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Leuprolide Acetate, a GnRH Agonist, Holds Up Neurodegeneration in an Experimental Glaucoma Model*

Acetato de leuprolida, agonista de la GnRH, retrasa la neurodegeneración en un modelo experimental de glaucoma

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ABSTRACT
Glaucoma is the main cause of irreversible blindness worldwide. In short, it is a multifactorial progressive optic neuropathy that correlates with retinal ganglion cell death, optic nerve head disturbances, and visual field disorders. Leuprolide acetate has recently been reported to have neurotrophic properties; thus, the aim of this work was to determine whether its systemic administration holds up the neurodegenerative process in an experimental glaucoma model. Wistar rats divided into three groups were included: 1) a control group, 2) a hyaluronic acid-induced glaucoma group, and 3) a hyaluronic acid-induced glaucoma group treated with intramuscular leuprolide acetate. The eye electrical responses to light were recorded by simultaneous full-field electroretinography, and the eyes were processed for histological study. The results showed an improvement in the electrical activity, a recovery of fibers from the optic nerve as well, as a reduction of the reactive astrogliosis in the leuprolide acetate treated group. In short, leuprolide acetate is a new potential alternative treatment in glaucoma, as it holds up the neurodegenerative process.

Keywords: Blindness; low vision; neurotrophic; regeneration.

* Artículo de investigación.

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RESUMEN

El glaucoma es la principal causa de ceguera irreversible en todo el mundo. En resumen, es una neuropatía óptica progresiva multifactorial que se correlaciona con la muerte de las células ganglionares de la retina, trastornos de la cabeza del nervio óptico y desórdenes del campo visual. Recientemente, se ha reportado que el acetato de leuprolida tiene propiedades neurotróficas; en consecuencia, el objetivo de este trabajo fue determinar si su administración sistémica retrasa el proceso neurodegenerativo en un modelo experimental de glaucoma. Se incluyeron ratas Wistar divididas en tres grupos: 1) un grupo de control, 2) un grupo de glaucoma inducido por ácido hialurónico y 3) un grupo de glaucoma inducido por ácido hialurónico tratado con acetato de leuprolida intramuscular. Las respuestas eléctricas oculares a la luz se registraron mediante electrorretinografía simultánea de campo completo, y los ojos se procesaron para el estudio histológico. Los resultados mostraron una mejora en la actividad eléctrica, una recuperación de fibras del nervio óptico, así como una reducción de la astrogliosis reactiva en el grupo tratado con acetato de leuprolida. En definitiva, el acetato de leuprolida es una nueva y potencial alternativa de tratamiento en el glaucoma, ya que frena el proceso neurodegenerativo.

Palabras clave: Ceguera; baja visión; neurotrófico; regeneración.

INTRODUCTION

Glaucoma is the main cause of irreversible blindness around the world (1). Such condition corresponds to a multifactorial and progressive optic neuropathy that correlates with peripheral visual field loss. Optic nerve head disturbances and retinal ganglion cell death are the structural distinctive features of the disease, although glaucoma is not only restricted to the inner retina; thus, recent reports had shown that abnormalities extend to the photoreceptors, and the outer and inner plexiform layers of the retina (2, 3). Further, alterations in several locations of central visual pathway such as lateral geniculate nucleus, superior colliculus, and primary visual cortex have been also reported (3, 4). The pathophysiology of cell death in glaucoma remains unclear, but apoptosis, oxidative stress, immune-mediated neuroinflammation, neurotrophic suppression by axoplasmic transport blockage, and nitric oxide and glutamate excitotoxicity have been described as the major associated mechanisms (5). Also, elevated intraocular pressure (IOP) is the main risk factor related to the onset of this neuropathy, and the only modifiable one associated to damage limitation (6).

It is worth to mention that retinal and optic nerve damage in glaucoma is considered a potentially incurable condition, because the mammalian central nervous system has no intrinsic regenerative capacity. Recently, several neurotrophic factors have been experimentally studied for their regenerative properties alone, or in synergy with other factors, some of them also tested in both retinal and optic nerve injury. Also, intravitreal administration of brain derived neurotrophic factor was successfully used to prevent retinal ganglion cells loss in acute ocular hypertension (7), whilst fibroblast growth factor-2 stimulates axon recovery in the optic nerve (8), and, in combination with neurotrophin-3 and the nerve growth factor, it has been reported to induce substantial regeneration (9, 10).

