Functional and Structural Effects of Erythropoietin Subconjunctival Administration in Glaucomatous Animals

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What Is It about?
Glaucoma is the leading cause of irreversible blindness worldwide. Erythropoietin (EPO) has revealed neuroprotective properties on the retina, preserving visual function in several glaucoma models. The present study assesses functional and structural benefits of EPO when administered subconjunctivally in the retina of glaucomatous albino rats using electroretinography and retinal thickness measurements. Subconjunctival EPO administration, a mini-invasive and safe periocular route, showed beneficial effects both on retinal structure and on retinal function. This neuroprotective effect should be applied in other animal species, and more studies should be performed to assess EPO kinetics when administered via a subconjunctival route in glaucoma conditions.

Keywords
Erythropoietin · Glaucoma · Subconjunctival route · Retinal ganglion cells · Rat

Abstract
Purpose: The present study aimed to assess functional and structural benefits of erythropoietin (EPO) when administered subconjunctivally in the retina of glaucomatous rats using electroretinography (ERG) and retinal thickness (RT) measurements. Methods: Glaucoma was experimentally induced in 26 Wistar Hannover albino rats. Animals were divided into 2 groups of 13 animals each: a treated group receiving a unique subconjunctival injection of 1,000 IU of EPO and a control group receiving a saline solution. In each group, 7 animals were used for retinal function evaluation (ERG) and 6 animals were used for retinal structural evaluation (histology). RT was measured, dorsally and ventrally, at 500 μm (RT1) and at 1,500 μm (RT2) from the optic nerve. Results: Retinal function evaluation: for both scotopic and photopic conditions, ERG wave amplitudes increased in the treated group. This increase was statistically significant ($p < 0.05$) in photopic conditions. Structural evaluation: for both locations RT1 and RT2, the retinas were significantly ($p < 0.05$) thicker in the treated group. Conclusion: Sub-
conjunctival EPO administration showed beneficial effects both on retinal structure and on retinal function in induced glaucoma in albino rats. This neuroprotective effect should be applied in other animal species.

Introduction

Glaucoma is the leading cause of irreversible blindness worldwide [1]. It is considered a progressive neurodegenerative eye disorder characterized in earlier stages by the degeneration and loss of retinal ganglion cells (RGCs) and their axons, leading to visual field loss [2, 3]. Presently, the available treatment strategies aim to reduce intraocular pressure (IOP) through medical, laser, or surgical methods [4]. Since the loss of function and death of RGCs is mainly by programmed cell death (apoptosis) [5, 6], the development of neuroprotective therapeutic approaches for those cells is crucial.

Erythropoietin (EPO) is a natural glycoprotein hormone, conventionally thought to be responsible only for producing red blood cells in our body. Additionally to its hematopoietic effect, this cytokine has demonstrated neuroprotective and neuroregenerative properties in the central nervous system [7–10]. Many preclinical studies have been conducted in several ocular diseases such as diabetic retinopathy, retinal detachment, glaucoma, retinopathy of prematurity, age-related macular degeneration, and optic neuritis [11]. EPO has been proven to prevent RGC apoptosis, preserving visual function in several glaucoma models [11–13] with very promising results [14, 15].

Previously, different ocular administration routes have been tested to achieve EPO therapeutic effects in the retina, namely systemic, intravitreal, and retrobulbar routes [12, 16, 17]. The systemic route may cause an undesired secondary effect, the increase in hematopoiesis, while the intravitreous and retrobulbar routes may lead to ocular complications, such as chorioretinitis, retinal detachments, cataracts, vitreitis, or even endophthalmitis [18, 19].

It has been previously demonstrated that EPO reached the RGC layer both in physiological and glaucoma conditions in a rat animal model when administered through the subconjunctival route [20, 21]. The subconjunctival route for EPO ocular administration has been demonstrated to be a safe and easy procedure with few associated risks [19, 22].

With this work, we intend to assess functional and structural potential benefits of EPO subconjunctival administration in glaucomatous rats using electroretinography (ERG) and histological evaluation.

Material and Methods

Animals

A total of 26 Wistar Hannover albino rats were included in this study: 14 females weighing 247 ± 28 g and 12 males weighing 378 ± 34 g. The animals were housed in type III boxes (1,195 cm²) with water and food ad libitum, and maintained in controlled conditions of temperature (20 ± 2°C), humidity (≈70%), and cyclic light (12 h light/12 h darkness). In order to be included in the study, all the animals underwent a complete ophthalmic examination and no ocular diseases were found. Unilateral glaucoma was induced on the right eye of each animal. Animals were divided into 2 groups of 13 animals each: a treated group (7 females and 6 males) and a control group (7 females and 6 males). Among these groups, 7 animals were used for functional evaluation (ERG) and 6 animals were used for structural evaluation (histology). The treated groups received a single subconjunctival injection of re-
combinant human EPO (rHuEPO), while the control groups received only saline. The contra-
lateral eye (left eye) remained untouched in all animals.

