UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

# STUDY ON ERYTHROPOIETIN SUBCONJUNCTIVAL ADMINISTRATION IN A GLAUCOMA ANIMAL MODEL

ANA PAULA SIMÕES NUNES DE RESENDE

ORIENTADOR:
Doutora Esmeralda Sofia da Costa Delgado
CO-ORIENTADOR:
Doutora Berta Maria Fernandes Ferreira São Braz

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Clinica

2018

UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

# STUDY ON ERYTHROPOIETIN SUBCONJUNCTIVAL ADMINISTRATION IN A GLAUCOMA ANIMAL MODEL

ANA PAULA SIMÕES NUNES DE RESENDE

ORIENTADOR:
Doutora Esmeralda Sofia da Costa Delgado
CO-ORIENTADOR:
Doutora Berta Maria Fernandes Ferreira São Braz

JÚRI:
Presidente: Doutor Rui Manuel de Vasconcelos e Horta Caldeira
Vogais:
- Doutor António José Leitão Neves Almeida
- Doutora Catarina Falcão Trigoso Vieira Branco Lavrador
- Doutora Sandra Maria da Silva Branco
- Doutor Carlos Marques Neves
- Doutora Esmeralda Sofia da Costa Delgado

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Clinica

2018

*To my father, Jorge and my children, Sofia and Manuel*

*In memory of my mother, Paula*
*I miss your unique smile every day of my life*

# Acknowledgements

This PhD was a great opportunity and a truly life-changing experience for me and it would have never been possible without the support and guidance that I received from many people. For this reason, I wish to express my sincere gratitude:

To my advisor Professora Doutora Esmeralda Delgado for all the advices, ideas, moral support and patience in guiding me through this project. She helped me and steered me in the right direction. More than my adviser she is a friend!

To my co-adviser Professora Doutora Berta São Braz for her support and encouragement since my first years in this faculty…this is our third thesis together! Her help over the years was very important to all the final achievements.

To Doctor Serge Rosolen for sharing his knowledge about ERG, for his invaluable advices on writing the scientific articles and for encouraging me in research. His passion for science is contagious! I´m special grateful for the opportunity he gave me of making part of this amazing work group, European Network in Veterinary Ophthalmology & Animal Vision (REOVVA).

To Professor Doutor Luís Tavares for being the first one to propose me this challenge. Besides all his support as FMV president and CIISA coordinator, I'm grateful for his trust.

To Professor Doutor Paulo Souza Ramalho for giving the motto for this thesis!

To Professora Doutora Conceição Peleteiro for the hospitality in the Pathologic Anatomy facilities and for her support and advices in histological observations.

To Sandra Carvalho for the technical support with histological sections and for helping me optimizing my immunohistochemistry assays. I will never forget our long journeys working on the microtome! To D. Maria do Rosário Luís for being always a kind help at the laboratory.

To Professor Doutor António Ferreira for kindly receiving me in the hospital facilities.

To Professor Doutor António Almeida and Doutora Lídia Gonçalves, for the work undertaken during *in vitro* studies in FFUL facilities. I hope that there will be many more collaborations between us in the future.

To Telmo Nunes for his statistical assistance, moral support and his hard questions which led me to widen my research to obtain better answers.

To Drª Belmira for all the kind words during this years and to Inês. Both ensure the welfare of all the animals included in this study.

To Diogo Guerra for the fantastic illustrations. It was a pleasure to work with him! To Victor Pedro for kindly helping the final formatting and editing this thesis. Their work was a valuable contribution to final manuscript.

To all people that kindly helped me in FMV. A special thanks to Beatriz Silva for her contribution in *in vitro* study, to Nuno Félix for collecting samples that allowed the optimization of immunohistochemistry techniques, to Alexandre Trindade for helping me with the immune-fluorescent microscope and to Sara Martins for being an extra pair of hands in the ERG examinations.

To my colleagues, nurses and technicians from Vet Visão, for providing support and friendship that I needed to finish this thesis and for constantly demanding the best of me, which makes me grow as a clinician.

To my longtime friends, Maria João, Madalena, Ana e Lucinda, who always took the time to listen. Their sense of humor, patience, optimism and advice was more valuable than they could ever imagine!

To my brother David, my sister in law Filipa and all my family (fortunately they are too many to name here!) for all their love, encouragement, support and for having always been there for me. I am very lucky to have all them in my life! A special thanks to Zé for his friendship and dedication that made possible all my adventures in academic life.

To my daughter Sofia and my son Manuel who are the joy of my soul and the love of my life. They add meaning to my existence!

Lastly, but most importantly, I wish to thank my parents for their unconditional love. My gratitude to them is beyond words…

Thank you all!

# Financial Support

This work was supported by:
Fundação para a Ciência e Tecnologia (FCT), through my PhD fellowship (SFRH/BD/65793/2009); Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA) da Faculdade de Medicina Veterinária, Universidade de Lisboa (UID/CVT/00276/2013) and European Society of Veterinary Ophthalmology Research Grant (ESVO-2012-004).



# Resumo

**Título: Estudo da administração subconjuntival de eritropoietina num modelo animal de glaucoma.**

O glaucoma é a principal causa de cegueira irreversível no mundo. A morte das células ganglionares da retina (RGC) causada por hipóxia e isquémia resulta numa progressiva perda de visão. Apesar do glaucoma ser uma doença neurodegenerativa multifatorial, as únicas opções terapêuticas visam o controle da pressão intraocular, não havendo atualmente um tratamento eficaz para prevenir a apoptose das RGC. Estudos recentes demonstraram que a eritropoietina (EPO), uma glicoproteína sintetizada maioritariamente em resposta a estados de hipóxia, tem ação neuroprotetora e neuroregenerativa em várias doenças oculares, tendo revelado resultados promissores em vários modelos animais de glaucoma. Nos estudos experimentais em que a EPO foi utilizada como substância neuroprotetora, foi administrada pelas vias sistémica, intravítrea ou retrobulbar para se obterem concentrações terapêuticas na retina. No entanto, a administração sistémica prolongada de EPO pode produzir efeitos secundários adversos relacionados com o aumento da hematopoiese, enquanto que as administrações intravítrea ou retrobulbar são procedimentos invasivos suscetíveis de causar várias complicações tais como endoftalmite, descolamento de retina, vitreite, retinite, coroidite ou catarata.

Esta tese teve por objetivo estudar uma via de administração periocular de EPO não invasiva, segura, eficaz e sem efeitos adversos. Assim, este trabalho avaliou a via subconjuntival como uma alternativa para a administração ocular de EPO em condições de glaucoma. No primeiro estudo *in vitro*, quantificou-se a permeação da EPO nos tecidos perioculares. O restante trabalho, desenvolvido em modelos *in vivo*, testou a permeação ocular da EPO após administração subconjuntival, tanto em condições fisiológicas como de glaucoma. O estudo contemplou ainda os efeitos morfológicos e fisiológicos da EPO ao nível da retina em animais glaucomatosos.

Os resultados obtidos demostraram que a EPO, quando administrada pela via subconjuntival, pode permear as principais barreiras oculares e atingir as RGC, em ambas as condições testadas, fisiológicas e de glaucoma, sem efeitos adversos locais ou sistémicos apreciáveis. Além disso, revelaram que a administração subconjuntival de EPO parece ter efeitos benéficos estruturais e funcionais na retina após a indução de glaucoma experimental em ratos.

**Palavras chave:** eritropoietina, glaucoma, via subconjuntival, células ganglionares da retina, rato

# Abstract

**Thesis Title: Study on erythropoietin subconjunctival administration in a glaucoma animal model**

Glaucoma is the number one cause of irreversible vision loss worldwide. Death of retinal ganglion cells (RGC), which results in the progressive loss of visual function, occurs in glaucoma and other ocular diseases caused by hypoxia and ischemia. Although glaucoma is a multifactorial neurodegenerative disease, the only currently method of treatment involves reduction of intraocular pressure and, at the present, there is no effective treatment to prevent RGC apoptosis. Notably, it has been reported that erythropoietin (EPO), a cytokine hormone produced in response to hypoxia, has significant neuroprotective and neuroregenerative properties in several types of ocular disorders. Pre-clinical studies in glaucoma animal models involving EPO have yielded very promising results. All studies involving EPO ocular administrations have used systemic, intravitreal or retrobulbar administration to reach retinal desired EPO concentrations. However, EPO chronic systemic administration can lead to adverse side effects related with haematopoiesis stimulation, while intravitreal or retrobulbar administrations are invasive procedures that can induce several complications such as endophthalmitis, retinal detachment, vitreitis, retinitis, choroiditis or cataracts.

This thesis aims to clarify if EPO's neuroprotection could be achieved by a non-invasive and safe periocular administration route without adverse effects. Being so, this work evaluates the subconjunctival route as an alternative for EPO administration in glaucoma disease. After the first *in vitro* study, where the permeation of EPO across the periocular tissues was quantified, all work was developed in *in vivo* models. EPO's ocular permeation after subconjunctival administration was tested, both in physiological and glaucomatous conditions. Furthermore, both retinal morphological and physiological effects of EPO administered by this route were assessed in glaucomatous animals.

Results showed that EPO, when administered subconjunctivally, can permeate the main ocular barriers and reach RGC layers, in both physiological and glaucomatous conditions, without significant local or systemic side effects. More than showing that EPO can reach the retina by this route, results also concluded that subconjunctival EPO administration seems to have structural and functional beneficial effects on the retina after glaucoma induction in rats.

**Keywords:** erythropoietin, glaucoma, subconjunctival route, retinal ganglion cells, rat

# Table of Contents


Acknowledgements ..... iii
Financial Support ..... v
Resumo ..... vii
Abstract ..... ix
Table of Contents ..... xi
List of Figures ..... xv
List of Tables ..... xxi
Acronyms and Abbreviations ..... xxiii
List of Publications and Awards ..... xxvii

**CHAPTER 1**

**General introduction ..... 1**

**1. GLAUCOMA ..... 3**

1.1 Aqueous humor dynamics and intraocular pressure ..... **5**
1.2 Definition and classification of glaucoma ..... **9**
1.3 Glaucoma in Veterinary Medicine ..... **10**
1.4 Inheritance of the glaucoma ..... **19**
1.5 Mechanisms of retinal ganglion cells death in glaucoma ..... **20**
1.5.1. Neurotrophic factors deprivation ..... **20**
1.5.2. Activation of apoptosis ..... **21**
1.5.3. Neurotoxicity ..... **23**
1.6 Glaucoma treatment ..... **26**
1.6.1 Traditional glaucoma treatment ..... **26**
1.6.2. Neuroprotection in glaucoma treatment ..... **30**

**2. ERITHROPOIETIN ..... 33**

2.1 The Expression of EPO in the Retina ..... **35**
2.2 The protective effects of EPO on the retina ..... **36**

2.2.1 EPO neuroprotective signaling pathways ..... 36
2.2.2 EPO protective effects on the retina ..... 38
**3. OCULAR DRUG DELIVERY ..... 43**
3.1 Blood-ocular barriers ..... 44
3.2 Ocular delivery routes ..... 46
3.2.1 Topical administration ..... 47
3.2.2 Systemic administration ..... 48
3.2.3 Intravitreal drug administration ..... 48
3.2.4 Periocular drug administration ..... 48

**CHAPTER 2 ..... 53**
**Rational, hypothesis and study aims ..... 53**
Rational ..... 55
Hypothesis ..... 55
Aims ..... 55

**CHAPTER 3**
**Ex vivo permeation of erythropoietin through porcine conjunctiva, cornea and sclera ..... 57**
Abstract ..... 59
Introduction ..... 60
Materials and Methods ..... 62
Results ..... 64
Discussion and Conclusions ..... 67

**CHAPTER 4**
**Alternative route for erythropoietin ocular administration ..... 71**
Abstract ..... 73
Introduction ..... 74
Materials and Methods ..... 75
Results ..... 77
Discussion and Conclusions ..... 80

**CHAPTER 5**
**Ocular erythropoietin penetration after subconjunctival administration in glaucomatous rats ..... 83**
Abstract ..... 85
Introduction ..... 86

