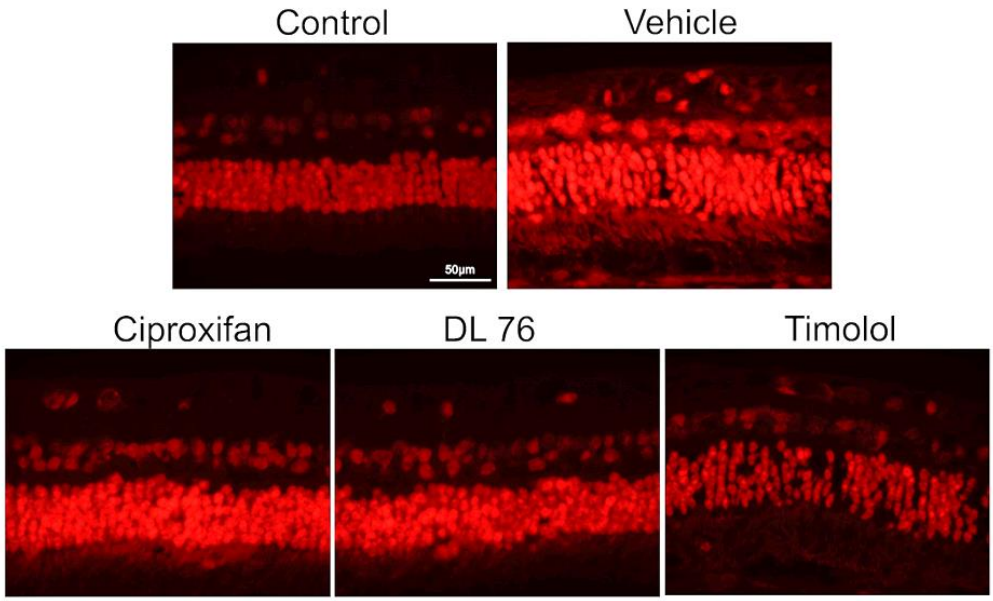


Figure 5.22. Representative images of DHE stained histological sections of retinae.

The 8-OHdG assay quantitatively confirmed the data obtained with DHE staining evaluation. The findings confirm that H_3 receptor antagonists and timolol treatments were effective in preventing ischemia-induced oxidative stress (Figure 5.23).

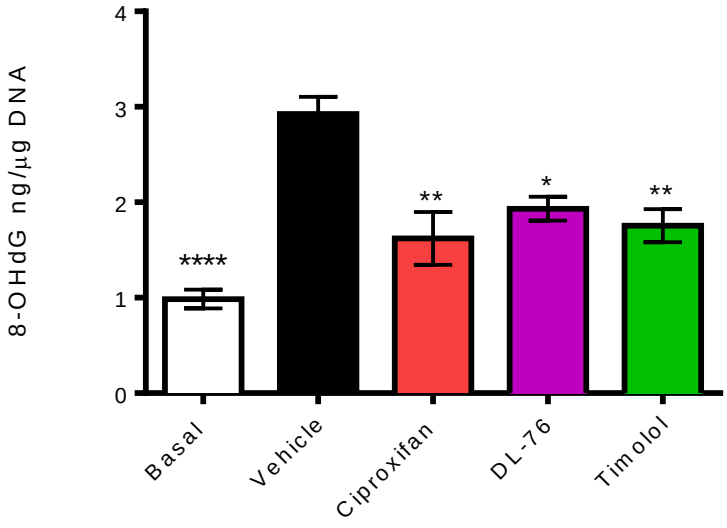


Figure 5.23. 8-OHdG level measurements. Data are expressed as mean±SEM (n=5). ****p<0.0001 basal (naïve animal) *versus* vehicle (glaucomatous animal). **p<0.01 ciproxifan and timolol 1%; p<0.05 DL76 *versus* vehicle. One-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

5.4.4 Measurement of retinal hypoxia modulation

The histological sections of induced glaucoma animals showed an intense GFAP positivity in the RGCs layers if compared to those of control animals. The increased level of glial fibrillary acidic protein (GFAP) is a reliable marker of gliosis. This result strongly supports the datum of RGCs loss observed in hematoxylin/eosin-stained retina, suggesting a replacement of apoptotic RGCs by glial cells. Gliosis is less evident in treated eyes.

Furthermore, chronic treatment with H_3 receptor antagonists modulated the hypoxia-induced expression of VEGFA. The red signal intensity is increased in vehicle *versus* basal sections and reduced compared to the representative images from the eyes of animals treated with H_3 receptor antagonists and timolol (Figure 5.24).