A substantial recovery of myelinated axons in sciatic nerve complete transection correlated with increased electromyographic activity in rat model, evidenced the neurotrophic properties of gonadotropin-releasing hormone (GnRH) and its synthetic analog leuprolide acetate, independently of their role in the reproductive system (11). Leuprolide acetate also modified the gray and white matter areas, decreased the scar area, promoted the recovery of spared tissue, and improved the locomotion in spinal cord injured rats (12). Furthermore, leuprolide administration induces an increase in the axonal diameter of spinal cord fibers of rats with experimental autoimmune encephalomyelitis (13).

Leuprolide acetate is a synthetic nonapeptide, and a GnRH agonist (14), less susceptible to enzymatic proteolysis and with higher affinity to its receptor, which increases its biological effect. Also, leuprolide acetate is a safe and tolerable drug with little side-effects, used in the treatment of prostatic cancer, endometriosis, uterine fibroids, precocious puberty, pulmonary edema, myocadiac infarction, *in vitro* fertilization, and spinal cord complications, such as bowel disease and neurogenic bladder (15). The best-known function of leuprolide acetate is the suppression of the secretion of gonadotropic luteinizing hormone and follicle-stimulating hormone, and the consequently suppression of gonadal sex steroids (14). Recently, it has been demonstrated that leuprolide acetate also has neurotrophic properties such as neuritic outgrowth, increase of the nervous fiber diameter, and increase in the expression of neurofilaments (13). The extra pituitary presence and non-reproductive role of GnRH and its receptor has been described in several tissues, including hippocampal and cortical brain neurons. In the human eye, the GnRH receptors have been reported to be present in the corneal epithelium, conjunctival epithelium, ciliary body non-pigmented epithelium, ciliary muscle, trabecular meshwork, lens epithelium, and abundantly in the optic nerve and the neural retina (16). The aim of this study was to determine whether the administration of leuprolide acetate holds up the neurodegenerative process of the retina and optic nerve in an experimental glaucoma model in rats evaluated through histological and electroretinographic analysis. In such case, a new research line on the effect of neurotrophic factors in glaucoma could be developed.

METHODS

Animals

Male Wistar rats (n = 30) of 10 weeks of age weighting 270 to 300 g were used in this study. They were housed in a temperature of 25 ± 2 °C and a light controlled room (12-hour light/dark cycle) with *ad libitum* supply of food and water. All animals were orchiectomized to avoid the possible neuroprotective effect of sex hormones, since leuprolide acetate can induce an increase in these hormones. The rats were divided into three groups: 1) a control group, which underwent sham surgery (n = 10); 2) a group with untreated glaucoma (n = 10), injected in the anterior chamber with hyaluronic acid and hypromellose (HA/HPMC); and 3) a group with treated glaucoma (n = 10), injected in the anterior chamber with hyaluronic acid, hypromellose and intramuscular leuprolide acetate (HA/HPMC+LA). For ethical considerations, the animals were managed in accordance with the Technical Specifications for the Production, Care and Use of Laboratory Animals of the Ministry of the Environment and Natural Resources (Semarnat, from 2011), and the Institutional Regulation of Animal Welfare of the Autonomous University of Aguascalientes.

Treatments

The right eyes of the groups 2 and 3 were treated with an intracameral injection of HA/HPMC every 96 hours for a period of 2 weeks to induce glaucoma, while the left eyes were used as control. Intraocular pressure was monitored by rebound tonometry, and the electrical responses were recorded by simultaneous full-field flash electroretinography. In the following 2 weeks, no treatment was administered, and from the 5th week, intramuscular leuprolide acetate (10 mg/kg, every 3 days) was given for a further 4 weeks (group 3). The eyes were enucleated 2 weeks after the leuprolide acetate treatment conclusion, and were processed for histologic study of the retinal and optic nerve.

Anesthesia

Surgical anesthesia was induced by intraperitoneal administration of sodium pentobarbital at a dose of 40 mg/kg, and ocular anesthesia was performed instilling tetracaine hydrochloride ophthalmic solution drops (0.5%).

Tonometry

Intraocular pressure was measured every 96 hours by rebound tonometry (I-care ® Tonovet, TV 01) in conscious unsedated rats for 4 weeks before the beginning of the model of glaucoma, and until the end of the study, always at the same hour (12 hrs) to avoid circadian variations, according to manufacturer indications. The study proceeded carefully, since the intraocular pressure can change because of the pulse, breathing, body position, stress, and self-defense maneuvers.