Materials and Methods ..... 87
Results ..... 89
Discussion and Conclusions ..... 93

**CHAPTER 6**
**Functional and structural effects of erythropoietin subconjunctival administration in glaucomatous animals ..... 97**
Abstract ..... 99
Introduction ..... 100
Material and methods ..... 101
Results ..... 103
Discussion and conclusions ..... 108

**CHAPTER 7**
**General discussion, conclusions and future directions ..... 111**
General discussion ..... 113
Conclusions ..... 118
Future directions ..... 119

**REFERENCES ..... 121**

**ANNEXES ..... 143**

# List of Figures

**Figure 1** - Anatomical organization of the eye (A) and the retina (B). Visual information flows from the photoreceptors, cones (1) and rods (2), via the bipolar cells (3) to the retinal ganglion cells (4), which project their long axons out of the eye in the optic nerve towards the brain. Horizontal cells (5) and amacrine cells (6) can modify this direct circuitry via lateral connections. © Diogo Guerra. 2017. ..... **4**

**Figure 2** - Theoretic diagram of aqueous production based on the gradient osmotic flow model. Through the passive (A) and the active (B) processes, three mechanisms are involved in aqueous humor formation: diffusion, ultrafiltration and active secretion © Diogo Guerra. 2017. ..... **6**

**Figure 3** - Schematic diagrams illustrating the trabecular meshwork conventional outflow pathway (A) and the uveoscleral outflow pathway (B). © Diogo Guerra. 2017. .... **7**

**Figure 4** - Primary glaucoma in a 5-year-old Siberian Husky (originals). ..... **12**

**Figure 5** - Secondary glaucoma caused by anterior uveitis. Note the corneal oedema and hypopyon in the ventral anterior chamber in this dog, associated with a systemic disease (original). ..... **12**

**Figure 6** - Anterior uveitis may develop secondary pupillary occlusion from a total annular posterior synechiae (iris to lens capsula), thus resulting in iris bombé (original). ... **12**

**Figure 7** - Anterior lens luxation may cause secondary glaucoma due to the pupillary obstruction and the compression of the iridocorneal angle and ciliary cleft (original). ..... **13**

**Figure 8** - Posterior or vitreal lens luxation with aphakic crescent. Note the ocular signs of glaucoma: severe episcleral congestion and pupil dilatation (original). ..... **13**

**Figure 9** - Secondary glaucoma can be caused by intraocular neoplasia in dog. A ciliary body melanoma can be visualized extending into the pupil (arrow) (original). ..... **13**

**Figure 10** - Hyphema, filling the anterior chamber, induced secondary glaucoma in this dog (original). ..... **13**

**Figure 11** - Unilateral congenital glaucoma in a domestic shorthair kitten (original). ..... **14**

**Figure 12** - Secondary glaucoma in cat can be caused by anterior uveitis. Two examples of anterior uveitis are presented: chronic (A) and idiopathic anterior uveitis (B) (originals). . **15**

**Figure 13** - Advanced stages of a diffuse iridal melanoma in cat (melanotic – A and amelanotic – B) (originals). The diffuse iridal melanotic melanoma starts with a progressive pigmentation of the iris which occurs over months to several years. The pigmentation may develop simultaneously in several areas on the anterior iridal surface and increase with time. Secondary glaucoma occurs when there is infiltration of the iridocorneal angle by malignant cells. ..... **15**

**Figure 14** - Secondary glaucoma caused by chronic or recurrent uveitis. Note the miosis, posterior synechiae and cataract (original). ..... **16**

**Figure 15** - Despite being a rare condition in horses, the anterior lens luxation caused secondary glaucoma in this horse's right eye (original)..... **16**

**Figure 16** - Some examples of clinical signs of glaucoma (originals): A - latent stage of PACG in dog (mydriasis, transient corneal oedema history); B - acute glaucoma with conjunctival hyperemia and scleral injection, diffuse corneal oedema and an unresponsive, dilated pupil in dog. C – Note the conjunctival hyperemia and scleral injection. Chronic glaucoma can present buphthalmos (D) and Haab's striae (dog - E and horse - F) that are caused by continuous stretching of the eye. ..... **18**

**Figure 17** - The intrinsic and extrinsic apoptotic pathways. © Diogo Guerra. 2017. ..... **22**

**Figure 18** - Hypothetical mechanisms leading to excitotoxicity in glaucoma. Adapted from Seki (2008). ..... **24**

**Figure 19** - Appearance of the tube of the Ahmed gonioshunt in a dog's anterior chamber (original). ..... **29**

**Figure 20** - In veterinary medicine, enucleation is a frequent option to relieve the pain of blind eyes in medically refractory glaucoma (original). ..... **29**

**Figure 21** - Also evisceration with an intrascleral silicone prosthesis can be an option to relieve the pain of these patients. Appearance of intrascleral silicone prosthesis in a dog's right eye (original). ..... **29**

**Figure 22** - Schematic diagram of the possible actions of EPO in neuroprotection, ''systemic protection'' and cardioprotection. Adapted from Bogoyevitch (2004). ..... **35**

**Figure 23** - EPO neuroprotective signalling pathways. The production of EPO is mainly evoked by HIF, and this up-regulation results in a sequence of downstream signalling activations, which will protect retinal cells.©Diogo Guerra. 2017. ..... **37**

**Figure 24** - Squematic representation of the EPO protective effects on the retina and its pathways. © Diogo Guerra. 2017. ..... **39**

**Figure 25** - Squematic representation of blood-ocular barriers: anterior blood-aqueous barrier (A) and blood-retinal barrier (B). © Diogo Guerra. 2017. ..... **45**

**Figure 26** - Squematic representation of the ocular routes commonly used for retinal drug delivery: topical (A), systemic (B), intravitreal (C), sub-conjunctival (D) and retrobulbar (E). © Diogo Guerra. 2017. ..... **47**

**Figure 27** - Schematic presentation of the kinetic phases of sub-conjunctival injections for retinal drug delivery. Adapted from Del Amo 2017. ..... **50**

**Figure 28** - Cumulative amount of EPO permeated (mIU/cm$^2$) of each ocular membrane - conjunctiva (n=10), cornea (n=10) and sclera (n=10) – at each time point. ..... **64**

**Figure 29** - Immunohistochemistry for cell nucleus marker of the conjunctiva, cornea and sclera (DAPI, blue) and EPO antibody (red), six hours after the administration into the donor phase. Note erythrocytes inside a blood vessel in control conjunctiva membrane due to auto-fluorescence staining (arrow). (x 10). ..... **66**

**Figure 30** - Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CH, Choroid. ***a***: control group. In the control group, EPO was not detectable. ***b***: at 12 hr after EPO administration the protein expression was more evident in the retinal pigment epithelium (white arrows) in spite of starting to appear in the RGC (green arrow). Note a blood vessel with erythrocytes cells (yellow arrow). ***c***: at 24 hr after EPO administration, the protein was detected in all retinal cell layers. ***d***: at 36 hr after EPO administration, the signal was more concentrated in the RGC layer (white arrows). Bar 30 µm. ..... **77**

**Figure 31** - ***a***: Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer 60 hr after EPO administration. ***b***: The same cells are contrasted with PGP 9.5 antibody (green), showing EPO inside a retinal ganglion cell. Bar 12 µm. ..... **78**

**Figure 32** - Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and PGP 9.5 antibody (green) showing EPO in others neuroretinal cells, probably a bipolar or amacrine cell once is localized in inner nuclear layer

(arrow). Note the strong signal in RGC layer and in nerve fiber layer 60 hr after EPO administration. NFL, nerve fiber layer; RGC, retinal ganglion cell layer; INL, inner nuclear layer. Bar 12 µm. ..... **78**

**Figure 33** - Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and a higher concentration (1/200 ab72911; 1/200 ab6724) of PGP 9.5 antibody (green) 60 hr after EPO administration. PGP9.5 stains neurorretinal cells in the outer plexiform layer, inner nuclear layer and retinal ganglion cell layer (yellow arrows). Note the strong EPO signal in RGC layer, outer plexiform layer and in nerve fiber layer (white arrows). NFL, nerve fiber layer; RGC, retinal ganglion cell layer; INL, inner nuclear layer. Bar 25 µm. ..... **79**

**Figure 34** - Heamatocrit variation after subconjunctival EPO administration. In the x axis we represent Time in days after subconjunctival administration and in the y axis Heamatocrit levels in percentage. The values changed from 54,8±2,9% on day 0 to 55,8±2,1% (p=0,456) on day 7, to 54,0±1,8% on day 14 (p=0,512), to 53,3±2,8% (p=0,246) on day 21 and to 53,2±2,5% on day 28 (p=0,215) showing no significant differences ..... **79**

**Figure 35** - IOP variation under general anaesthesia on day 0, before and one hour after coagulation of episcleral veins. The x-axis represents two time points: A- before and B- one hour after the surgery and in the y-axis we represent IOP values in mm Hg (n=15). ..... **90**

**Figure 36** - IOP variation during the study. The x-axis represents time in days and the y-axis the IOP values in mm Hg. Day 1 (n=15), day 3 (n=12), day 7 (n=9) and day 14 (n=6) represents the number of days after the glaucoma induction. ..... **90**

**Figure 37** - Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Operated (right) eyes: ***a.*** one day after glaucoma induction and rHuEPO administration, the protein expression was more evident in the RGC layer (white arrows). ***b.*** Seven days after glaucoma induction and rHuEPO administration, the protein was still detected in retinal ganglion cells layer (white arrow) and was also detectable in the inner nuclear layer (green arrow). ***c.*** Fourteen days after glaucoma induction and rHuEPO administration, the signal was residual but still present in all retinal cell layers. ***d.*** Retinal sections fourteen days after glaucoma induction in a non-treated animal (saline solution). Note the significant decrease of retinal thickness. Non-operated (left) eyes: ***e***, ***f***, ***g*** and ***h.*** Bar 25 µm ..... **91**

**Figure 38** - Immunohistochemistry for cell nucleus marker of the retina (DAPI, blue) and EPO antibody (red). Seven days after glaucoma induction and rHuEPO administration a detail of EPO expression located at the RGC layer is shown. Bar 10 µm ..... **92**

**Figure 39** - Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and neuronal cell cytoplasm marker (PGP 9.5 antibody, green). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. ***a.*** One day after glaucoma induction and rHuEPO administration, EPO protein signal was strongly evident in RGC layer (white arrows). Due to the high intraocular pressure and the hemato-retinal barrier breakdown, erythrocytes are present and overspread on ONL (green arrow). ***b.*** The use of the anti-PGP9.5 antibody allowed the identification of neuronal cell bodies (orange arrows). Bar 25 µm. ..... **93**

**Figure 40** - H&E staining of rats' retinas at the optic nerve level. At the distance of 500 µm from the optic nerve (ON) (red trace) ventral and dorsal retinal thickness were measured. ..... **103**

**Figure 41** - H&E staining of rats' retinas at the optic nerve level. The distance of 1500 µm were measured from the optic nerve (ON) (red trace) and three measurements were done in the same visual field. ..... **103**

**Figure 42** - IOP variation before and 1 hour after coagulation of 3 episcleral veins (n=26) with a significant increase on right eyes that underwent surgery. ..... **104**

**Figure 43** - 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. For each graphic is shown the curves obtained on day 0 (before surgery), day 7 (after surgery) and finally on day 21 (at the end of the study). ..... **106**

**Figure 44** - Rats retinas measured at 1500 µm from the optic nerve. Retinas from the glaucomatous eyes (right eye): control group (A) and treated group (B) and a normal retina from a non-glaucomatous eye (left eye) (C) (H&E; Scale bar: 100 µm). ..... **107**

**Figure 45** - Retinal thickness at 500 µm and 1,500 µm from the optic nerve head, on the control and treated groups. Bars represent median score; boxes include 25th to 75th percentile; whiskers represent ±1.58 Interquartile range/sqrt (n). ..... **107**