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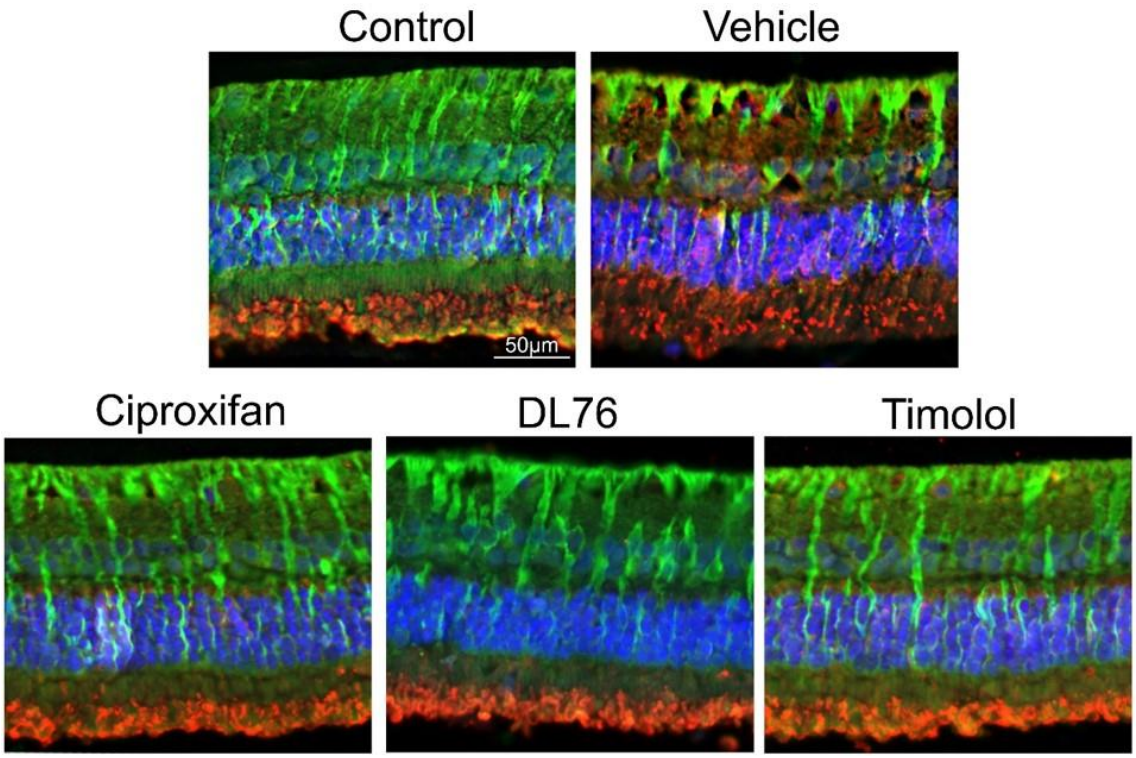

Figure 5.24. Representative images of double-labeled retinae. In green GFAP (glial cells) in red VEGFA, in blue DAPI (nuclei).

5.4.5 Analysis of retinal microglial modulation

Chronic treatment with H_3 receptor antagonists modulated the microglial activation in the retina. The immunofluorescence analysis of CD11b, a reliable marker of activated microglia, revealed a profound increase of activated microglial cells in a retinal section of glaucomatous eyes. The intensity of red i.e activated state of microglial cells was reduced in the representative images of retinal samples of animals treated with H_3 receptor antagonists and timolol. An opposite expression profile was found in the immunofluorescence reaction of IBA1, a marker of resting microglia (Figure 5.25).

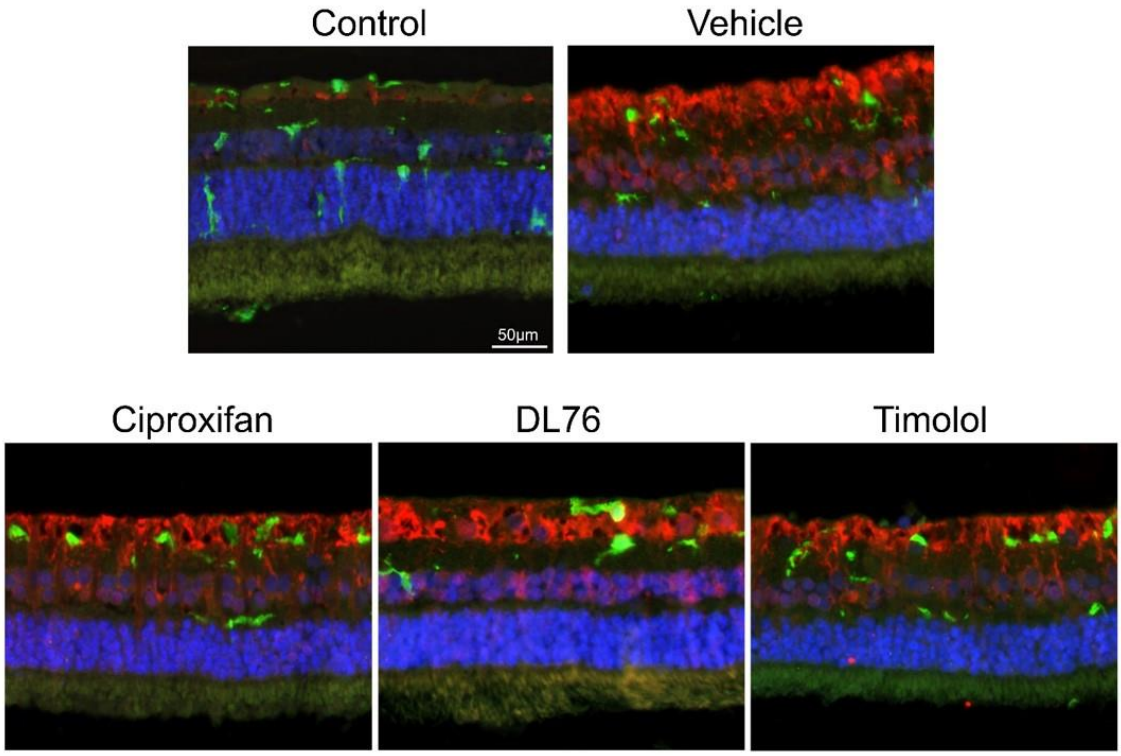

Figure 5.25. Representative images of double-labeled retinae. In red CD11B (activated microglia, in green IBA1 (resting microglia), in blue DAPI (nuclei).

5.4.6 Histaminergic receptor subtypes expression in retina and TM in chronic ocular hypertension

The western blot analysis revealed a decreased level of H_1R expression in animals with OHT treated with vehicle The H_3R antagonism was able to restore the H_1R expression at the basal level, showing a baroprotective effect. No difference in expression was noticed for retinae with this receptor subtype. H_3 receptor expression appeared to be increased in TM of vehicle-treated OHT animals. Once again, the administration of H_3R antagonists restored the receptor expression close to naïve/basal level. The H_4R expression was barely detectable in the TM of all experimental groups without significative differences (Figures 5.26 and 5.27).