Experimental glaucoma

The experimental model of glaucoma was based on the method described by Moreno and contributors (17). Briefly, intracameral injections (25 µl) of HA/HPMC (80 mg/ml) were administered every 96 hours, alternating an 1 hour clockwise in each application, to reduce corneal trauma, allow healing, and prevent aqueous humor leakage. The solution was loaded in a syringe (30-gauge needle), the needle was tangentially approached to the sclerocorneal limbus parallel to the iris surface with the bevel up. A self-sealing wound was made gently inserting the needle, until the full bevel was into the corneal stroma, then the needle was slightly tilted, and 45 degrees rotated, while it was pushed until the full bevel was inside the anterior chamber, keeping the needle away from the iris, the lens, and the corneal endothelium preventing bleeding, cataract, or edema. Next, the plunger was gently depressed at a speed of approximately 25 µl/min, until the intraocular pressure raised up to 30 mmHg; the syringe was then slowly withdrawn in the same trajectory, and the eye was covered with tobramycin ointment for prophylaxis, and to avoid ocular surface desiccation.

Tissue preparation

The eyes were enucleated under general and local anesthesia. 1 drop of vasoconstrictor (0.1% naphazoline) was instilled to reduce the conjunctival bleeding. The ocular conjunctiva was dissected around the sclerocorneal limbus, preserving the nictitating membrane, to identify the tissue orientation. Next, near to the sclera, the extraocular muscle bundle was removed, and the optic nerve was carefully dissected from the orbit apex. For the histological sections, tissues were fixed in a 4% paraformaldehyde solution.

Histological analysis

Sagittal sections (10 µm) of the retina from the prelaminar region and transverse sections of the optic nerve from 1 mm behind the posterior pole were obtained (Leica RM2125RT Microtome™), embedded in epoxy resin and stained with the standard hematoxylin-eosin and toluidine blue protocol. For histological analysis the slides were visualized (Zeiss Axioscope™), captured (AxioVision MR3™ camera), and processed (Image Pro Plus Software 4.5.1™), to evaluate the structural integrity of the retina, as well as its overall, inner, and outer thickness. The integrity of the optic nerve was assessed in terms of the glial profile, and axon density, counted by a semiautomated method, as described by Quigley and Broman (1), with minor modifications. In brief, the cross-section area was measured at low magnification (10x), non-neural elements were digitally removed from the image (Fiji/ImageJ free distribution), the axons per square millimeter was calculated in 1 central and 4 peripheral fields of 160 μm^2 at high magnification (100x), and then multiplied for the area, to obtain the mean axon number.

Electroretinography

Electrical responses to single flash stimulation (-3.0 log cd.s/m^2) delivered by a digital stimulator (STM200 Biopac Systems, Inc. USA) located in the top of a Ganzfeld-like dome were recorded by full field electroretinography from both, the right and left eyes simultaneously, in previously general and ocular anesthetized rats, and under scotopic conditions as described by Gallego-Ortega and contributors (5). In brief, the animals were dark-adapted for a period of 3 hours and placed

in a thermal pad to maintain body temperature, and the pupils were dilated with tropicamide (0.5%) and phenylephrine (2%) to achieve full field retinal stimulation. All the recordings were performed at the same hour (22:00 hrs) in a room under dim red-light illumination. One drop of lubricant (hypromellose 0.5%) was instilled to enhance conductivity and avoid the risk of exposure keratopathy. The reference electrode was placed on the forehead, the ground electrode on the tail, and the active recording electrodes in contact with the central cornea (15). 15 continuous pulses (1.0 Hz, 6 ms) were allowed and averaged. The electrical signals were digitized using a high-speed data acquisition and analysis system (MP-150 BIOPAC Systems, Inc. / Acqknowledge ® 4.1, USA). Also, ERG a-b wave, b/a ratio and latency were analyzed in accordance with the standards of the International Society for Clinical Electrophysiology of Vision.

Statistical analysis

The statistical analyses were performed using the GraphPrism 6.0 software ™ (San Diego, CA, USA). Descriptive results were presented as the mean ± SEM. Differences between 2 and more groups were determined by the unpaired 2-tailed Student's t-test and one-way Anova followed by the Tukey's *post hoc* test, respectively. The threshold for significance was $p < 0.05$.