# List of Tables

**Table 1** - Types of glaucomas in dogs ..... **10**

**Table 2** - Canine breeds predisposed to primary glaucoma..... **11**

**Table 3** - Types of glaucomas in cats. ..... **14**

**Table 4** - Mechanisms of surgical treatments for the glaucomas ..... **28**

**Table 5** - Summary of current research strategies employed to study neuroprotection in glaucoma previously applied in CNS diseases ..... **31**

**Table 6** - Cumulative amount of EPO in the receptor phase of each ocular membrane - conjunctiva (n=10), cornea (n=10) and sclera (n=10) – at each time point. Data are presented as mean± standard deviation (mIU/cm$^2$) and percentage (%) of the total amount of rHuEPO instilled in the donor phase. ..... **65**

**Table 7** - IOP under general anaesthesia (mm Hg) ..... **90**

**Table 8** - IOP in awake condition (mm Hg) ..... **90**

**Table 9** - Amplitude of b-waves in photopic flash, photopic flicker and scotopic examinations of the treated and control groups, at day 0, day 7 and day 21. Results are reported as median [min;max] (* p< 0,05). ..... **105**

**Table 10** - Retinal thickness of the treated and control groups at 500 µm and 1500 µm from the optic nerve (ON). Results are reported in µm as median ± standard error (* p< 0,05)..... **107**

# Acronyms and Abbreviations

| | |
|---|---|
| **AH** | aqueous humor |
| **AIF** | apoptosis-inducing factor |
| **AKT** | protein kinase B |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **Apaf-1** | apoptotic protease-activating factor-1 |
| **ATP** | adenosinetriphosphate |
| **BAB** | blood-aqueous barrier |
| **Bad** | Bcl-2-associated death protein |
| **Bax** | Bcl-2-asssociated x protein |
| **BBB** | blood brain barrier |
| **Bcl-2** | B-cell leukaemia lymphoma 2 |
| **Bcl-xL** | B-cell leukaemia lymphoma xL |
| **BDNF** | brain-derived neurotrophic factor |
| **BIRC-4** | baculoviral IAP repeat-containing protein 4 |
| **BRB** | blood-retinal barrier |
| **$Ca^{2+}$** | calcium ions |
| **CG** | control group |
| **cIAP** | cellular inhibitor of apoptosis |
| **$C_{max}$** | maximum concentration |
| **CNS** | central nervous system |
| **CNTF** | ciliary neurotrophic factor |
| **$CO^2$** | carbon dioxide |
| **Cop-1** | Copolymer-1 |
| **COX-2** | cyclooxygenase-2 |
| **CRP** | C-reactive protein |

| | |
|---|---|
| **DAPI** | 4’,6-diamidino-2-phenylindole |
| **DNA** | deoxyribonucleic acid |
| **e.g.** | *exempli gratia* |
| **ELISA** | enzyme-linked immune-sorbent assay |
| **EndoG** | endonuclease G |
| **EPO** | erythropoietin |
| **EPOR** | erythropoietin receptor |
| **ERG** | electroretinography |
| **ERK** | extracellular signal-regulated kinase |
| **ERK-1/2** | extracellular signal-related kinase-1/2 |
| **ET-1** | endothelin-1 |
| **Fas** | Fibroblast-associated |
| **FasL** | Fas Ligand |
| **GDNF** | glial cell line-derived neurotrophic factor |
| **GSK-3** | glycogen synthase kinase 3 |
| **$H^+$** | hidrogen ion |
| **$H_2O$** | water |
| **$H_2O_2$** | hydrogen peroxide |
| **$HCO_3^-$** | bicarbonate |
| **Hg** | mercury |
| **HIF-1** | hypoxia-inducible factor-1 |
| **HSPs** | heat shock proteins |
| **i.e.** | *id est* |
| **i.p.** | intraperitoneal |
| **i.v.** | intravenous |
| **IAP** | Inhibitors of apoptosis protein |
| **IL** | interleukins |
| **IOP** | intraocular pressure |
| **IU** | international unit |
| **JAK-2** | Janus tyrosine kinase 2 |
| **$K^+$** | potassium ion |
| **MAPK** | mitogen-activated protein kinase |
| **$Mg^{2+}$** | magnesium ions |
| **MK801** | dizocilpine |
| **MMPs** | metalloproteinases |
| ***n*** | number of experimental units |
| **$Na^+$** | sodium ion |
| **NF-κB** | nuclear factor Kappa B |
| **NGF** | nerve growth factor |
| **NMDA** | N-methyl-D-aspartate |
| **NO** | nitric oxide |

| | |
|---|---|
| **NT-3** | neurotrophin-3 |
| **NT-4/5** | neurotrophin-4/5 |
| **$^1O_2$** | singlet oxygen |
| **$O^{2-}$** | superoxide anion |
| **OH*** | hydroxyl radical |
| **OMI/HTRA2** | high-temperature-requirement protein A2 |
| **ON** | optic nerve |
| **p** | probability value |
| **$p75^{NTR}$** | p75 receptor |
| **PACG** | primary angle-closure |
| **Papp** | apparent permeability coefficients |
| **PBS** | phosphate-buffered saline |
| **PGE2** | Prostaglandin E2 |
| **PHD** | prolyl hydroxylase |
| **PI3K** | phosphatidylinositol-3-kinase |
| **PLR** | pupillary light reflexes |
| **POAG** | primary open-angle glaucoma |
| **Qt** | cumulative amount of permeated |
| **RGC** | retinal ganglion cells |
| **rHuEPO** | human recombinant erythropoietin |
| **ROS** | reactive oxygen species |
| **RPE** | retinal pigment epithelium |
| **SAA** | serum amyloid A |
| **SD** | standard deviation |
| **SMAC/DIABLO** | second mitochondria-derived activator of caspases |
| **STAT** | signal transducer and activator of transcription |
| **TG** | treated group |
| **TM** | trabecular meshwork |
| **TNF** | tumor necrosis factor |
| **TRAIL** | TNF-related apoptosis inducing ligand |
| **Trk** | tropomyosin related kinase |
| **VEGF** | vascular endothelial growth factor |
| **XIAP** | X-linked IAP |

# List of Publications and Awards

**The research work conducted in this thesis originated the following papers published in international refereed and indexed journals:**

Resende, A. P., São-Braz, B., & Delgado, E. (2013). Alternative route for erythropoietin ocular administration. *Graefe's Archive for Clinical and Experimental Ophthalmology*, 251(8), 2051–2056. http://doi.org/10.1007/s00417-013-2367-7

Resende, A. P., São-Braz, B., & Delgado, E. (2016). Ocular Erythropoietin Penetration after Subconjunctival Administration in Glaucomatous Rats. *Ophthalmic Research, 56*(2), 104–110. http://doi.org/10.1159/000444327

Resende, A. P., Silva, B., São-Braz, B., Nunes, T., Gonçalves, L., & Delgado, E. (2017). *Ex vivo* permeation of erythropoietin through porcine conjunctiva, cornea, and sclera. *Drug Delivery and Translational Research*, 7(5), 625–631. http://doi.org/10.1007/s13346-017-0399-y

Resende, A. P., Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E. (2018). Functional and structural effects of erythropoietin subconjunctival administration in glaucomatous animals. *Biomed Hub, 3, 488970.* http://*doi: 10.1159/000488970*

**The research work resulted in the following posters and oral presentations:**

Resende, A. P., São-Braz, B., & Delgado, E. (2011). "Erythropoietin reaches the retina after subconjunctival administration". Poster session presented at *the Champalimaud Neuroscience Symposium*, September 18-19, Lisboa, Portugal. (Annexe I)

Resende, A. P., São-Braz, B., & Delgado, E. (2011). "Expression patterns of erythropoietin in the retina after subconjunctival administration". Oral comunication presented at the *Annual meeting of the European Society of Veterinary Ophthalmology*, Octobre 14-16, Prague, Czech Republic. (Annexe II)

Resende, A. P., São-Braz, B., & Delgado, E. (2013). “Immunohistochemistry of the rast’s retina after subconjunctival erythropoietin administration”. Oral comunication presented at the *Annual meeting of the European Society of Veterinary Ophthalmology*, Octobre 17-20, Bucareste, Romenia. (Annexe III)

Resende, A. P., São-Braz, B., & Delgado, E. (2014). “Eritropoietina por via subconjunctival: alternativa promissora no glaucoma canino?”. Oral comunication presented at the *XXII Congresso Nacional da A.P.M.V.E.A.C.*, May 17-18, Lisboa, Portugal. (Annexe IV)

Resende, A. P., Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E. (2014). “Electroretinographic changes after erythropoietin subconjunctival administration in glaucomatous animals”. Oral comunication presented at the *Annual meeting of the European Society of Veterinary Ophthalmology - Nordic eye,* September 4-7, Malmo, Sweeden. (Annexe V)

Resende, A. P., São-Braz, B., & Delgado, E. (2015). “Alterações na electrorretinografia em ratos glaucomatosos após administração subconjuntival de eritropoietina”. Oral comunication presented at the *XI Congresso Hospital Veterinário Montenegro/V Congresso Enfermagem Hospital Veterinário Montenegro*, February 21-22, Sta Maria da Feira, Portugal. (Annexe VI)

Silva, B., Resende, A. P., Gonçalves, L., Nunes, T., São-Braz, B., & Delgado, E. (2016). “Permeabilidade de três membranas oculares à eritropoietina num modelo *in vitro* de olho de porco”. Oral comunication presented at the *XII Congresso Hospital Veterinário Montenegro/ VI Congresso Enfermagem Hospital Veterinário Montenegro*, February 20-22, Sta Maria da Feira, Portugal. (Annexe VII)

Silva, B., Resende, A. P., Gonçalves, L., Nunes, T., São-Braz, B., & Delgado, E. (2016). “Permeability of ocular membranes to topic human recombinant erythropoietin using a pig eye *ex vivo* model” Oral comunication presented at the *Annual meeting of the European Society of Veterinary Ophthalmology - SFEROV*, October 6-9, Toulouse, France. (Annexe VIII)

Resende, A. P., Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E. (2017). “Did subconjunctival administration of erythropoietin induce a neuroprotective effect in glaucomatous rats?” Oral comunication presented at the *Annual meeting of the European College of Veterinary Ophthalmolog*, May 18-21, Estoril, Portugal. (Annexe IX)

**The research work also resulted in the following oral presentations by invitation:**

Resende, A. P. (2016). “Erythropoietin as an alternative for neuroprotection in glaucoma”. Oral comunication by invitation presented at the *Annual meeting of the European College of Veterinary Ophthalmolog meeting - SFEROV*, October 6-9, Toulouse, France.

**The parts of the research work conducted in this thesis has been distinguished with the following awards:**

Best oral presentation award of the European Society of Veterinary Ophthalmology at the 2013 ESVO meeting, held in Bucareste, Romenia, with the theme: “Immunohistochemistry of the rat´s retina after subconjunctival erythropoietin administration”, **Resende, A. P.**, São-Braz, B., & Delgado, E.

Best oral presentation award of the European Society of Veterinary Ophthalmology at the 2014 ESVO-Nordic eye meeting, held in Malmo, Sweeden, with the theme: “Electroretinographic changes after erythropoietin subconjunctival administration in glaucomatous animals”, **Resende, A. P.**, Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E.

Best oral presentation award of the XI Congresso Hospital Veterinário Montenegro / V Congresso Enfermagem Hospital Veterinário Montenegro, 2015, held in Sta Maria da Feira, Portugal, with the theme: “Alterações na electrorretinografia em ratos glaucomatosos após administração subconjuntival de eritropoietina”, **Resende, A. P.**, Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E.

CHAPTER 1

# GENERAL INTRODUCTION

# 1. GLAUCOMA

The eye is the interface between the visual system and the outer world. The vision starts with the eye capturing light, emitted or reflected by objects, in the environment. Light enters through the cornea and passes the anterior chamber, the pupil surrounded by the iris, the lens and the vitreous body, and finally reaches the retina (Bear, Connors & Paradiso, 2007).

The retina is considered part of the central nervous system and is organized in 10 layers, from the sclera to the vitreous: retinal pigment epithelium, photoreceptor layer, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and inner limiting membrane (Fig. 1).