**Anesthesia**

Anesthesia was performed by intraperitoneal (i.p.) administration of 75 mg ketamine/kg body weight (b.w.) (Imalgene 1000®, Merial Portuguesa, Rio de Mouro, Portugal) and 1 mg medetomidine/kg b.w. (Domitor®, Orion Corporation, Espoo, Finland).

**Subconjunctival EPO Injections and Glaucoma Induction**

Under general anesthesia, 1,000 IU of rHuEPO (NeoRecormon 5000®, Roche Diagnostics GmbH, Mannheim, Germany) was administered by subconjunctival injection, to the right eye of each animal belonging to the treated groups, and an equal volume of saline solution was administered, by the same route, to the control groups.

Forty-eight hours after subconjunctival injections, glaucoma was induced on the same eye, to all groups, by coagulation of 3 episcleral veins, using a technique described by Shareef et al. [23]. For these procedures a surgical microscope (Zeiss Opmi Visu Series/S7 Micro-
scope, Munich, Germany) and the coagulation device from the phacoemulsification system (Laureate, Alcon Laboratories, Lake Forest, CA, USA) for hemostasis were used. The two inclusion criteria for the animals in the study were postexperimental glaucoma induction high IOP and non-measurable ERG traces on day 7 in their right eyes.

**Intraocular Pressure Measurements**

IOP measurement was performed under general anesthesia to all groups, on both eyes, before and 1 h after coagulation, using rebound tonometry (Tonolab, Icare®, Helsinki, Finland).

**Electroretinography**

Flash ERGs were performed in 14 animals (treated = 7 animals; control = 7 animals) according to previously published methods [24] for functional evaluation. Under general anesthesia, binocular full-field (Ganzfeld) ERGs were recorded before glaucoma induction and at 7 and 21 days after glaucoma induction. After 12 h of a dark adaptation period and in scotopic settings, rod function was tested by stimulating the retina with dim flashes (intensity: –3.02 log cds/m²). In photopic settings, cone function was tested by stimulating the retina with flashes (0.98 log cds/m²) and 6.2 Hz flicker. ERG results were acquired for both eyes at the same time. The left eyes' traces were used as the control in each examination to confirm good technical procedures.

**Euthanasia and Enucleation**

Animals were euthanized 21 days after glaucoma induction. Euthanasia was performed by administration of an overdose of pentobarbital sodium (60 mg/kg b.w.) through the i.p. route. Before enucleation, the dorsal, ventral, and lateral points were carefully painted along the coronal plane using tissue-marking dyes (Cancer Diagnostics Inc., Morrisville, NC, USA). Both eyes were enucleated immediately after death and stored in 4% paraformaldehyde in phosphate buffer (0.1 M, pH of 7.4) for additional processing.

**Histologic Examination**

Structural evaluation was performed in 12 animals (treated = 6 animals; control = 6 animals). Both eyes were fixed for 24 h and routinely processed for histological diagnosis. Paraffin sections of 3 μm were cut through the globe, along the anterior-posterior axis, and stained with hematoxylin and eosin. The retinal sections were analyzed and assessed by optical microscopy (Olympus BX51 microscope and a DP21 digital camera). Retinal thickness
(RT) was measured at 500 μm (Fig. 1) and at 1,500 μm, dorsally and ventrally from the optic nerve (ON) head in all retinal sections. At 1,500 μm from the ON, the mean of 3 measurements was considered for statistical analysis (Fig. 2).

Data Analysis
Data were analyzed with GraphPad (InStat version 3.10 for Windows, GraphPad Software, USA), Excel (Microsoft, USA), and R 3.3.2 (R Core, Vienna, Austria) [25]; results are reported as mean ± standard error for variables with a normal distribution, and as medians (min.; max.) for variables with a non-normal distribution. Repeated measures analysis of variance was used to test for significant differences of the interaction between group and time on b-wave amplitude on electroretinography examinations between days 7 and 21. Independent samples Student’s t test was used to compare RT between the test and control groups. Homogeneity of variances was verified by Fisher’s F test.

Results

Intraocular Pressure Values
Results of EPO measurements are presented in Figure 3. Before the coagulation of the episcleral veins (n = 26), median IOP values in right eyes and left eyes were 10.27 ± 1.53 and
10.06 ± 1.09 mm Hg, respectively. One hour after coagulation of the episcleral veins (n = 26), the median IOP was 56.65 ± 11.21 mm Hg in right eyes and 10.52 ± 1.34 mm Hg in left eyes (p < 0.001). IOP values showed a significant statistical difference between the right eyes and the left eyes after surgery.