RESULTS

Intraocular pressure

The average IOP (figure 1) of right eyes injected for 2 weeks with HA/HPMC significatively exceeded that of left eyes injected with saline solution for the same period (29.1 ± 0.54; 8.6 ± 0.26 mmHg; $p < 0.001$). The administration of the saline solution in the group of control did not significantly affect the IOP values.

Electroretinography

A scotopic ff-ERG was performed to identify the functional status of the inner retina. The average amplitude of the a-b wave and the b/a ratio (b-wave amplitude divided by the a-wave amplitude) were determined. No significant differences were found in the initial amplitude of the a-b wave and the b/a ratio (figure 2) of the right eyes of the control group (421 ± 31 µv; 1.813 ± 0.076), the HA/HPMC group (429 ± 30 µv; 1.808 ±0.076), and the HA/HPMC+LA group (424 ± 38 µv; 1.804 ± 0.052).

For 15 days, the right eyes of the groups 2 and 3 were injected in the anterior chamber to induce glaucoma. 2 more weeks after the injections, that is 30 days since de beginning of the study, the amplitude of the a-b wave and the b/a ratio (figure 3) of

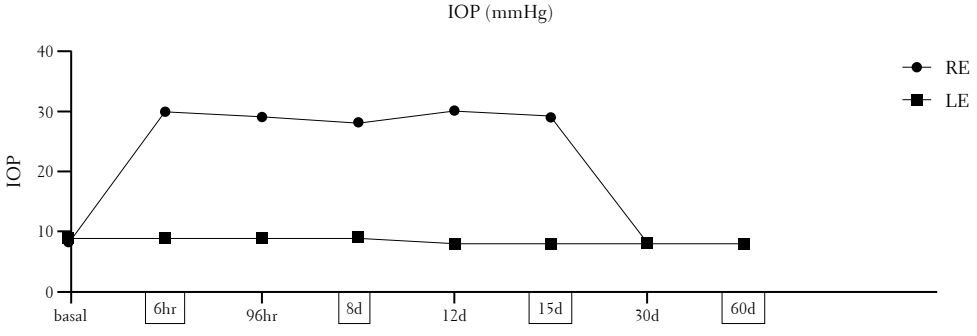

FIGURE 1. **Average intraocular pressure of right and left eyes injected for 2 weeks with HA/HPMC and saline solution respectively**

Source: own work

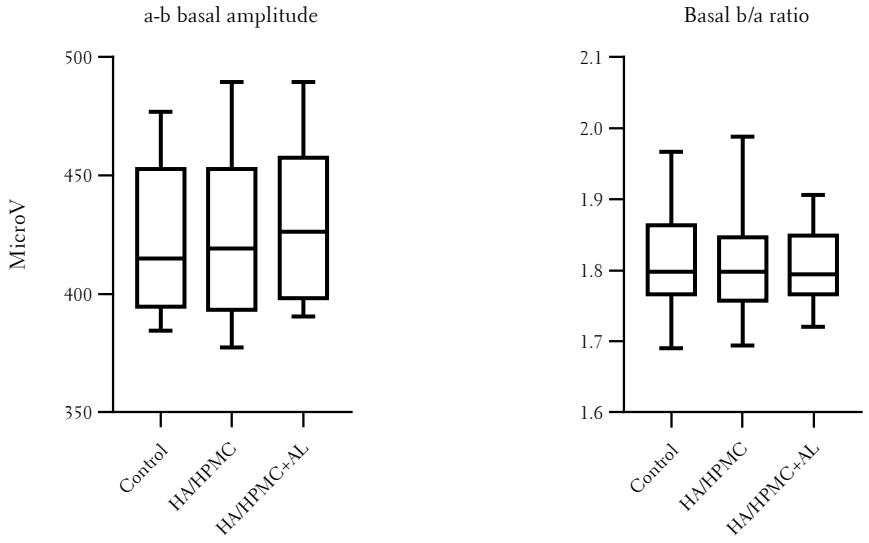

FIGURE 2. **The initial amplitude of the a-b wave of control, HA/HPMC, and HA/HPMC+LA groups do not show significant differences. The basal b/a ratio evidence a balance between the a-wave and b-wave of the electroretinogram in all groups.**