The vertebrate retina has a large diversity of component cells that form morphologically and functionally distinct circuits that work in parallel, and in combination, to produce a complex visual output. There are five major neuronal cell classes: photoreceptors, horizontal cells, bipolar cell, amacrine cells and ganglion cells (Fig. 1) with Müller glial cells providing metabolic and homeostatic support (Hoon, Okawa, Della Santina & Wong, 2014).

Light passes the relatively transparent inner layers and is captured by photo pigment in the outer segments of the photoreceptor cells. Then is converted into electrochemical neural signals witch travel from the cones and rods to the retinal ganglion cells (RGC). The visual information leaves the retina via RGC long axons, the optic nerve (ON), towards the visual cortex in the brain (Bear *et al.*, 2007).

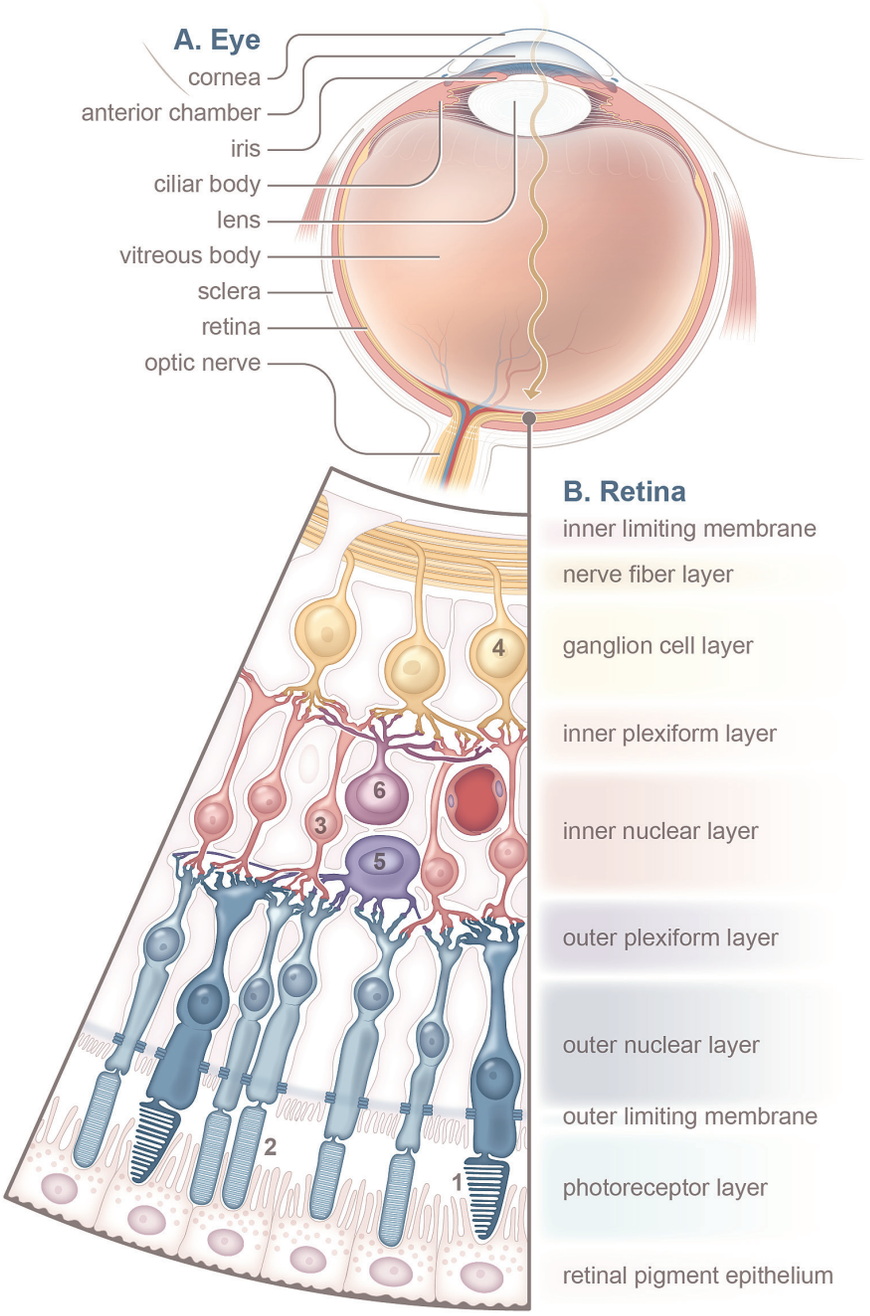


**Figure 1** - *Anatomical organization of the eye (A) and the retina (B). Visual information flows from the photoreceptors, cones (1) and rods (2), via the bipolar cells (3) to the retinal ganglion cells (4), which project their long axons out of the eye in the optic nerve towards the brain. Horizontal cells (5) and amacrine cells (6) can modify this direct circuitry via lateral connections. © Diogo Guerra. 2017.*

## 1.1 AQUEOUS HUMOR DYNAMICS AND INTRAOCULAR PRESSURE

Aqueous humor (AH) is a transparent fluid that fills the anterior and posterior chambers of the eye. With a refractive index of 1.335, its density is slightly greater than that of water (Gelatt, Gilger & Kern, 2013). The lens and cornea must remain clear to allow light transmission, and therefore cannot have vasculature. The AH resembles an ultrafiltrate of plasma for these avascular structures and provides nutrition, removes excretory products from metabolism, transports neurotransmitters, stabilizes the ocular structure and contributes to the regulation of the homeostasis of these ocular tissues (Gelatt *et al.*, 2013; Goel, Picciani, Lee & Bhattacharya, 2010).

Intraocular pressure (IOP) is partly regulated by the rate of AH formation, which normally equals the rate of outflow (Quigley, Dunkelberger & Green, 1989). In the human healthy eye, flow of AH against resistance generates an average IOP of approximately 15 mmHg (Goel *et al.*, 2010). IOP is affected by factors such as specie, age, mean arterial pressure, central venous pressure, blood osmolality, and episcleral venous pressure (Maggs, Miller & Ofri, 2008).

In dogs, mean normal values for IOP are estimated at 19.2 ± 5.5 mmHg (Gelatt & MacKay, 1998) (by applanation tonometry) and between 10.8 ± 3.1 mmHg (Knollinger, La Croix, Barrett & Miller, 2005) and 9.1 ± 3.4 mmHg (Leiva, Naranjo & Peña, 2006) (by rebound tonometry). In cats, mean reported values of normal IOP are 18.4 ± 0.67 mmHg (by applanation tonometry) and 20 ± 0.48 mmHg (by rebound tonometry) (Rusanen, Florin, Hässig & Spiess, 2010). In horses the normal IOP described are 23.3 ± 6.89 mmHg (by applanation tonometry) (Miller, Pickett & Majors, 1990).

Disruption of the equilibrium between production and drainage of AH may result in elevation of IOP, which is the major risk factor in the pathogenesis of glaucoma (Goel *et al.*, 2010).

The two main structures related to AH dynamics are the ciliary body, where it is produced, and the trabecular meshwork, where it is drained (Goel *et al.*, 2010).

### Aqueous humor production

The ciliary body attaches to the scleral spur. Occupying the inner and anterior portion of this structure, in a region called pars plicata, are the ciliary processes. It is in the ciliary processes that the AH production takes place (Goel *et al.*, 2010). The epithelium of the ciliary processes has two layers: an inner, non-pigmented layer in contact with the AH in the posterior chamber, and an external, pigmented layer in contact with the ciliary process stroma. The apical surfaces of the two layers lie in apposition to each other. Both sympathetic and parasympathetic nerves supply the ciliary body (Kiel, Hollingsworth, Rao, Chen & Reitsamer, 2011).

Three mechanisms are involved in AH formation: diffusion, ultrafiltration and active secretion (Fig. 2). The first two processes are passive and do not entail active cellular participation.

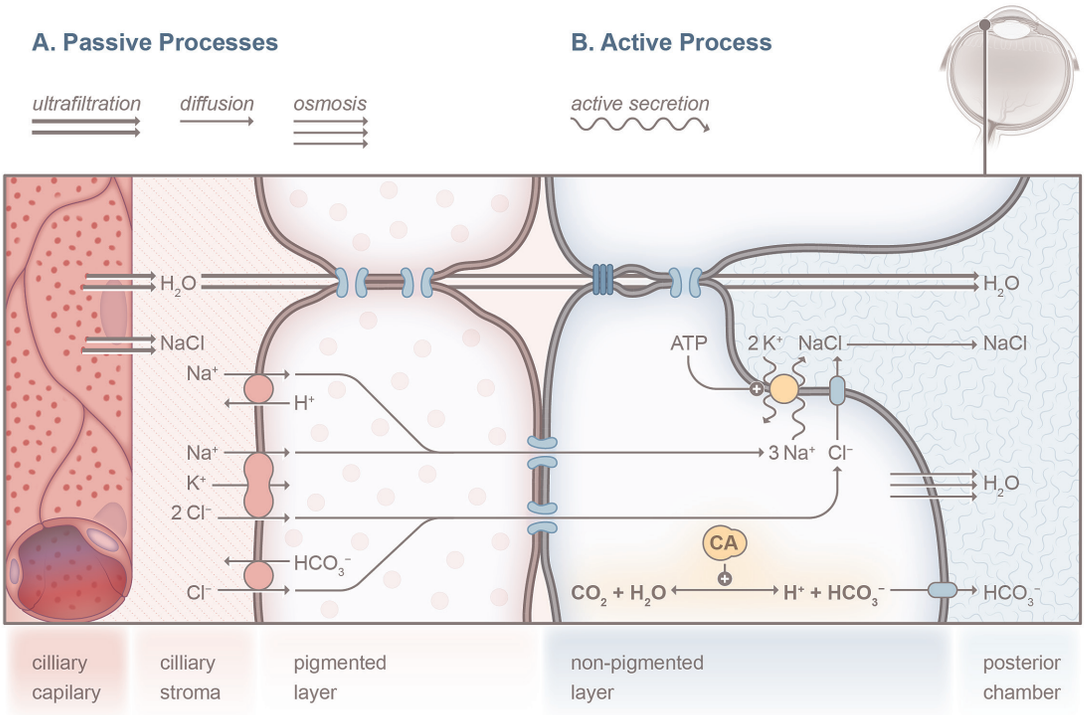


**Figure 2** - *Theoretic diagram of aqueous production based on the gradient osmotic flow model. Through the passive (A) and the active (B) processes, three mechanisms are involved in aqueous humor formation: diffusion, ultrafiltration and active secretion © Diogo Guerra. 2017.*

Diffusion occurs when solutes, especially lipid soluble substances, are transported through the lipid portions of the tissue's membrane between the capillaries and the posterior chamber, proportional to a concentration gradient across the membrane (Civan & Macknight, 2004).

Ultrafiltration is the flow of water and water-soluble substances, limited by size and charge, across fenestrated ciliary capillary endothelia into the ciliary stroma, in response to an osmotic gradient or hydrostatic pressure (Civan & Macknight, 2004).

Active secretion, the major contributor to AH formation, is responsible for approximately 80% to 90% of the total AH formation (Mark, 2010). This process requires energy (usually ATP) to secrete material against a concentration gradient through two mechanisms:

1. The major constituent actively transported from blood to the AH is the $Na^+$ ion. An enzyme complex, $Na^+$/ $K^+$ - ATPase, is an active transport system present in the non-pigmented ciliary epithelium; this enzyme complex is membrane-bound and is found in the highest concentrations along the lateral interdigitation of these cells (Maggio, 2015).
2. Carbonic anhydrase is the enzyme present in the non-pigmented epithelial cells responsible for the formation of bicarbonate, from carbon dioxide and water, and its active transport across the ciliary epithelium. It catalysis the reaction:

$$CO_2\ H_2O \leftrightarrow HCO_3^- + H^+$$

Entry of $Na^+$ ion and bicarbonate into the aqueous is associated with entry of water into the posterior chamber (Fig.2) (Gelatt *et al.*, 2013).