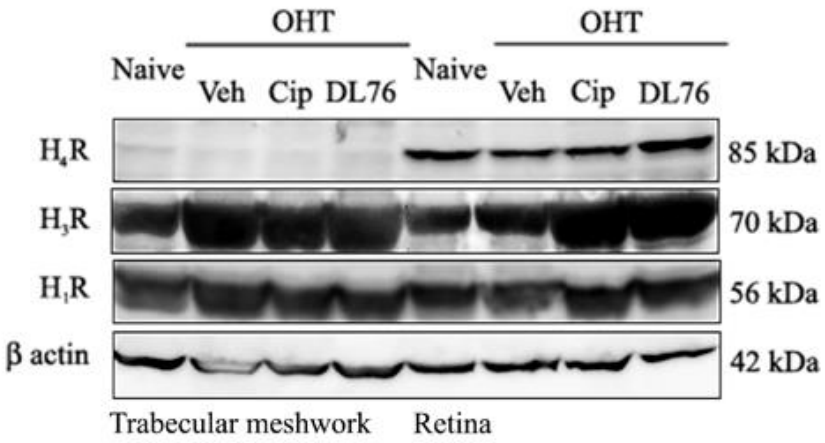

Figure 5.26. Western blot analysis of histaminergic receptors in trabecular meshwork and retina in naïve and glaucomatous conditions belonging to vehicle, ciproxifan and DL76 groups. OHT: ocular hypertension.

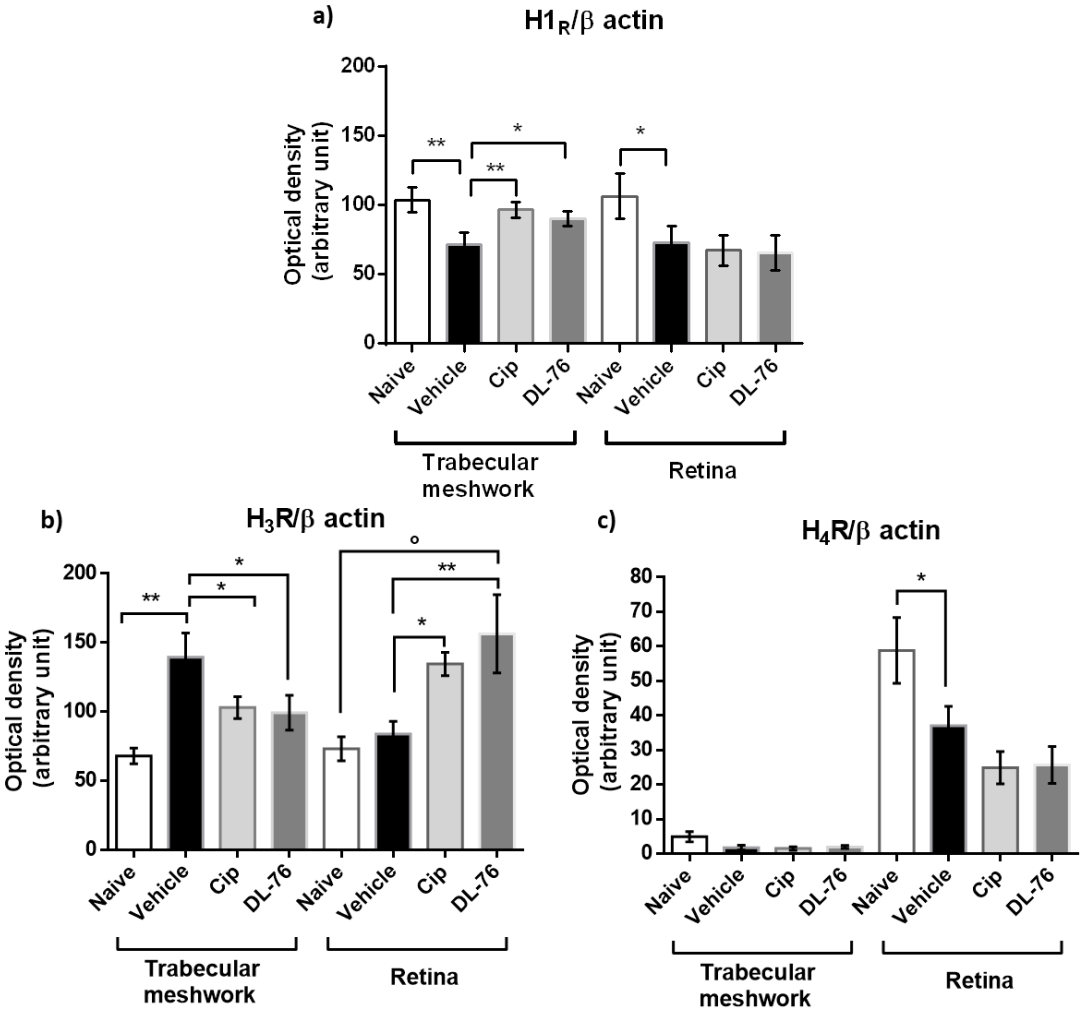

Figure 5.27. Optical densitometry of protein expression of histaminergic receptors in trabecular meshwork and retina in naïve and glaucomatous conditions (OHT) belonging to the vehicle, ciproxifan, and DL76 groups. Data are expressed as mean±SEM (n=3).

Panel a): H_1 receptor expression: **p<0.01 naïve and ciproxifan *versus* vehicle; *p<0.05 DL76 *versus* vehicle in the trabecular meshwork. *p<0.05 naïve *versus* vehicle in the retina. Panel b): H_3 receptor expression: **p<0.01 naïve *versus* vehicle; *p<0.05 DL76 and ciproxifan *versus* vehicle in the trabecular meshwork. **p<0.01 DL76 *versus* vehicle; *p<0.05 ciproxifan *versus* vehicle and p<0.05 naïve *versus* vehicle in retina *p<0.05 ciproxifan *versus* vehicle and p<0.05 naïve *versus* vehicle in the retina. Panel c): *p<0.05 naïve *versus* vehicle in the retina. One-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

5.5 H_3R antagonist and NO-donor combined chronic treatment

These experiments demonstrated that the pharmacological combination of an H_3R antagonist with an NO-donating compound had a boost like effect on IOP reduction and significantly reduced the Resistivity Index.