Source: own work

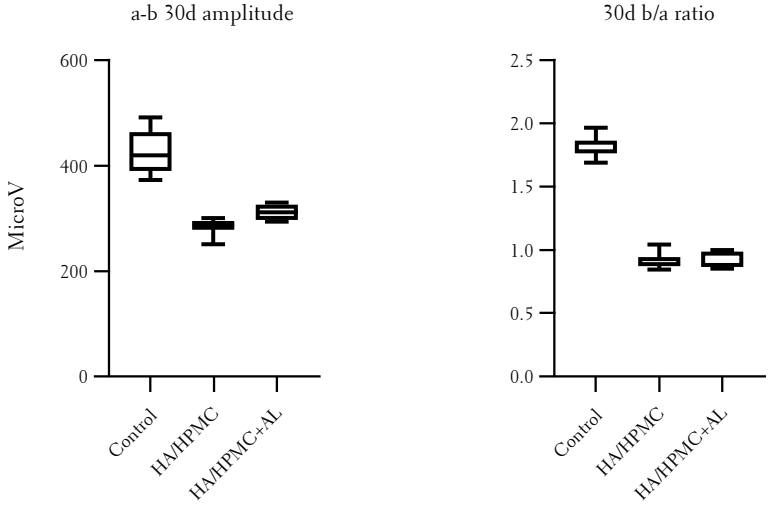

FIGURE 3. **The a-b wave amplitude and the b/a ratio diminished in the HA/HPMC and the HA/HPMC+LA groups 2 weeks after the ocular hypertension induction.**

Source: own work

the control group remained apparently unaltered (426 ± 43 µV; 1.813 ± 0.076), whilst a reduction of the b-wave was observed in the electroretinogram of the other groups, decreasing both the amplitude of the a-b wave and the b/a ratio of the HA/HPMC group (283 ± 14 µV; 0.912 ± 0.058) and the HA/HPMC+LA group (299 ± 7 µV; 0.914 ± 0.060).

The treatment with intramuscular leuprolide acetate started from 31-day, and was extended for 4 weeks; 2 more weeks were left without any treatment, and, then, the last ff-ERG was recorded. The control group, with a-b amplitude and b/a ratio (figure 4), seemed to be kept stable (413 ± 34 µV; 1.813 ± 0.076), whilst the HA/HPMC group

still presented a significant reduction in such parameters (260 ± 23 µV; 0.724 ± 0.027). Instead, the HA/HPMC+LA group showed a significant increase in the b-wave, which determined a considerable increase in the a-b amplitude and the b/a ratio (368 ± 15 µV; 1.485 ± 0.026).

HISTOLOGICAL ANALYSIS

Sagittal section of the retina

The average raw and normalized thickness of retinal sagittal sections were analyzed (figure 5). In the control group (A), a normal total retinal thickness, and a well-balanced distribution of the inner and outer retina was seen (173 ± 2 µm; 100 ± 3.11 µm; 69.1 ± 0.31 µm), as well as a normal arrangement and uniform trajectory of its layers. In contrast, the HA/HPMC group (B) presented shrinkage in practically all the retinal layers, and a significative thinning ($p < 0.001$) of the inner retina, beside the breakdown of the inner nuclear layer and the outer nuclear layer, in comparison with those of the control group (115 ± 1.45 µm; 57.7 ± 1.06 µm; 60.3 ± 0.67 µm). Instead, the HA/HPMC+LA treated group (C) showed a significative escalate ($p < 0.001$) of the total and inner retinal thickness regarding the HA/HPMC group; although there was no significative difference in the outer retinal thickness between this groups (144.4 ± 2.22 µm; 80.7 ± 0.95 µm; 61.8 ± 1.39 µm), a healthier structure of the treated group results evident.

Transverse sections of the optic nerve

Figure 6 shows a general overview of the optic nerve in the experimental subjects. The control group presented a healthy morphology, a uniform pattern of staining in the central and peripheral regions, without glial-profile areas (A). Also, a normal and non-activated astrocytic population proper of this portion of the emerging optic nerve is seen, with discrete and thin astrocyte processes and a reduced astrocytic area (1.2%) (B). The nerve axons were tightly grouped (12,579 ± 512 fibers/mm^2) into bundles, and separated by a thin septae (C).