Since the AH is formed mostly by active secretion (Shahidullah, Wilson, Yap & To, 2003), the carbonic anhydrase inhibitors are among the most effective drugs decreasing IOP in medical management of glaucoma (Maggio, 2015).

Aqueous humor circulates within the anterior chamber because of the temperature difference between the air-cooled cornea and the iris, process known as thermal circulation (Goel *et al.*, 2010). Then, AH leaves the eye via the anterior chamber angle, also called iridocorneal angle, the structure formed by the joining of the iris and the cornea.

**Aqueous humor outflow**

The AH is drained both through conventional and unconventional pathways (Fig. 3).

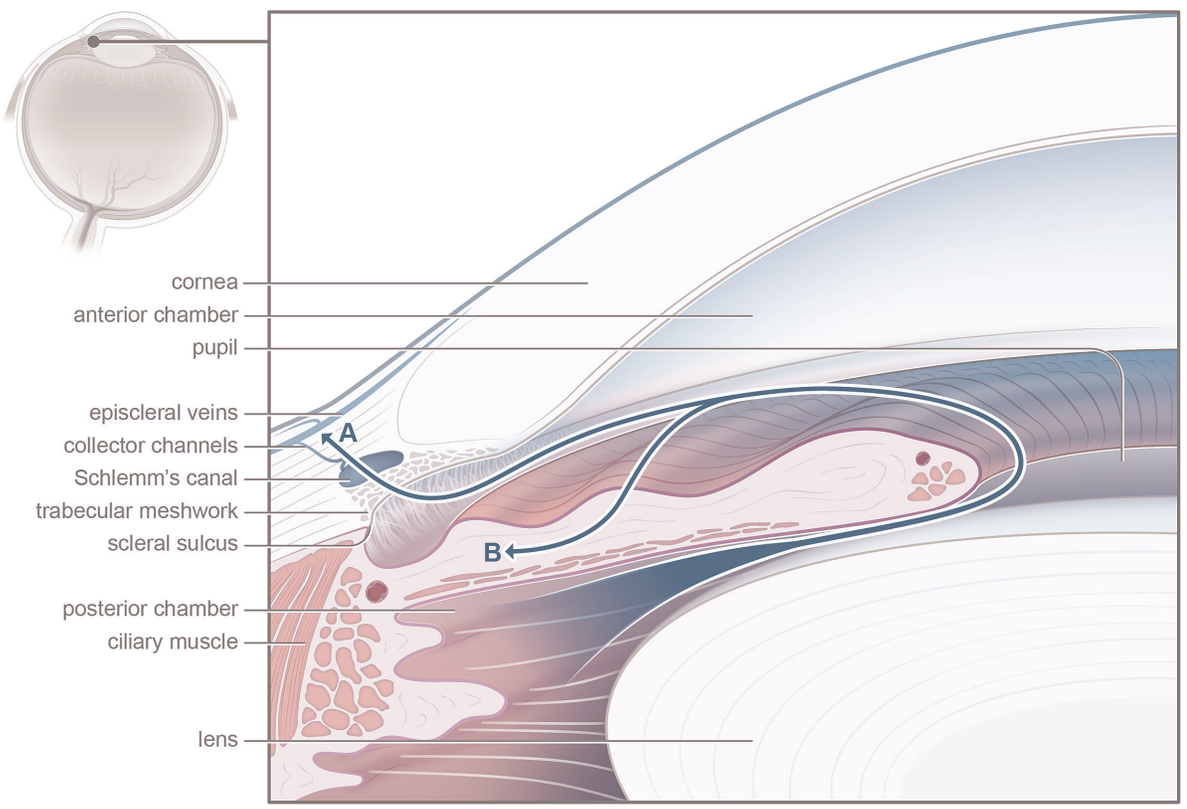


**Figure 3** - *Schematic diagrams illustrating the trabecular meshwork conventional outflow pathway (A) and the uveoscleral outflow pathway (B). © Diogo Guerra. 2017.*

The corneoscleral trabecular meshwork (TM) is the structure that overpasses the scleral sulcus and converts it into a circular channel, called Schlemm's canal. In the TM conventional outflow pathway, the AH flows out through the TM into the Schlemm's canal and is subsequently absorbed into the episcleral veins via the collector channels (Fig. 3 A). In the uveoscleral outflow pathway, the AH flows from the face of the ciliary body and iris to the ciliary muscle and suprachoroidal space to either veins in the choroid and sclera or through scleral pores to episcleral tissue (Fig. 3 B) (Goel *et al.*, 2010). The amount of AH drained through this pathway, the unconvencional, varies between species. In cats, it only accounts for 3% of the total AH drainage, 15% in dogs but in horses it can reach a much greater percentage (Samuelson, Smith & Brooks, 1989; Smith, Samuelson, Brooks & Whitley, 1986). Morover, it is influenced by the contraction of the ciliary body muscle and by the difference in hydrostatic pressure between the anterior chamber and the suprachoroidal spaces (Maggio, 2015).

**Variations in Intraocular Pressure**

The IOP can suffer variations during the day and also with age, blood flow and inflammation.

In some species, IOP varies slightly during the day, being higher in the morning and gradually declining over the day (e.g. humans and dogs). The opposite variation is observed in cats (Del Sole, Sande, Bernades, Aba & Rosenstein, 2007), rabbits, and nonhuman primates (Maggs *et al.*, 2008).

The rate of AH production in humans is significantly lower at night, attributed to decreased endogenous circulating catecholamine levels, which may partly explain the decreased nocturnal efficacy of topical beta blockers (Topper & Brubaker, 1985).

Both production and outflow of AH tend to decline with age resulting in a slight decrease of the IOP during life. In humans, AH production and IOP tend to decline by 15-35% between the age of 20 and 80 years (Gabelt & Kaufman, 2005), although it varies considerably with ethnic background and the presence of other diseases, such as systemic hypertension and obesity (Quaranta, Katsanos, Russo & Riva, 2013). Similarly, IOP in cats is considerably lower in geriatric cats than in young cats, declining approximately 1 mmHg per year, after 7 years of age (Kroll, Miller & Rodan, 2001).

Disorders associated with substantially lower blood flow to the eye (e.g., dehydration, hypovolemic shock, cardiogenic shock) tend to result in lower IOP. Also, intraocular inflammation, especially uveitis, is able to drastically decrease AH production and IOP (Gelatt *et al.*, 2013; Maggs *et al.*, 2008).

## 1.2 DEFINITION AND CLASSIFICATION OF GLAUCOMA

Glaucoma is a group of diseases characterized by progressive dead of RGC and their axons, which causes structural changes at the level of the lamina cribrosa in the ON head and irreversible vision loss (European Glaucoma Society, 2008; Gottanka, Johnson, Martus & Lutjen-Drecoll, 1997; Hernandez & Pena, 1997).

The modern definition of glaucoma reflects the acknowledgment that this disease is a progressive neurodegenerative process, where an elevated IOP, considered the major risk factor, is only one of the involved mechanisms (Calkins, 2012; Heijl *et al.*, 2002).

Besides elevated IOP (Agis, 2000; Gordon *et al.*, 2002; Leske *et al.*, 2007), other risk factors have been proposed to contribute to glaucoma progression such as age (Leske *et al.*, 2001), genetic background (Wolfs *et al.*, 1998), thinner corneal thickness (Medeiros *et al.*, 2013) and vascular dysregulation (Leske, 2009). The existence of any of these factors might determine an individual's risk to develop glaucoma, but they are not necessarily the cause of this condition. For example, people with elevated IOP do not necessarily develop glaucoma and a number of glaucomatous patients do not seem to suffer from ocular hypertension (Calkins, 2012; Heijl *et al.*, 2002). A progressive loss of vision can occur in those patients with normal tension as well as in patients whose IOP is controlled by drugs (Burgoyne, Downs, Bellezza, Suh & Hart, 2005; Burgoyne & Downs, 2008).

In humans, glaucoma is often classified into primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), secondary angle-closure glaucoma, secondary open-angle glaucoma, congenital glaucoma and juvenile glaucoma (Goel *et al.*, 2010). The most common type may differ from one region of the world to another. For instance, PACG is more prevalent in certain regions in Asia, whereas POAG is more equally distributed throughout the world and is the most common form of the disease (Quigley & Broman, 2006).

POAG is characterized by a progressive cupping of the optic disc resulting from compression, stretching, and remodelling of the connective tissue (extracellular matrix) of the lamina cribrosa. Astrocytes are the major cell type in the optic nerve and may participate actively in the remodelling of the extracellular matrix. There is individual variability in the composition, structure or reactive processes of the tissue supporting the axons in the ON. That may explain the variations in the nature and degree of cupping in response to intraocular pressure and in the progression of the neuropathy (Hernandez & Pena, 1997). Increasing severity of ON damage in POAG is accompanied by an increase in the amount of sheath-derived plaque material in the TM. Sheath-derived plaques mainly consist of section through the cribriform elastic net and the connecting fibrils. In POAG, these fibrils contain an additional sheath of VI collagen and various proteoglycans (Gottanka *et al.*, 1997).

PACG is a result of anatomical disorders that lead to obstructing the drainage flow of the AH through the TM. These disorders can be caused by abnormalities in the relative or absolute sizes or positions of the anterior segment elements, abnormal forces in the posterior segment

(that alter the anatomy of the anterior segment), or any combination of them. The most common form of angle-closure glaucoma is the pupillary block, an impedance to the flow of AH between the lens and the iris, from the posterior chamber to the anterior chamber. The pupillary block causes pressure in the posterior chamber to become higher which in turn compresses the anterior chamber (Ritch, Liebmann & Tello, 1995). This increases of the IOP can occur gradually or rapidly causing escalation of the glaucomatous damage.

## 1.3 GLAUCOMA IN VETERINARY MEDICINE

In veterinary practice, several species can be presented for ophthalmic examination but this section will only focus the canine, feline and equine glaucoma since are the most common ones. In dogs, as in humans, glaucoma is considered to be the most frequent cause of irreversible blindness (Cook, 1997) and almost invariably is the result of impaired AH outflow. There have been several reports of glaucoma epidemiology in veterinary patients. A study conducted at the University of Utrecht in 1985 reported that 8.6% of the total of canine and feline patients presented to their ophthalmology department were affected by glaucoma (Boevé & Stades, 1985a, 1985b). Canine glaucoma may be classified on the basis of the possible cause (primary, secondary, or congenital), the gonioscopic appearance of the iridocorneal angle (open or narrow/closed iridocorneal angle), and the duration or stage of the disease (acute or chronic) (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). The types of glaucomas in dogs are summarized on table 1.

| | |
|---|---|
| Primary glaucomas | Open angle: Acute / chronic |
| | Narrow / closed angle: Acute / chronic |
| Secondary glaucomas | Uveitis |
| | Lens-associated: |
| | Lens luxations |
| | Intumescent cataract |
| | Hyphema |
| | Intraocular neoplasia |
| | Aphakic |
| | Malignant / ciliary block |
| | Melanocytic / Pigment cell proliferation |
| | Pigment cell exfoliation / anterior uveal cysts |
| Congenital glaucoma | Pectinate ligament dysplasia |
| | Goniodysgenesis |

**Table 1** - *Types of glaucomas in dogs*

The glaucoma classification based on the cause includes the primary, secondary and congenital glaucomas.

Primary glaucomas (Fig.4) develops without concurrent ocular or systemic diseases, is hereditary in many canine breeds, and has a bilateral potential for development.