5.5.1 IOP reduction with ciproxifan and molsidomine chronic treatment

IOP rose from 14.6±3.6 to 39.5±4.3 48 hours after carbomer injection and remained stable for 2 weeks in vehicle-treated animals**.** The combined treatment with ciproxifan and molsidomine both at 0.5% showed a statistically significant reduction of IOP starting from day 5 *versus* vehicle. IOP reduction was stable during all the experimental session (Figure 5.28)

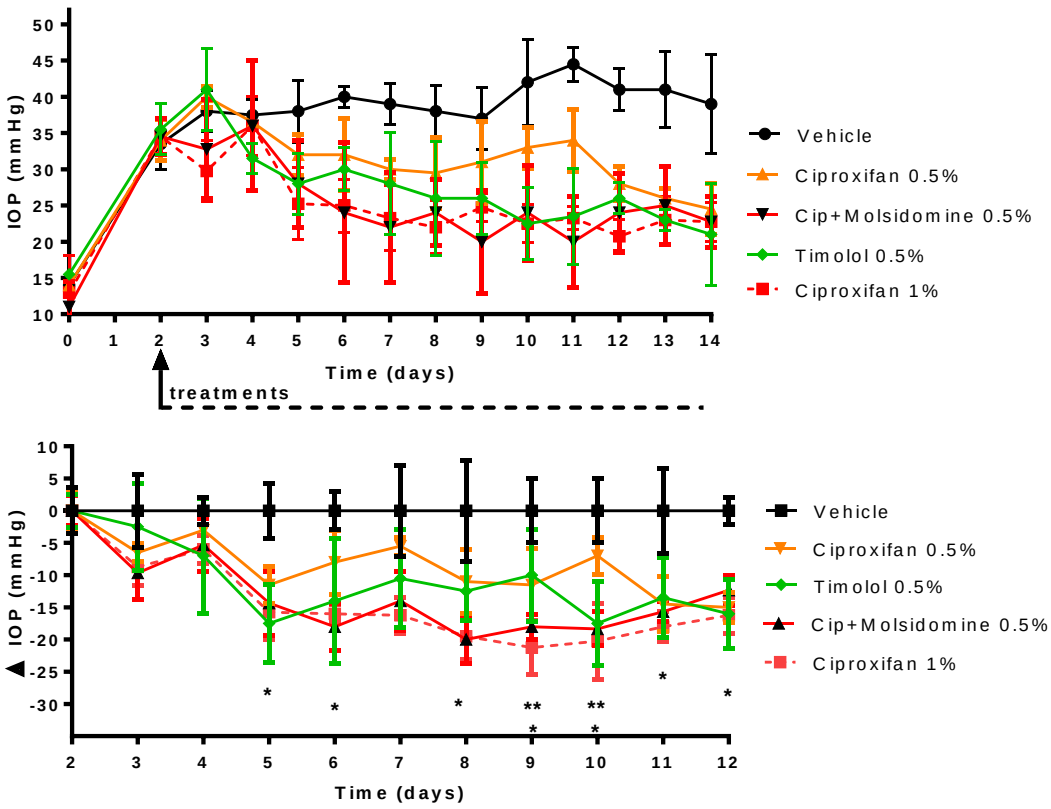

Figure 5.28. IOP time course. Data are expressed as mean±SEM (n=5). **p<0.001 ciproxifan 1%, cip+molsidomine at day 10; *p<0.05 timolol at day 5, 10, cip+molsidomine 0.5% at day 6, 8, 9, 10; ciproxifan 1% at day 8, 11, Timolol at day 10 *versus* vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test.

5.5.2 Analysis of vascular performance of retinal central artery

Resistivity Index (a ratio) resulted to be 0.32 in naive animals and 0.50 in glaucomatous vehicle-treated animals. The co-treatment with ciproxifan and molsidomine resulted in a more powerful statistically significant reduction of RI in ameliorating the vascular performance of the retinal central artery (Figure 5.29).

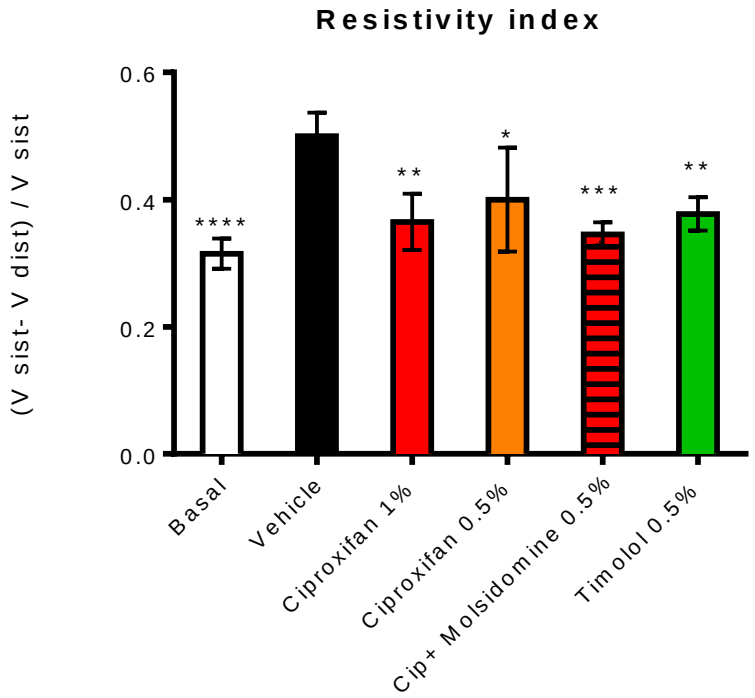

Figure 5.29. Pourcelot or resistivity index. Data are expressed as mean±SEM (n=6). ****p<0.0001 basal (naïve animals) *versus* vehicle (glaucomatous animals). ***p<0.001 ciproxifan+molsidomine 0.5%; **p<0.01 ciproxifan 1%; *p<0.05 ciproxifan and timolol 0.5% *versus* vehicle. One-way ANOVA followed by Bonferroni multiple comparison *post hoc* test.