The optic nerve of HA/HPMC group showed an evident loss of integrity, and an irregular staining pattern with numerous pallor areas (D). Additionally, significant reactive astrogliosis and hypertrophic astrocytic processes, presence of degenerative

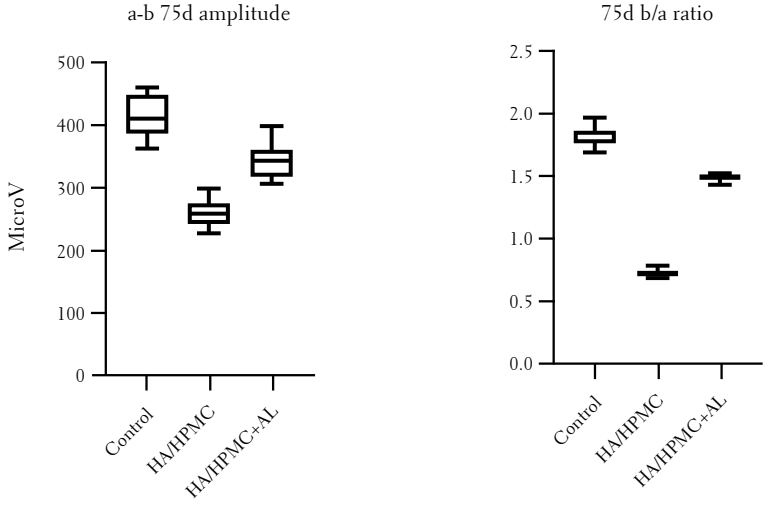

FIGURE 4. **The a-b wave amplitude and the b/a ratio of the HA/HPMC+LA group increased significatively after the leuprolide acetate treatment.**

Source: own work

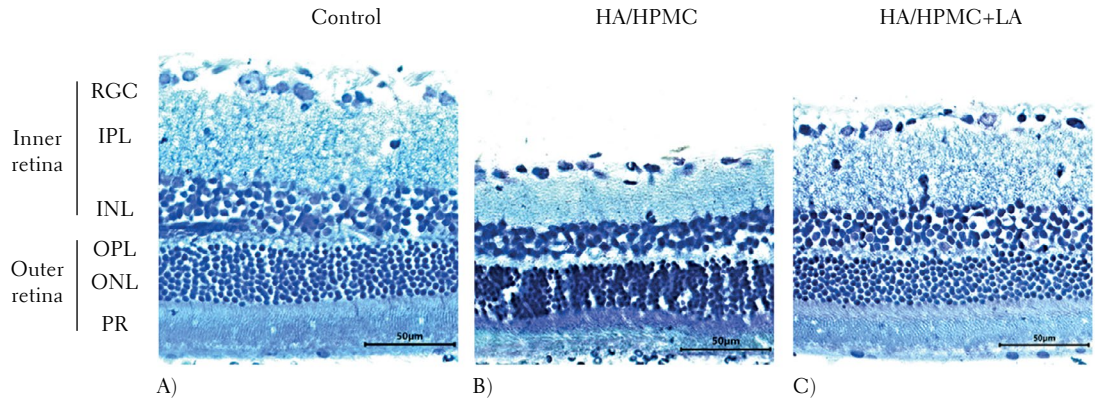

FIGURE 5. **Microphotographs of sagittal section of the retina (40x)**

* A) The control group shows a uniform thickness and disposition among the different retinal layers, as well as a balanced proportion between the inner (RGC, IPL, INL) and the outer retina (OPL, ONL, PR). B) The HA/HPMC group presents shrinkage in practically all the retinal layers, while the inner nuclear and inner plexiform layers present the most severe affectation. C) The HA/HPMC group exhibits a significant difference in thickness and layer arrangement. (RGC: retinal ganglion cells; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; PR: photoreceptors).