Primary glaucoma is subdivided into two main forms, primary open-angle glaucoma, in which the drainage angle appears gonioscopically normal (presumably because the impediment to aqueous outflow is deep to the pectinate ligaments) (Bedford, 2016) and primary angle-closure glaucoma, in which the drainage angle appears gonioscopically narrowed or closed. Pectinate ligament dysplasia or the consolidation of adjacent pectinate ligaments into broad sheets (initially termed mesodermal dysgenesis) in iridocorneal angle generally occur in middle-aged to older dogs (i.e., 6–10 years) (Gelatt *et al.*, 2013). PACG is, at least, eight times more common than POAG and acute PACG is also two times more common in female dogs than in male dogs (Maggs *et al.*, 2008). Breeds that appear to be predisposed to primary glaucoma are listed in table 2 (Gelatt *et al.*, 2013).

| Breeds | |
|---|---|
| Akita | Italian Greyhound |
| Alaskan Malamute | Lakeland Terrier |
| Basset Hound | Maltese |
| Beagle | Miniature Pinscher |
| Border Collie | Miniature Schnauzer |
| Boston Terrier | Norfolk Terrier |
| Bouvier des Flandres | Norwegian Elkhound |
| Brittany Spaniel | Norwich Terrier |
| Cairn Terrier | Poodle-Toy / Miniature |
| Cardigan Welsh Corgi | Samoyed |
| Chihuahua | Scottish Terrier |
| American Cocker Spaniel | Sealyham Terrier |
| Dachshund | Shih Tzu |
| Dalmatian | Siberian Husky |
| Dandie Dinmont Terrier | Skye Terrier |
| English Cocker Spaniel | Smooth Fox Terrier |
| English Springer Spaniel | Tibetan Terrier |
| German Shepherd | Welsh Springer Spaniel |
| Giant Schnauzer | Welsh Terrier |
| Greyhound | West Highland White Terrier |
| Irish Setter | Wire Fox Terrie |

**Table 2** - *Canine breeds predisposed to primary glaucoma*

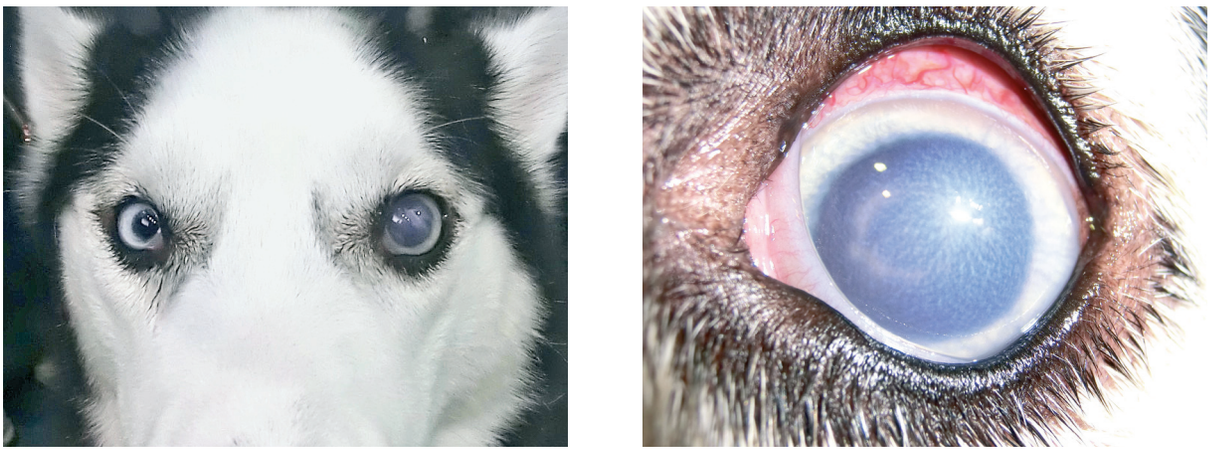

**Figure 4** - *Primary glaucoma in a 5-year-old Siberian Husky (originals).*

The secondary glaucomas are associated with other ocular or systemic disorders that alter aqueous humor dynamics and cause IOP elevation. This type of glaucoma tends to be an unilateral condition and are not inherited. Some of the conditions that may initiate these forms of glaucoma, however, may be genetically determined in certain breeds, such as those with cataracts and lens luxation. Secondary glaucomas are at least twice as common as primary glaucomas in dogs and are divided according to cause, as well as by an open, narrow/closed iridocorneal angle at gonioscopy (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). A recent study from the University of Zurich in 2011 (Strom, Hässig, Iburg & Spiess, 2011a, 2011b) reported that the secondary canine glaucomas are associated with anterior uveitis (23.0%), lens luxation (22.6%), intraocular surgery (13.4%), intraocular neoplasia (10.6%), unspecified trauma to the globe (8.3%), ocular melanosis (6.9%), hypermature cataract (6.9%), and hyphema (3.23%). The figures 5 to 10 illustrate some examples of secondary glaucoma in dog.

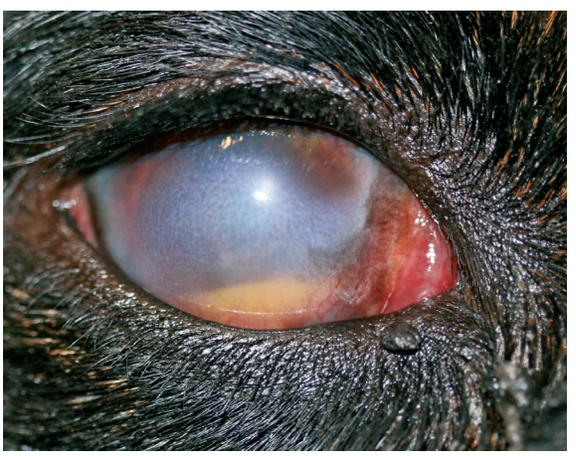

**Figure 5** – *Secondary glaucoma caused by anterior uveitis. Note the corneal oedema and hypopyon in the ventral anterior chamber in this dog, associated with a systemic disease (original).*

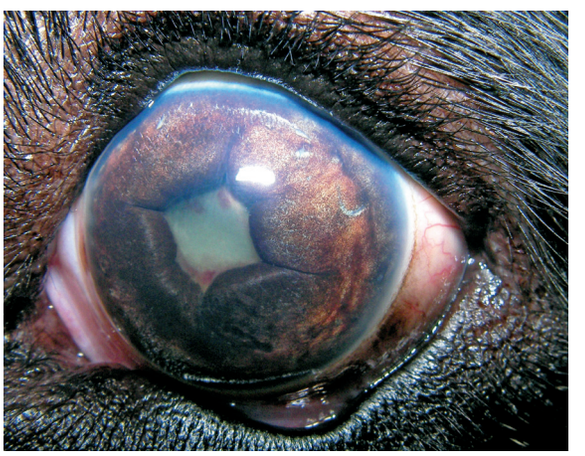

**Figure 6** – *Anterior uveitis may develop secondary pupillary occlusion from a total annular posterior synechiae (iris to lens capsula), thus resulting in iris bombé (original).*

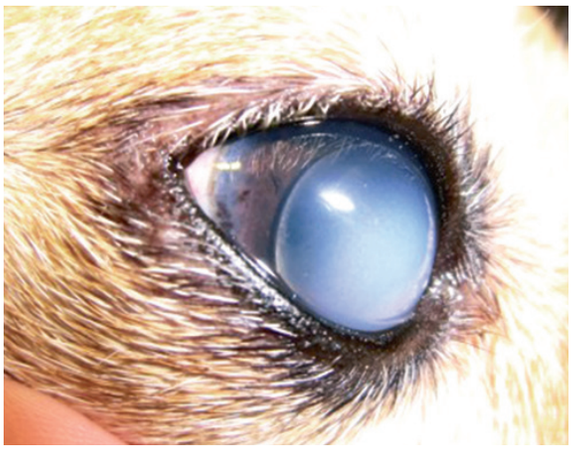

**Figure 7** – *Anterior lens luxation may cause secondary glaucoma due to the pupillary obstruction and the compression of the iridocorneal angle and ciliary cleft (original).*

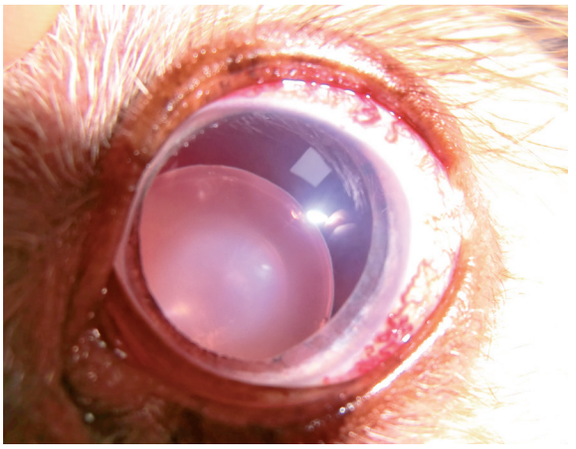

**Figure 8** – *Posterior or vitreal lens luxation with aphakic crescent. Note the ocular signs of glaucoma: severe episcleral congestion and pupil dilatation (original).*

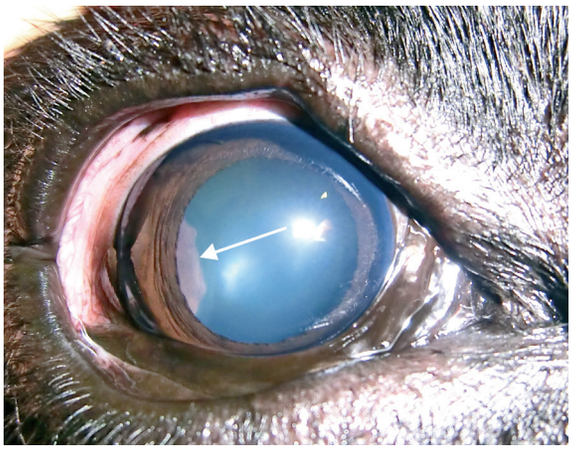

**Figure 9** – *Secondary glaucoma can be caused by intraocular neoplasia in dog. A ciliary body melanoma can be visualized extending into the pupil (arrow) (original).*

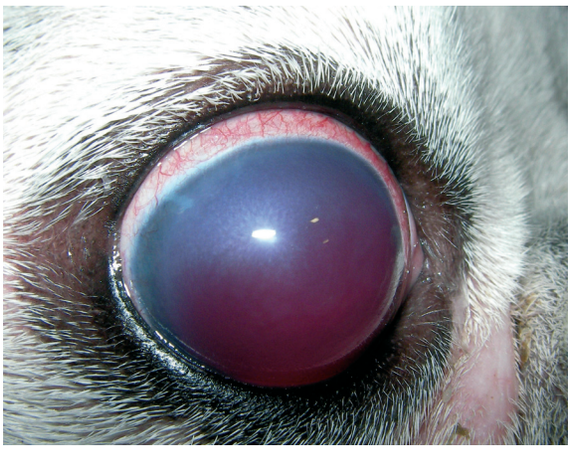

**Figure 10** – *Hyphema, filling the anterior chamber, induced secondary glaucoma in this dog (original).*

Congenital glaucomas develops at birth or a few weeks to months later (<1 year of age) (Fig. 11). It is quite rare and usually caused by genetic defects of the iridocorneal angle leading to abnormalities of the aqueous humor outflow pathways (Kroeber *et al.*, 2010; Strom *et al.*, 2011a). The extent of the angle anomaly may affect the time of onset for glaucoma development: a more severe defect of the iridicorneal angle leads to a sooner IOP elevation (Gelatt *et al.*, 2013).

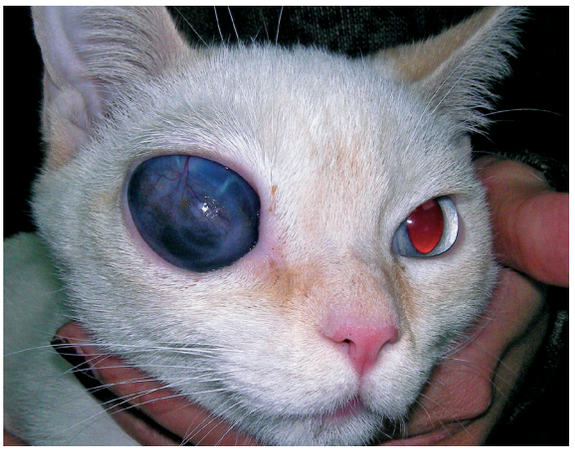

**Figure 11** – *Unilateral congenital glaucoma in a domestic shorthair kitten (original).*

On the basis of investigative research, classification of glaucoma according to its stage is less relevant, but very important for its therapeutic approach.