5.6. Preliminary investigation on the mechanism of action of H_3R antagonist ciproxifan on TM cells

The results presented in this last subchapter are to be considered as preliminary. Once the cells were isolated and stabilized with four passages, they showed features similar to those described for these cells (Weinreb *et al.*, 1977). TM cells were identified by immunofluorescence analysis of the positive and negative markers. Furthermore, H_3R was found on the cells and the administration of an H_3R antagonist produced a modification of intracellular calcium concentration. This effect was recorded in 4 different experimental settings but still has to be considered not conclusive.

5.6.1 TM cells isolation and characterization

The morphological evaluations carried out on the TM isolated cells included the evaluation of the cellular shape that resulted to be similar to the one described in the literature for human-derived TM cells (Stamer and Clark, 2017). More importantly these cells were found positive for the α-SMA, aquaporin 1, H_3R, and negative for MYHC vimentin (Figure. 5.31 and 5.32).

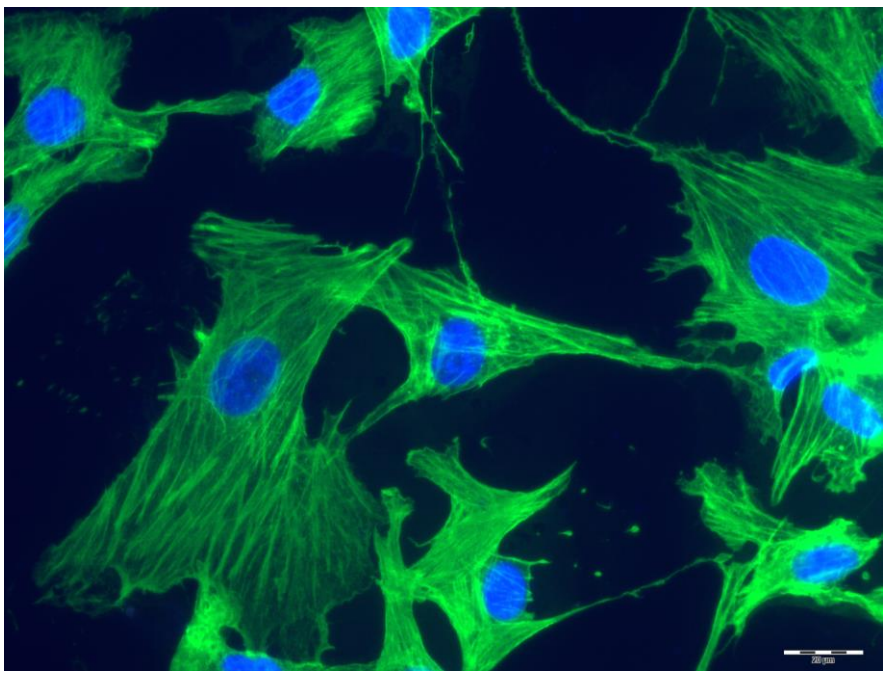

Fig. 5.30**.** Immunofluorescence staining of TM cells: α-SMA signal (green), DAPI (blue) nuclei. 60x magnification.

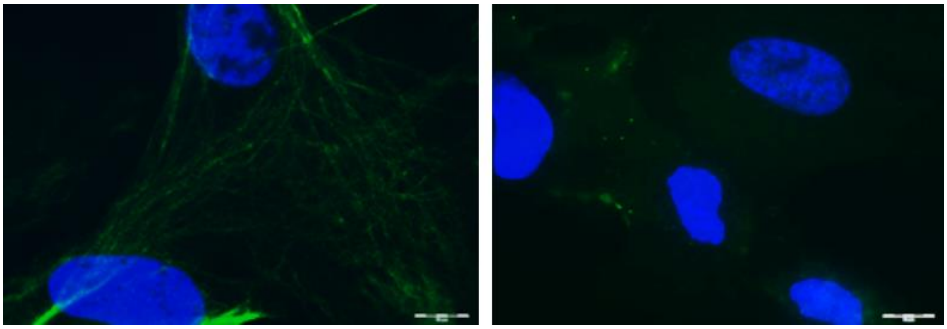

Figure 5.31. Immunofluorescence staining of TM cells: left panel) aquaporin1 (green), DAPI (blue) for nuclei; right panel) H_3R (green), DAPI (blue) for nuclei. 100x magnification.

To characterize the primary culture cells isolated from trabecular meshwork I tested their ability to express myocilin by exposing them to dexamethasone (DEXA) for 5 days. The expression of myocilin was revealed with western blot analysis. As reported in Figure 5.32, myocilin expression was dose-dependent.

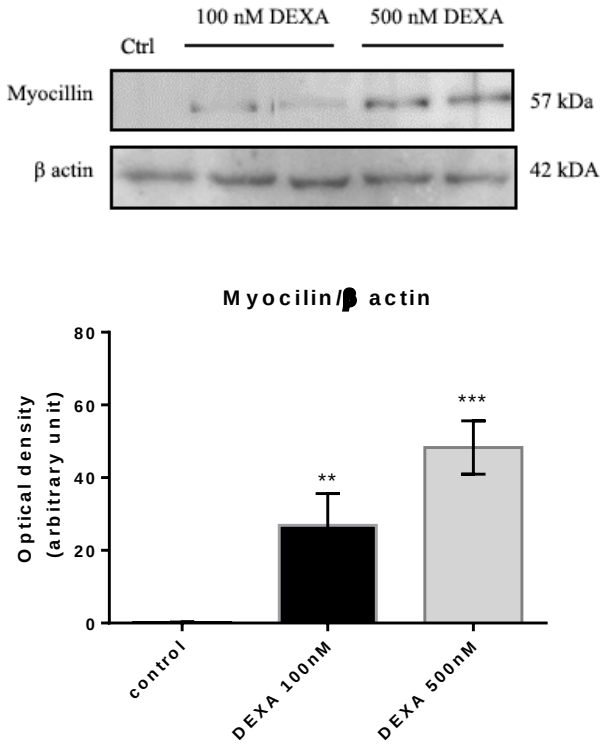

Figure 5.32. Optical densitometry of protein expression of myocilin in 100 nM and 500 nM DEXA conditioned TM cells normalized with β-actin. ***p<0.001 Dexamethasone treated cells 500nM *versus* control; **p<0.01 Dexamethasone treated cells 100nM *versus* control.