Source: own work

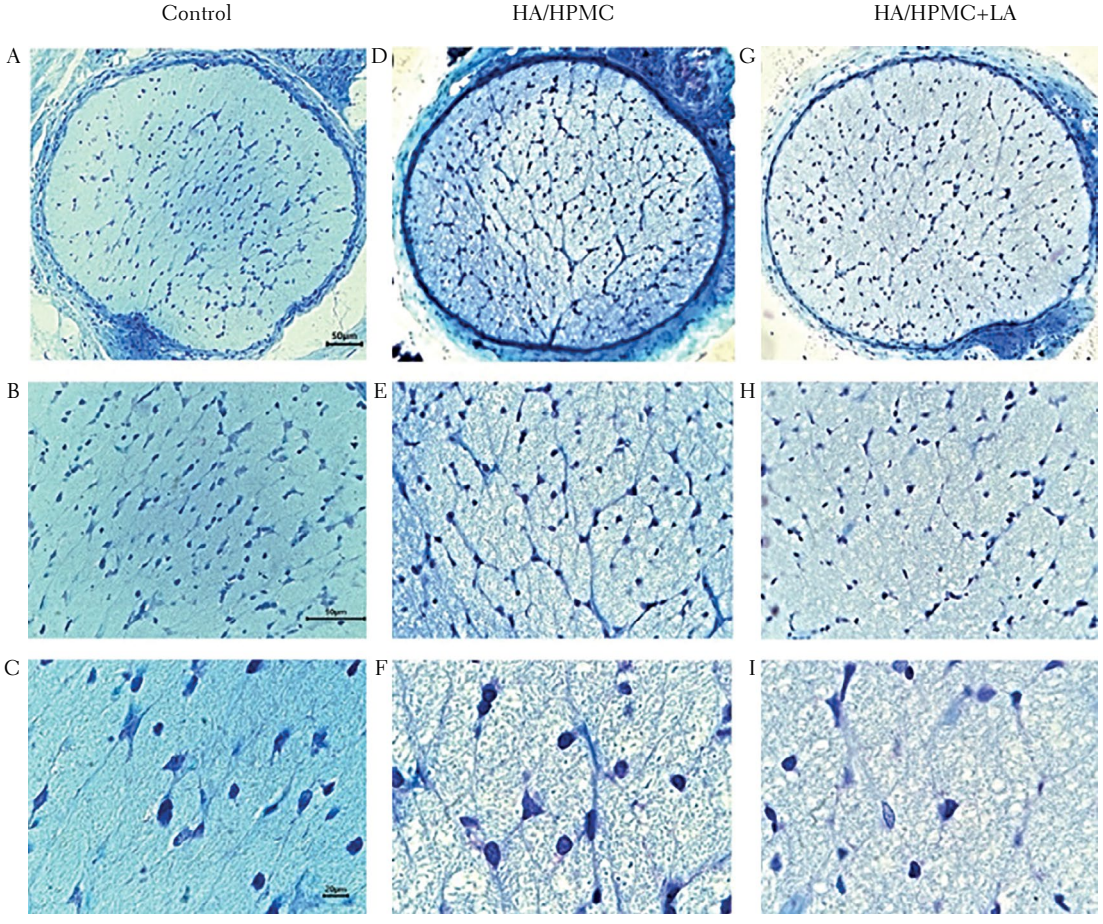

FIGURE 6. **Micrographs of optic nerve transverse section (10x, 40x, 100x). The control group subdivisions (A, B, C) present a healthy morphology of neural and no-neural elements. The HA/HPMC subgroup (D, E, F) manifests several signs of neurodegeneration, such as reactive astrogliosis and axonal loss. The HA/HPMC+LA subgroup (G, H, I) shows a less reactive glia and a healthier neural profile.**

Source: own work

axon black dots, and greater spacing between fibers are evident (E). Furthermore, fewer nerve fibers (5,686 ± 312 fibers/mm^2; $p < 0.001$), axon edema and an important astrocytic area (2.8%) are also observed (F).

In contrast, the optic nerve of HA/HPMC+LA group showed a more regular staining and less pale areas (G), less astrocyte activation and minor astrocytic area (1.3%) (H), few areas of glial scarring and a quantity of fibers significantly higher (9641 ± 290 fibers/mm^2; $p < 0.001$) (I).

DISCUSSION

Elevated IOP is a common thread that connects most forms of glaucoma, and is the major risk factor for the disease. The final common pathway of tissue damage in glaucoma is the axonal damage that manifests as optic nerve atrophy, causing the progressive visual field loss that eventually led to blindness.

Different experimental models of glaucoma in animals have been reported. These include those based on the administration of hypertonic saline solution, cauterization of episcleral veins, photocoagulation of the trabecular meshwork, injection of red blood cells, genetic mutations, topical application of steroids, autoimmune models, and injection of latex and magnetic microspheres (19, 20). In this study, a model based on the administration of hyaluronic acid in the anterior chamber using a viscoelastic agent as vehicle was used. In this model, the IOP remained more than 3-fold the control value, like that obtained by other authors (21, 17). With this procedure, it was possible to induce an important affectation of both the retina and the optic nerve.

Typically, it has been described that glaucoma is a disease that affects the retinal ganglion cells and the nerve fiber layer (22). In the HA/HPMC group, as in other studies, we found that it can include other cell types or layers, such as the photoreceptor cell layer, the inner nuclear layer, and the inner and outer plexiform layers of the retina affected by glaucomatous disease (4, 17).