Glaucoma in cats is less common than in dogs and 95–98% cases appear to be secondary. The secondary feline glaucomas are associated with antecedent ocular or systemic disease, such as uveitis, neoplasia, trauma, and intraocular haemorrhage. Anterior uveitis (Fig. 12 A and B) and intraocular neoplasia (Fig. 13 A and B) are the most frequent causes. Table 3 summarizes the types and causes of feline glaucoma (McLellan & Miller, 2011).

| | |
|---|---|
| Primary glaucoma | Open angle, with and without collapsed ciliary cleft |
| | Narrow / closed angle (Burmese) |
| | Pectinate ligament dysplasia (Siamese) |
| Secondary glaucoma | Anterior uveitis (chronic) |
| | Lens luxations (trauma / primary / cataract) |
| | Phacolytic / phacoclastic uveitis (lens perforation) |
| | Hyphema (systemic hypertension, bleeding disorders, trauma) |
| | Intraocular neoplasia (primary / secondary neoplasms) |
| | Aqueous humor misdirection syndrome (shallow anterior chamber and anteriorly displaced lens) |
| Congenital glaucoma | Secondary to structural outflow anomalies |

**Table 3**- *Types of glaucomas in cats.*

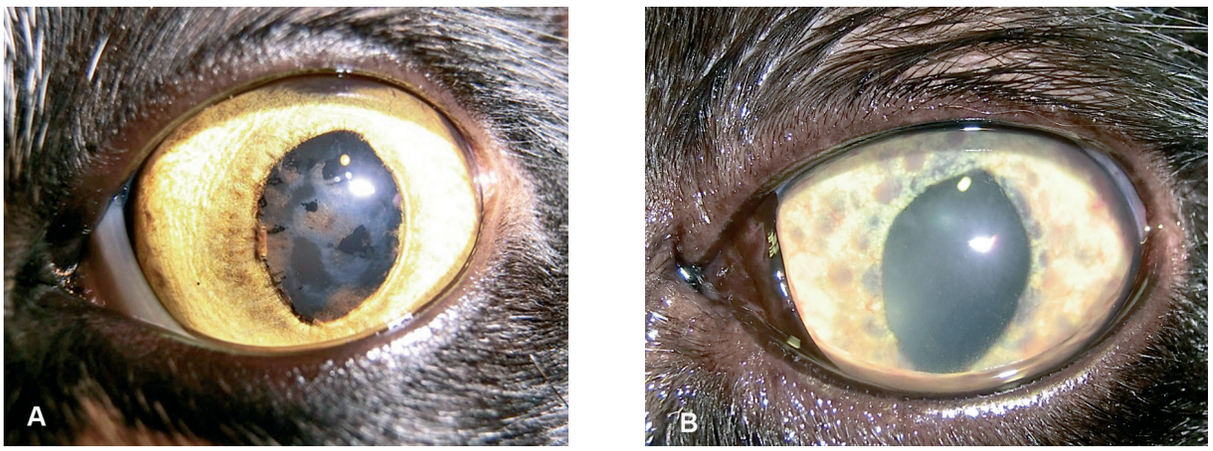


**Figure 12** – *Secondary glaucoma in cat can be caused by anterior uveitis. Two examples of anterior uveitis are presented: chronic (A) and idiopathic anterior uveitis (B) (originals).*

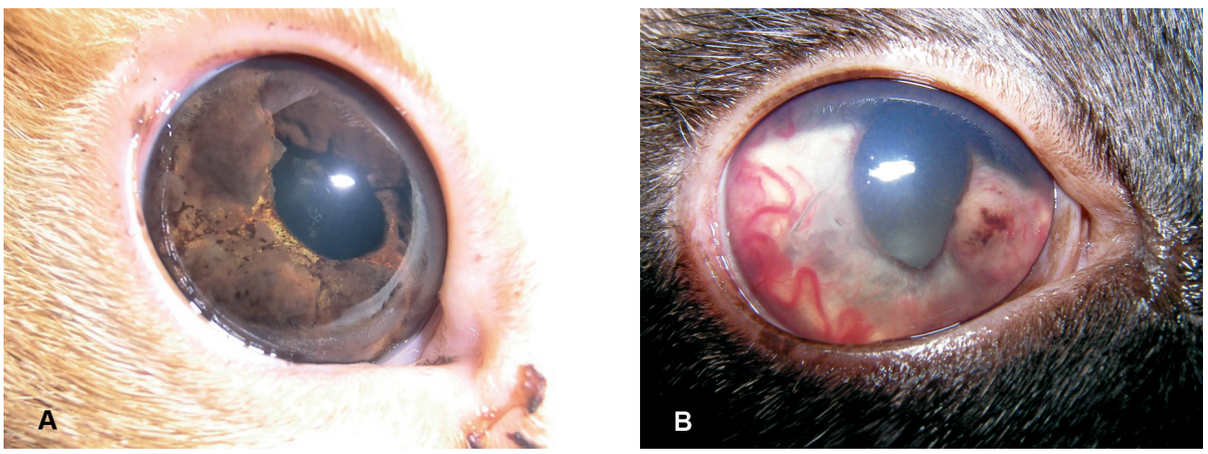


**Figure 13** – *Advanced stages of a diffuse iridal melanoma in cat (melanotic – A and amelanotic – B) (originals). The diffuse iridal melanotic melanoma starts with a progressive pigmentation of the iris which occurs over months to several years. The pigmentation may develop simultaneously in several areas on the anterior iridal surface and increase with time. Secondary glaucoma occurs when there is infiltration of the iridocorneal angle by malignant cells.*

The glaucoma in horses has a low prevalence (0.07% in the United States) and is also categorized into primary, secondary and congenital types. The secondary glaucomas, due to chronic or recurrent uveitis (Fig. 14), is the most common type of glaucoma in horses. Congenital glaucoma caused by developmental anomalies of the iridocorneal angle has been reported in foals (Gilger, 2005).

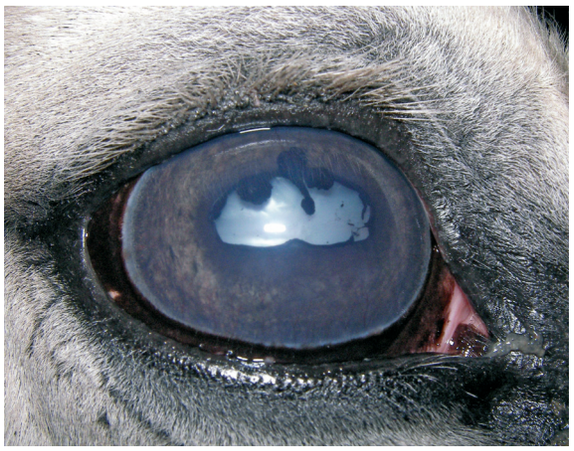

**Figure 14** – *Secondary glaucoma caused by chronic or recurrent uveitis. Note the miosis, posterior synechiae and cataract (original).*

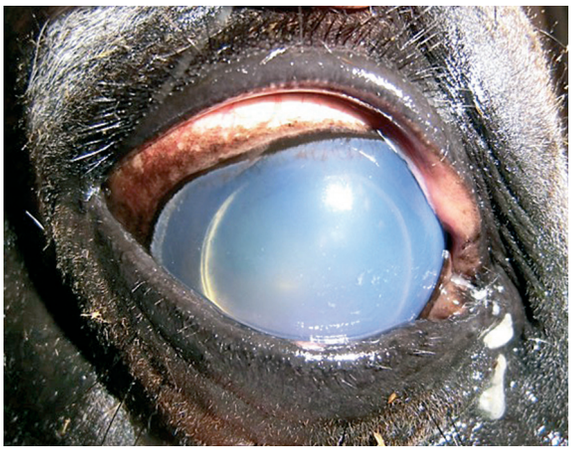

**Figure 15** – *Despite being a rare condition in horses, the anterior lens luxation caused secondary glaucoma in this horse's right eye (original).*

**Clinical signs**

Glaucoma is a disease of constant and progressive changes, and consists of five stages (Ritch, Shields & Krupin, 1996):

(1) an initial event or series of events that progressively reduces the function of the aqueous humor outflow pathways;

(2) physical changes causing aqueous humor outflow obstruction;

(3) elevated IOP that is too high for normal optic nerve axoplasmic flow and blood flow;

(4) RGC dysfunction with resulting optic nerve degeneration and atrophy;

(5) and progressive visual field loss and blindness.

Clinical signs of glaucoma can differ according to species, and with the stage and type of glaucoma.

Primary glaucoma is a bilateral disorder, but usually both eyes are not simultaneously affected. POAG in dogs is characterized by a slow progression and the disease is usually insidious and asymptomatic. In the earliest phase, IOP varies between 25 and 40 mmHg with mydriasis, variable corneal oedema and episcleral congestion. In those cases where vision is still present and the dog's eye is not appreciated as abnormal, patients are often present only in later stages of the disease, when vision is already compromised. With the progression of the disease, IOP rises with consequent globe enlargement (megaloglobus, buphthalmos), lens subluxation or total luxation, retinal degeneration, optic disc cupping and blindness (Gelatt *et al.*, 2013; Maggio, 2015).

The PACG is the most common type of primary glaucoma in dogs and the clinical signs also vary according to the stage. The clinical stages of canine PACG can be divided into latent

or predromal; intermittent; acute congestive or high-pressure; postcongestive; and chronic glaucoma (Gelatt *et al.*, 2013). In the first stages of the disease (latent and intermittent), signs range from none to mild mydriasis, with a variable degree of episcleral injection and transient corneal edema (Fig. 16 A). Usually, the affected dog is often presented as an emergency at the congestive stage with an acute glaucoma. The clinical sings include severe ocular pain (blepharospasm, epiphora and protrusion of the third eyelid), conjunctival hyperemia and scleral injection, diffuse corneal edema and, when visible, the pupil is moderately dilated and usually unresponsive (Fig. 16 B and C). Fundic examination is not always possible at that stage. On the contrary, chronic glaucoma is not usually a reason for emergency presentation. Patients present chronic discomfort, normally confirmed by the evident relief after globe removal; however, all the signs of acute pain are absent. Corneal edema is variable, from moderate to absent, and the eye normally presents buphthalmia (Fig. 16 D), with or without Haab's striae (Fig. 16 E and F). Both buphthalmos and Haab's striae (due to the rupture of corneal Descemet's membrane) are caused by the stretching of the eye, in response to the persistent increased IOP and are pathognomonic sings of chronic glaucoma.

Clinical signs of secondary glaucoma are usually similar to those of the primary disorder along with associated signs of the underlying problem (e.g. aqueous flare, swollen iris, hypopyon, lens dislocation, iridal mass, etc.) (Strom *et al.*, 2011b) and they vary from acute to progressive/chronic, according to the etiopathogenesis of the disease (Gelatt *et al.*, 2013; Maggio, 2015) (Fig. 5-10 and 12-15).

Feline glaucoma shows subtler clinical signs and therefore easily overlooked. Usually the increase in IOP is slow and progressive and no evident conjunctival hyperemia or corneal edema are initially present. Vision is also preserved for a much longer time than in dogs. Frequently, patients are presented when glaucoma has reached a chronic stage, with mild to moderate corneal edema, mydriasis, mild episcleral vessels injection, lens luxation/subluxation duo to a buphthalmia, retinal degeneration and blindness (Gelatt *et al.*, 2013; Maggio, 2015).

Equine clinical signs of glaucoma are usually related with multiple episodes of intraocular inflammation since the recurrent uveitis is the most common underlying cause. Besides the high intraocular pressures (40–80 mmHg), a diffuse corneal edema is normal present associated with signs of chronic intraocular inflammation, such as posterior synechia (adhesions), a miotic pupil and cataract. Haab's striae are relatively common in chronic glaucoma in horses (Gilger, 2005) (Fig. 16 F).