5.7.2 Effects of H_3R antagonism on intracellular calcium concentration in TM cells

Ciproxifan treatment (100μM) of TM cells suggests a direct effect on the change of intracellular concentration of calcium. At this concentration, the pharmacologic effect of ciproxifan can be nonspecific. No effects were observed with solutions 10 μM histamine, 10 μM Acetylcholine, 10–100 μM carbachol or 0.1% DMSO (vehicle). The cells responded with a full signal to the positive control ionomycin 5μm. These results are preliminary for the low number experimental sets and the absence of a G protein associate agonist or antagonist to use as a positive control.

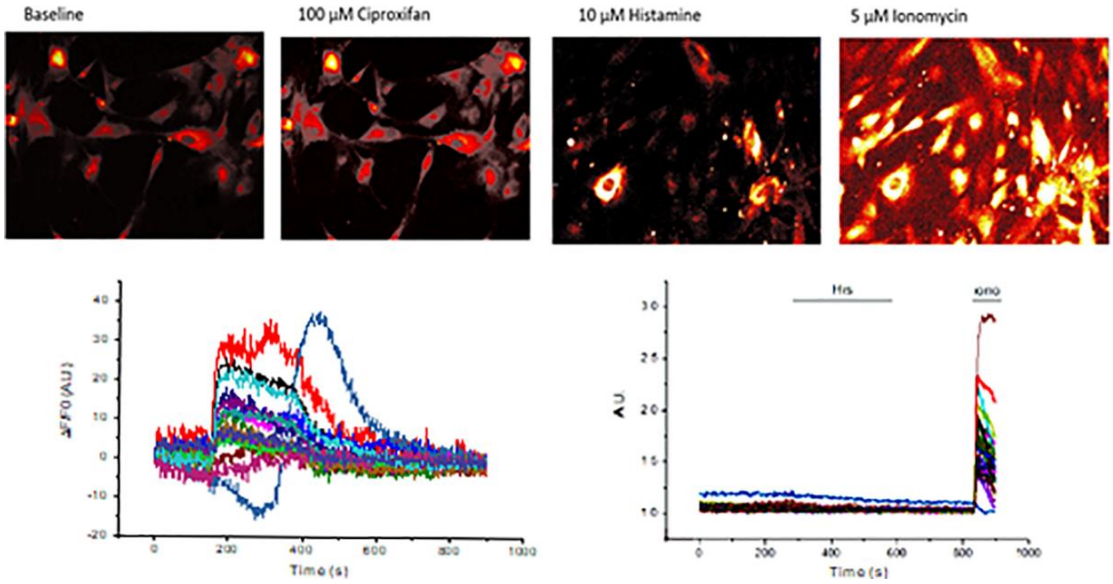

Figure 5.33: Fluo 3-AM response to different treatments. Calcium fluorescence quantification as ΔF/F (graphs below). Each line corresponds to a different single cell response.

6. DISCUSSION

Many different pharmacological approaches are available for the treatment of glaucoma, a very common ocular pathology that affected 60.5 million people in 2010 worldwide, representing the most common preventable cause of blindness (Quigley and Broman, 2006). Despite the high prevalence, relatively little progress has been made in glaucoma management over the past decades. Beta-blocking agents, such as timolol, prostaglandin derivatives, as $PGF_{2\alpha}$ synthetic analog latanoprost, and finally carbonic anhydrase inhibitors, as dorzolamide, are effective pharmacologic treatments for many patients but not for all of them and side effects, even severe ones, limit their use in therapy. Many patients with glaucoma require more than one ocular hypotensive medication to achieve and/or maintain their target IOP and the use of multiple topical glaucoma preparations is associated with factors that may decrease treatment efficacy and increase adverse effects (Holló *et al.*, 2014).

Therefore, an increasing number of patients request surgical interventions and novel pharmacological treatments are needed (Bettin and Di Matteo, 2013). One major advance on was made in 2013 with the introduction in the Japanese Pharmacopeia of Rho-associated kinase (ROCK) inhibitors (Inoue and Tanihara, 2013). ROCK inhibitors lower IOP by a unique mechanism, namely the depolymerization of intracellular actin in the conventional outflow tissues and suppress the production of extracellular matrix by TM cells, representing a novel and alternative and method to reduce IOP. Considering that conventional outflow is a dominant pathway in humans, IOP-lowering ROCK inhibitors are able to treat the glaucomatous eye. Ripasudil hydrochloride was the first ROCK inhibitor approved in Japan for clinical use for glaucoma and ocular hypertension. The efficacy of ripasudil has been confirmed in clinical trials but still, this drug is used as an adjunctive medication to prostaglandin analogs and/or adrenergic β-receptor antagonists (Inoue and Tanihara, 2017). This suggests that even this idealistic approach is limited in clinical practice by the presence of nonresponders and that combined pharmacological approach is always on the horizon.

Baroprotection is not easy to achieve and 24 hour IOP regulation is not always obtained especially when glaucoma is treated with selective laser trabeculoplasty (Kiddee and Atthavuttisilp, 2017). In patients enrolled in the Advanced Glaucoma

Intervention Study (AGIS) long-term IOP circadian fluctuation is associated with disease progression (Caprioli and Coleman, 2008). IOP has a circadian rhythm, aqueous humor production decreases during the night in diurnal mammals like rabbits, drainage facility decreases as well and, as a result, IOP increases at night (Smith and Gregory, 1989)

The posterior hypothalamus is involved in circadian rhythm regulation and histamine is one of the neuro-hormones implicated in the arousal system (Haas *et al.*, 2008)**.** Knowledge on the importance of histamine in the central nervous system is constantly on the rise from circadian rhythm regulation (Lin *et al.*, 2011), to migraine (Yuan and Silberstein, 2017), cognitive functions (Passani *et al.*, 2017), feed behavior (Provensi *et al.*, 2016) but a little is known about histaminergic system in the eye.