**Electroretinography Evaluation**

The ERG results are presented in Table 1. At the end of the study (day 21), ERG wave amplitudes increased in the treated group in both scotopic and photopic conditions. In photopic conditions, the median b-wave amplitudes (min; max.) of the treated group were 54.3 µV (21.5; 271.0) on day 7 and 189.0 µV (106.0; 319.0) on day 21, and the control group had median values of 97.4 µV (7.0; 158.0) on day 7 and 182.0 µV (44.0; 259.0) on day 21 (p = 0.79). In photopic conditions, median b-wave amplitudes for flash examination of the treated group were 0.0 µV (0.0; 0.0) on day 7 and 73.4 µV (55.9; 101.0) on day 21, and the control group had median values of 0.0 µV (0.0; 45.3) on day 7 and 47.8 µV (38.3; 60.1) on day 21 (p = 0.006). In flicker examination, the treated group had median values of 17.0 µV (3.1; 49.6) on day 7 and 60.9 µV (30.4; 85.9) on day 21, and the control group had median values of 33.5 µV (10.0; 82.2) on day 7 and 42.4 µV (26.5; 76.5) on day 21 (p = 0.02).

These differences were statistically significant in photopic conditions for flash and flicker examination. The presented results concern the examination of the right eyes. The results for the left eyes were within normal values. Figure 4 is a representative ERG obtained in photopic conditions.
Fig. 4. Representative ERG obtained in photopic luminance conditions: flash (a) and flicker (b), and scotopic luminance conditions (c) in an animal from the treated group and in another animal from the control group. In each trace the right eye (operated eye) is represented in black and the left eye (non-operated eye) is represented in grey. Each graph shows the curves obtained on day 0 (before surgery), day 7 (after surgery), and finally on day 21 (at the end of the study).
luminance conditions: flash (Fig. 4a) and flicker (Fig. 4b), and scotopic luminance conditions (Fig. 4c) in an animal from the treated group and in another animal from the control group.

**Table 2.** Retinal thickness of the treated and control groups at 500 and 1,500 μm from the optic nerve (ON)

| Retinal thickness, μm | 500 μm from the ON | 1,500 μm from the ON |
|-----------------------|---------------------|----------------------|
| Treated group         | 206.3 ± 14.8*       | 175.9 ± 21.2*        |
| Control group         | 176.9 ± 20.1        | 154.0 ± 15.4         |

Results are reported as medians ± standard error. *p < 0.05.

**Discussion**

Recently, EPO has revealed neuroprotective properties on the retina in addition to its hematopoietic effect [11, 26]. Several investigations have addressed EPO properties to mediate protection against retinal damage through different pathways, including increasing resistance to inflammation, oxide-induced damage, ischemia, degeneration, and permeability [13, 27]. Concerning RGCs, which are especially affected in glaucoma disease, EPO provides protection against apoptosis by activation of STAT5, MAPK/ERK, and PI3K/Akt. EPO also assists these cells in mitigating inflammatory injury through activation of NF-κB, which suppresses inflammatory factors such as TNF-α and IL-1β [13]. Another study demonstrated that one of the protecting mechanisms of EPO to the injuries that a retina suffers caused by
chronic intraocular hypertension is through the HIF-1α/NOS signal conduct path. EPO inhibits the activation of HIF-1α through negative feedback that inhibits the transcription of iNOS to avoid neurotoxicity caused by oversynthesis of iNOS [28]. For these reasons, EPO is actually considered a promising neuroprotective agent in glaucoma.

With encouraging neuroprotection results, recombinant EPO has already been tested clinically for autoimmune optic neuritis (NCT00355095), traumatic optic neuropathy (NCT01783847), methanol-associated optic neuropathy (NCT02376881), and retinopathy of prematurity (NCT00910234), with all the patients being treated through systemic administration [11]. Apart from formal clinical trials, EPO therapy using intravitreal injections has shown promise in several other retinal diseases [11].