It has been proposed also that the injured axons of the retinal ganglion cells may present a certain degree of regeneration induced by neurotrophic factors, such as those produced by Schwann cells or the immune system in a peripheral nerve transplant (23, 24).

We have previously reported that leuprolide acetate administration induces the recovery of myelinated axons in the sciatic nerve with complete transection and an increase in the axonal diameter of spinal cord injured rats (13, 11). Considering that leuprolide acetate may have neurotrophic properties, it is possible that this phenomenon is also occurring in the retina and the retinal ganglion cell axons. Leuprolide acetate could be also inducing an increase in the survival period of damaged cells, as occurs by the neurotrophic brain derived nervous factor, after optic nerve injury (25).

Moreover, the fibrotic scar has been referred to prevent axon growth in the optic nerve lesions, because astrocytes establish an inhibitory physical barrier (26). Furthermore, activation of astrocytes after the injury increases the expression of pro-apoptotic factors, and they secrete inflammatory cytokines, which directly impact neuronal function (27). Our results show a significant reduction in the astrocytic area after treatment with leuprolide acetate, which could improve the conditions of the injury site, as occurs in the scar reduction in injured spinal cord rats when leuprolide acetate is administered (12).

Electroretinogram studies in different glaucoma models reveal dramatic changes in the morphology of several retinal layers, associated with functional disturbances (28, 20). In our study, histological analysis showed a healthy morphology of the retina and the optic nerve in the right eyes of the control group, while the HA/HPMC group had an important affectation. The ff-ERG

revealed that the amplitude of the a-wave, dependent on the outer retina (outer plexiform layer, photoreceptors, and Müller cells) was preserved, while the b-wave dependent on the inner retina (inner plexiform layer and ON/OFF bipolar cells) was sensitive to ocular hypertension, as it happens in a true glaucoma model, thus affecting the b/a ratio. Meanwhile, the amplitude of the a-b wave and the b/a ratio of HA/HPMC+LA group showed a significant increase respect to the HA/HPMC group and was like that of the control group.

It is interesting to note a correlation between the ff-ERG changes and the trophic factor expression in retinal lesion models. It has been reported as well that suppression and recovery of the a-b wave correlates temporally with a transient upregulation of ciliary neurotrophic factor in the retina of nerve-sectioned eyes (29, 30, 31). Also, an action by this trophic factor on the ERG is suggested by experiments in which the protein was directly applied into the subretinal space (32); leuprolide acetate may be acting in a similar way.

It has been observed that application of exogenous growth factors alone is minimally effective because injured and mature retinal ganglion cells are not primed to respond to growth factor signaling (33, 34). However, leuprolide acetate administration could be acting more effectively through the activation of GnRH receptors, which have been reported in the eye of mammals, including the human, specifically in the neural retina, optic nerve, and nerve fiber layer (35, 36, 37)

Despite of the results obtained regarding the regenerative effect of a combination of nerve growth factor, neurotrophin 3-5, brain-derived neurotrophic factor and ciliary neurotrophic factor are partial or totally inconclusive (38, 10). In this work, we finally report that the use of leuprolide acetate seems to hold up the neurodegeneration process of the retina and the optic nerve, being thus a possible novel and friendly method that provides a non-invasive pharmacological treatment to reduce the effects of glaucomatous disease. Leuprolide acetate administered via intramuscular injection can cross the blood brain barrier as observed in previous studies (39), which avoids the possible topical application with surgical risks.

CONCLUSION

According to our current findings, the administration of leuprolide acetate partially improves the morphology and electrical activity of the retina, promotes the recovery of nerve fibers from the optic nerve, and significatively reduces the astrogliosis in a rat model of glaucoma; thus, the set of the neurotrophic factors should be considered as a new therapeutic approach for primary open angle glaucoma. However, this study is limited by factors such as a short-time window, that does not allow the assessment of cell viability and permanence throughout the time, the absence of an *in vivo* monitoring, and the lack of specific markers and immunofluorescence studies. Further research in this area is needed; for future studies we suggest experimental designs that allow short, middle, and long-time observations, as well as immunohistofluorescence and whole-mounted retinal studies, for a more detailed histologic analysis, and *in vivo* follow-up by spectral domain optical coherence tomography. Also, combination of leuprolide acetate with other neurotrophic factors and GnRH analogues as well as different administration pathways are also suggested.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of financial, academic, or personal interest.

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