Many induced glaucoma models have been developed over the decades to create the proper conditions for controlled experiments like: the posterior chamber injection of α-chymotrypsin, the laser-induced TM blockage or the subconjunctival injection of betamethasone (A. Bouhenni *et al.*, 2012). Among these models, the carbomer model was chosen in order to examine both the onset and the pathological progression in a controlled, reproducible manner (Xu *et al.*, 2002; Fabrizi *et al.*, 2012). There are some limitations making rabbits unsuitable for glaucoma research and these include the cost of the animals and the fact that most of antibodies available on the market to perform immunological-based assays are developed in this species. It is not easy to find the suitable antibodies developed in hosts different from rabbits. The choice of using rabbits was led by the fact that the rabbit eye is relatively large, which makes it a good model for eye research. Furthermore, the dimension of the eyes in these animals are very similar to those of humans and this is a very useful feature to study the pharmacokinetics of a new compound.

The results strongly indicate that the histaminergic system plays an important role in IOP regulation. In fact, histamine receptors resulted to be expressed in a neuronal and nonneuronal compartment in the eye, as well as, in TM derived cells.

The results presented in this thesis clearly indicate that topical treatments with H_3 receptor antagonists are effective in reducing IOP both in transient and stable ocular hypertension animal models with a chronic treatment experimental setting. Topical treatments with both imidazolic molecule, ciproxifan, and non-imidazole ones, such as compounds DL76 and GSK189254, were effective in decreasing IOP at 60 and 120

minutes beyond the 25% reduction target, in a dose-dependent way, in transient OHT model. This effect was counteracted by pre-treatment with the H_3 agonist imetit, suggesting that the pharmacologic mechanism was specific for the H_3 receptor subtype.

Histamine H_1 receptor antagonism was also able to block ciproxifan induced IOP reduction, suggesting that the observed effect could be mediated by endogenous histamine release in this para-physiologic IOP elevation model. To confirm this hypothesis and to investigate the source of histamine, I depleted mast cells in the choroid tissue of their granule content with compound 48/80 (Masini *et al.*, 1991) and, as expected, I could not observe any decrease in IOP. These experiments suggested that mast cells act as histamine providing cells in this experimental setting. It is important to consider that, in the central nervous system, approximately 50% of HA belongs to the non-neuronal pool (Cacabelos, 1990). The presence of mast cells in meninges and perivascular locations of the brain blood barrier indicates that this cell type is relevant in neurovascular responses (Cacabelos *et al.*, 2016).

Histamine could interact with H_1 receptor, *via* Gq, phospholipase C, intracellular calcium increase and subsequently cell contraction. It has been previously demonstrated (Markwardt *et al*., 1996; 1997) that histamine, via H_1 receptor, stimulates the production of inositol phosphate and mobilization of intracellular calcium in cultured human ciliary muscle cells reducing IOP, and it is important to consider that this effect was observed only in a model of transient OHT. The transient OHT model is far from naturally occurring OHT and glaucoma and the effects that can be measured in this model are limited to a very narrow window of observation. This model can be used easily to perform pharmacokinetic experiments but not for collecting biological samples to study and measure the effects of a treatment.

Naïve rabbits were used to evaluate the presence of histamine receptors that resulted to be expressed in retina, ciliary bodies both as proteins and mRNA, with the exception for H_2 receptor that was expressed as mRNA but as a protein. Moreover, I did not observe any biological action with the H_2 receptor agonist, amthamine. This histamine receptor subtype is localized in old world monkeys in a very specific spot in the retina, i.e. the cone pedicle (Vila *et al.*, 2012), but the protein expression was not quantified.

Confirming a previous result present in the literature, the H_1 receptor was localized in ciliary bodies, preferentially on endothelial cells (Marshall, 1984). Histamine H_3 receptors have been localized at the tips of ON-bipolar cell dendrites, in cone pedicles and rod spherules, closer to the photoreceptors than to the other neurotransmitter receptors (Gastinger *et al.*, 2006).

One major difficulty of this research was that antibodies are very hard to find if you use rabbits as an animal model of pathology. Moreover, all the antibodies to histamine receptors that were available are considered only partially reliable.

The demonstration of histamine H_3 receptor subtype in the ciliary body and trabecular meshwork, not only in the retina, was an original result. The trabecular meshwork is a specialized tissue responsible for draining the majority of AH from the anterior chamber of the eye, thereby controlling the IOP. The localization of H_3 receptor at this level is therefore particularly interesting.

Histamine H_3 receptors are expressed not exclusively in neurons and not only at a pre-synaptic level (Zhang *et al.*, 2013). Histamine H_3 receptors are expressed on distinct endocrine cell types in the rat fundi mucosa (Morini *et al.*, 2008); in the apical membrane near collecting duct cells, in the kidney of rats, and their expression is significantly increased in diabetic animals, suggesting its involvement in fluid homeostasis (Pini *et al.*, 2015).

The results here reported clearly indicate that the expression of H_3 receptor subtype was significantly increased in tissue samples obtained from animals with chronic ocular hypertension and ciproxifan and DL76 treatment modulates the overexpression of this receptor. It is important to emphasize that the IOP lowering effect observed in the chronic model of glaucoma was established starting from the third day of treatment, suggesting a mechanism of action that involves a medium-term process and not an immediate one in a state of ocular pathology. It is important to consider that the chronic model is much closer to human glaucoma than the transient OHT model but the stable model is difficult to obtain and 25% of rabbits will not develop OHT. Therefore, a limitation of this model is the elevated number of animals to use in each experimental setting.