Drug delivery and pharmacokinetics play important roles in current retinal therapeutics and the development of new medications [29]. The subconjunctival route for EPO ocular administration is an easy and safe procedure with minimal associated risks and without significant unwanted side effects related to hematopoiesis stimulation. The main ocular barrier to subconjunctival administrations are flow barriers (elimination to blood flow and lymphatic flow) and penetration barriers [29], with scleral tissue being the more important barrier to be considered. However, it has been proven that transscleral delivery of immunoglobulins and other large compounds to the choroid and retina is feasible. Ambati et al. [22] demonstrated that large molecules, such as IgG, diffuse across sclera in a manner consistent with porous diffusion through a fiber matrix. They also concluded that scleral permeability decreased with increasing molecular weight and the molecular radius [22]. We have previously demonstrated that rHuEPO can permeate porcine conjunctiva, sclera, and cornea in an ex vivo model [30]. Since glaucoma is a chronic disease, multiple treatments to protect RGCs are required, and using the subconjunctival route seems to be more feasible. Concerning subconjunctival EPO administration, we also demonstrated that rHuEPO reached the RGC layers when administered by this route both in physiological and glaucomatous conditions without significant unwanted side effects [20, 21].

Hence, it was of the utmost importance to assess both functional and structural benefits of subconjunctivally administered rHuEPO in the retina of glaucomatous rats, performing ERGs and RT measurements, which was the aim of the present study. To reduce the individual variability of each experiment, we separated animals by gender. A group of female rats were used to test visual function and a group of male rats were used to evaluate changes in RT.

### Table: Retinal thickness at 0.5 mm and 1.5 mm from the optic nerve

| Bar | Retinal thickness at 0.5 mm from optic nerve, µm | Retinal thickness at 1.5 mm from optic nerve, µm |
|-----|-----------------------------------------------|-----------------------------------------------|
| Control group | 220 | 175 |
| Treated group | 200 | 150 |

**Fig. 6.** Retinal thickness at 500 and 1,500 µm from the optic nerve head in the control and treated groups. Bars represent the median score; boxes show the 25th to 75th percentiles; whiskers represent ±1.58 interquartile range/square root (n).
Our glaucoma experimental model has been used before and has proven to be reliable and reproducible [31–33]. In the present study the IOP rise was confirmed 1 h after the surgical procedure in animals from both groups. Seven days after glaucoma induction, the injury of the retinas of the glaucomatous eyes secondary to the rise in IOP was confirmed by the ERG traces through a non-measurable or weak response on the b-wave curve on both scotopic and photopic examinations. Animals that presented with a measurable ERG trace on day 7 were excluded from the study based on the fact that glaucoma induction could have been unsuccessful and it was necessary to ascertain that the studied animals were in the same experimental conditions.

At the end of the study (day 21), the ERG results showed that the treated group experienced a better recovery than the non-treated group. In photopic examinations, both flash and flicker b-wave results showed a statistical difference ($p < 0.05$) between groups. In scotopic examinations, although the results were not statistically significant, a tendency for improvement was observed. This could possibly be due to an insufficient number of animals in each studied group associated to the large variability obtained on the ERG b-wave results.

Furthermore, the effect of rHuEPO subconjunctival injection on the thickness of the retina from glaucomatous animals was also evaluated. RT measurements at the ON level allowed the conclusion that retinas from the treated group were thicker than the ones from the non-treated group ($p < 0.05$).

One of the limitations of this study is the use of single EPO injections. Repeated EPO subconjunctival injections should be administered to evaluate both local and systemic potential side effects. The glaucoma model used is another limitation recognized by the authors. Although this glaucoma experimental model has been used before and has been shown to be reliable and reproducible [31–33], glaucoma is a multifactorial and very complex disease. Therefore, combining data from studies using several different glaucoma models is important for understanding the complete picture of EPO ocular neuroprotection in glaucoma disease. In spite of the limitations mentioned above, these studies open new perspectives concerning EPO administration for future studies targeting ocular neuroprotection, both in preclinical and clinical scenarios.

Since structure and function are highly correlated in the vertebrate retina [34], our findings suggest a neuroprotective effect of subconjunctival rHuEPO injection on the retinas of albino rats with induced glaucoma.

In conclusion, rHuEPO administered via the subconjunctival route after glaucoma induction showed beneficial effects both on cones, rods, and their outputs, and also on RT. However, more studies should be performed to assess EPO kinetics when administered via the subconjunctival route in glaucoma conditions.

**Acknowledgements**

The results of this study have been partially presented at the Congress of the European College of Veterinary Ophthalmology, Estoril, Portugal (2017): “Did subconjunctival administration of erythropoietin induce a neuroprotective effect in glaucomatous rats?” by A.P. Resende, S. Rosolen, T. Nunes, B. São Bras, and E. Delgado.

This work was supported by UID/CVT/00276/2013, Fundação para a Ciência e Tecnologia (SFRH/BD/65793/2009) and a European Society of Veterinary Ophthalmology Research Grant (ESVO-2012-004).
Disclosure Statement

The authors report no conflicts of interest. There are no financial interests in the equipment or methods described.

Statement of Ethics

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Faculty of Veterinary Medicine – University of Lisbon Ethical Committee.

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