The efficacy of the long-lasting treatment was sustained by the observation that retinal ganglionic cells, evaluated in hematoxylin/eosin-stained retinal sections and

quantified morphometrically, were physiologically maintained in the eyes of animals that achieved an adequate baroprotection during the experimental procedures. Moreover, I also observed an important reduction of oxidative stress. The presence of oxidative stress markers is one of the constant findings in the progression from OHT to optic neuropathy (Sorkhabi *et al.*, 2011). I demonstrated a significant reduction in 8-OHdG, a reliable biomarker of DNA oxidative stress (Hsieh *et al.*, 2014) in treated animals. Dihydroethidium (DHE) is another commonly used marker of *in situ* oxidative stress of nuclei in retinal layers (Song *et al.*, 2016). Oxidative stress in the retinae was reduced by baroprotective treatments in terms of reduction of fluorescence signal in the presence of DHE staining of nuclei of the neurons of retinal layers.

Morphology was useful to appreciate the glia and microglia involvement in the neuroinflammation process. There is a growing attention among the scientific community into the cross-talk between neuronal and non-neuronal cells in central (Kempuraj *et al.*, 2017) and peripheral nervous system diseases (Yafai *et al.*, 2014). I observed that both microglial activation and hypoxia-induced VEGF-A expression were reduced in animals treated with H_3 receptor antagonists or with timolol, with no differences between treatments. In the retinae of animals with chronic high IOP I observed an increase in glial cell number and an increase in the fluorescent signal of VEGF-A, suggesting that, at least in this initial inflammatory phase of glaucoma, hypoxia and ischemia/reperfusion-induced by mechanic damage due to ocular hypertension, are factors that could be modulated by IOP control. Microglia activation was attenuated as well, suggesting a less activated microglia driven inflammation process in less stressed, baroprotected retinae.

Microglia plays a major role in neuroprotection (Hamasaki *et al.*, 2017) and the observed change in the state of activation of these cells, confirmed that ocular hypertension is a trigger situation for the activation of microglia, oxidative stress, apoptosis and the loss of retinal ganglion cells. These morphological observations were accompanied by the execution of Eco Color Doppler functional test to study retro-bulbar hemodynamic parameters of the central retinal artery. This clinical test is very useful to evaluate the vascular performance of retro-bulbar arteries (Stalmans *et al.*, 2011). These aspects were studied in the chronic ocular hypertensive model, where the effects of ciproxifan, 50% less concentrated (0.5% instead of 1%), given in pharmacological

combination with the nitric oxide donor molsidomine (0.5%) were significantly increased.

It is important to remember that three variables are of interest in IOP balance: the rate of aqueous humor formation, the resistance to outflow, and the episcleral venous pressure. The rate of formation and resistance to outflow are the primary targets for the current drug therapy aimed at lowering IOP. Aqueous humor outflow is mediated through the trabecular meshwork (conventional outflow) and, through the uveoscleral region (uveoscleral outflow). The trabecular meshwork is responsible for approximately 75% of outflow resistance (Grant, 1958), but the implementation of the vascular performance is important to maintain an adequate perfusion of the posterior pole of the eye. Vascular impairment in the eye district is important in the pathogenesis of normotensive glaucoma.

The H_3 antagonist/nitric oxide donor combination resulted in improving the vascular performance, boosting the H_3 antagonism in the reduction of Pourcelot index acting on the third variable of interest in IOP balance. Prostaglandin/nitric oxide donor association is a very promising pharmacological combination for glaucoma treatment (Impagnatiello *et al.*, 2011) and is starting to be successfully received in the clinic (Medeiros *et al.*, 2016; Weinreb *et al.*, 2016).

The sad truth is that we are still far from fully understanding the pathogenic mechanisms leading to optic neuropathy and the only available therapeutic approach is baroprotection and neuroprotective strategies are not yet available (Doozandeh and Yazdani, 2016).

To have better insight into the mechanism of action of H_3 antagonist in reducing IOP I started a primary cell culture derived from TM cells, which are the most attractive pharmacological target for reducing IOP efficiently (Rocha-Sousa et al., 2013). These cells were characterized as trabecular meshwork cells (Stamer and Clark, 2017), and the H_3 receptor subtype was found to be expressed.

The calcium imaging experiment gave just preliminary results that are not ready to be interpreted clearly, as the concentration of ciproxifan used in the settings was too high to be a specific H_3 antagonistic effect the compound.

In conclusion, I can reasonably affirm that the histamine system plays a role in IOP regulation and histamine H_3 antagonists reduce IOP over the 25% therapeutic target.

This effect requires a functional histamine system to work. All the histamine receptor subtypes are expressed in the ocular structures. There seem to be a clear correlation between IOP alteration, impairment of retinal artery flow and hypoxia-induced inflammation (VEGF-A), with a resulting increase of activated microglial cells and oxidative stress. This process could be modulated by a baroprotective strategy involving H_3 receptor antagonism. Histamine H_3 receptor antagonists, alone or in association with NO donors, could be a promising therapy for glaucoma in the future.

It would be very interesting to further investigate and possibly understand the mechanism of action of H_3R antagonism in IOP reduction. Such investigations will be completed in the next future. Moreover, it would be interesting to study the fibrotic process that leads trabecular meshwork to functional impairment (Wang *et al.*, 2017) and to study the role of histamine H_4 receptor antagonists in the modulation of the fibrotic process in the eye. The antifibrotic effects of JNJ777240 have already been demonstrated in other models of fibrosis (Rosa *et al.*, 2014).

The connection that exists between microglia and histamine could represent an interesting field of investigations (Frick *et al.*, 2016). The immunofluorescence staining experiments were very useful as a method for future experiments investigating retinal inflammation. I would also like to find a convincing double-labeling technique to evaluate the colocalization of H_4 receptors and microglia. In fact, in my opinion, histamine modulation could play a role in glaucoma in terms of neuroprotection too, representing a completely novel strategy to combat RGC loss and glaucomatous blindness. This pharmacological approach, which could be used even after the damage has been established, would be a strategy to improve the resilience of the retinal nervous system through microglial modulation.

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