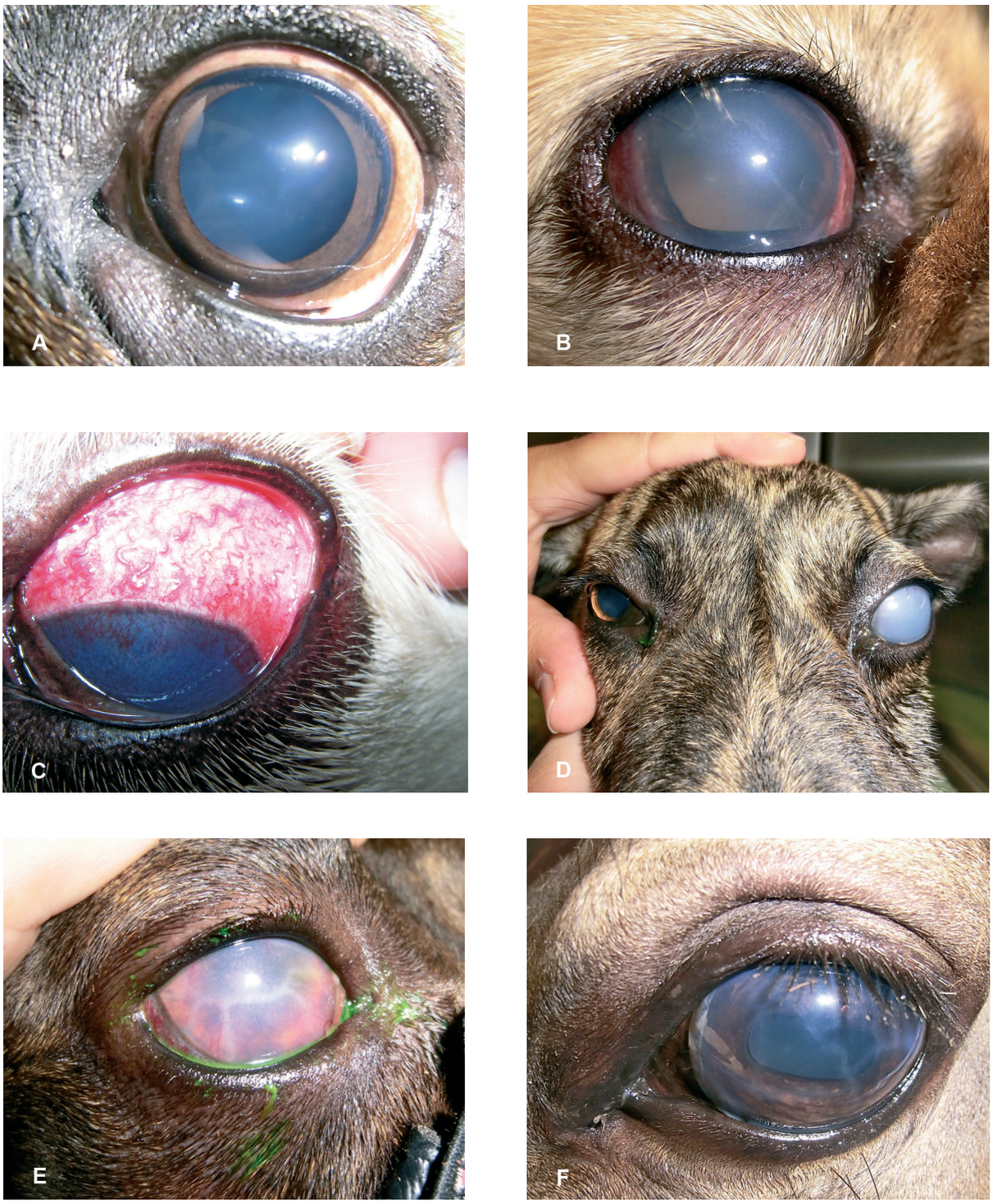


**Figure 16** – *Some examples of clinical signs of glaucoma (originals): A - latent stage of PACG in dog (mydriasis, transient corneal oedema history); B - acute glaucoma with conjunctival hyperemia and scleral injection, diffuse corneal oedema and an unresponsive, dilated pupil in dog. C – Note the conjunctival hyperemia and scleral injection. Chronic glaucoma can present buphthalmos (D) and Haab's striae (dog - E and horse - F) that are caused by continuous stretching of the eye.*

## 1.4 INHERITANCE OF THE GLAUCOMA

Despite decades of research, the mechanisms that generate trabecular outflow resistance in POAG in human are difficult to understand and still unclear. However, the heredity has an important role in glaucoma pathogenesis. Some of the earliest evidence came from reports in which glaucoma was passed down from generation to generation in a Mendelian pattern. Also, the fact that some animals, including the DBA/2J mouse (pigmentary glaucoma) and different dog breeds, have been documented to be afflicted by inherited glaucoma, support the idea of a genetic basis (Fingert, 2011).

### MYOC, OPIN, WDR36, NTF4 and CYP1B1 genes

The gene MYOC, encoding for myocilin, was the first reported to be linked to the increase in IOP in the most common subtype of glaucoma in humans, the POAG (Stone *et al.*, 1997). Mutated myocilin interferes with the normal outflow of aqueous humor through the TM. Patients with the MYOC mutation accumulate myocilin in the trabecular meshwork cells, with consequent destruction of the outflow pathway for AH, leading to higher IOP (Tamm, 2002).

OPTN was the second identified gene linked to hereditary POAG. Sequence alterations in OPTN were found in 16.7% of families with hereditary primary open angle glaucoma (Rezaie *et al.*, 2002) but in contrast to the elevated IOP found in MYOC-associated POAG, this gene is mainly mutated in families with normal IOP (De Marco, Buono, Troise & Diez-Roux, 2006). The OPTN mutation may exert its primary effect on RGC increasing their susceptibility to premature cell death (Morton, Hesson, Peggie & Cohen, 2008) under the infleunce of external stress factors (De Marco *et al.*, 2006).

The third gene involved in adult-onset POAG is WDR36 (Monemi *et al.*, 2005). WDR36 mutations may directly affect RGC's axon growth and lead to progressive retinal degeneration by apoptosis (Gallenberger *et al.*, 2011).

A more recent mutation in NTF4 gene was reported to be related to 1.7% of POAG in human patients (Pasutto *et al.*, 2009) but its role remains unclear.

Finally, mutations in CYP1B1 gene are a common cause of human primary congenital glaucoma due to severe ocular dysgenesis (Libby *et al.*, 2003).

### ADAMTS10 and SRBD1 genes

Concerning the veterinary patients, none of the genes previously described were found in dogs. Although inherited POAG have been reported in at least 45 dog's breeds, very limited information is available about the inheritance model in canine primary glaucoma.

However, the ADAMTS10 gene has been recently associated with POAG in Beagle (Kuchtey *et al.*, 2011). The animal model used in this study was a colony of Beagle dogs with an

inherited autosomal recessive trait of POAG (Gelatt & Gum, 1981). The elevation in IOP was due to an increased resistance to outflow of aqueous humor, despite normal appearing open iridocorneal angles. The ADAMTS10 gene contributes to formation of the extracellular matrix and may be involved in the formation of elastic microfiber structures. Its high levels expression in the TM suggests that an ADAMTS10 mutation could have particularly pronounced effects on AH outflow (Kuchtey *et al.*, 2011).

Another gene, SRBD1, with an important role in glaucoma pathology in both Shiba-Inus and Shih-Tzus was recently described (Kanemaki *et al.*, 2013). The results showed that SRBD1 polymorphisms have also been associated with normal- and high-tension glaucomas in humans (Mabuchi *et al.*, 2011).

## 1.5 MECHANISMS OF RETINAL GANGLION CELLS DEATH IN GLAUCOMA

Glaucoma is a complex, multifactorial disease, so it is likely that several molecular pathways converge to induce loss of RGC, the population of central nervous system (CNS) neurons with their soma in the inner retina and axons in the optic nerve. The mechanism is complex and a variety of molecular signals, acting alone or in cooperation, promote RGC death. These include: axonal transport failure, neurotrophic factor deprivation, toxic pro-neurotrophins, activation of intrinsic and extrinsic apoptotic signals, mitochondrial dysfunction, excitotoxic damage, oxidative stress, misbehaving reactive glia and loss of synaptic connectivity (Almasieh, Wilson, Morquette, Vargas & Di Polo, 2012).

### 1.5.1. Neurotrophic factors deprivation

Neurotrophins are diffusible trophic molecules that exert a potent survival effect on adult CNS neurons. They are a family of small peptides that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) in mammals (Huang & Reichardt, 2001; Segal & Greenberg, 1996). Among neurotrophins, BDNF has received particular attention because of its potent role on the survival of RGC (Almasieh *et al.*, 2012). BDNF is produced by cells in the ganglion cell layer and inner nuclear layer (Cohen-Cory & Fraser, 1994; Perez & Caminos, 1995; Vecino, Caminos, Ugarte, Martín-Zanca & Osborne, 1998) and together with other neurotrophic factors promote neuronal survival by inhibiting apoptotic pathways (Raff *et al.*, 1993). The biological effects of neurotrophins are mediated by two classes of cell surface receptors:

1) the tropomyosin related kinase (Trk), family of receptor tyrosine kinases, comprising TrkA, the receptor for NGF; TrkB, the receptor for BDNF and NT-4/5; and TrkC, the receptor for NT-3 (Kaplan, Hempstead, Martin-Zanca, Chao & Parada, 1991; Klein *et al.*, 1991; Lamballe, Klein & Barbacid, 1991);
2) the p75 receptor ($p75_{NTR}$) which binds all neurotrophins with similar affinity (Huang & Reichardt, 2003; Teng & Hempstead, 2004).

A current hypothesis is that blockade of axonal transport in glaucoma leads to deficits in the levels and availability of these factors and subsequent RGC death. Other neurotrophic factors such as ciliary neurotrophic factor (CNTF) (Wu, Zhang, Song Lu, & Hu, 2007) and glial cell line-derived neurotrophic factor (GDNF) (Koeberle & Ball, 2002; Yan, Wang, Matheson & Urich, 1999) also play a role in the endogenous response of retinal neurons to injury.

### 1.5.2. Activation of apoptosis

RGC have been shown to die by apoptosis in experimental glaucoma (Garcia-Valenzuela, Shareef, Walsh & Sharma, 1995; Quigley *et al.*, 1995) and in human glaucoma (Kerrigan, Zack, Quigley, Smith & Pease, 1997). Apoptotic RGC death has been confirmed by *in vivo* real-time visualization in ocular hypertensive rat eyes (Cordeiro *et al.*, 2004). The apoptotic process can be triggered by various stimuli and involves intrinsic and extrinsic pathways (Fig.17).

In the intrinsic pathway (also called “mitochondrial pathway”), apoptosis results from an intracellular cascade of events in which mitochondrial permeabilization plays a crucial role. Mitochondrial membrane permeabilization is frequently the decisive event that delimits the frontier between survival and death (Kroemer, Galluzzi & Brenner, 2007). Neurotrophic factor deprivation can trigger this pathway where pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members are activated (Kroemer *et al.*, 2007), leading to the release of cytochrome C from the mitochondria (Kluck, Bossy-Wetzel, Green & Newmeyer, 1997; Yang *et al.*, 1997). Among pro-apoptotic family members, Bax plays a pivotal role in the regulation of RGC death (Almasieh *et al.*, 2012). Once in the cytoplasm, cytochrome C, an essential protein component of the respiratory chain, binds to the apoptotic protease-activating factor-1 (Apaf-1) to form the apoptosome, a complex that recruits and activates procaspase-9, resulting in caspase-3 cleavage and activation (Li *et al.*, 1997).

In the extrinsic pathway (also known as “death receptor pathway”), apoptosis is triggered by the ligand-induced activation of death receptors at the cell surface. Such death receptors include the tumor necrosis factor (TNF) receptor-1, CD95/Fas (the receptor of CD95L/FasL), as well as the TNF-related apoptosis inducing ligand (TRAIL) receptors-1 and -2 (Kroemer *et al.*, 2007). Those active death receptor recruit procaspase-8 to yield active caspase-8, which in turn activates caspase-3. Other well-characterized intermembrane space proteins that are released from mitochondria during apoptosis include the second mitochondria-derived

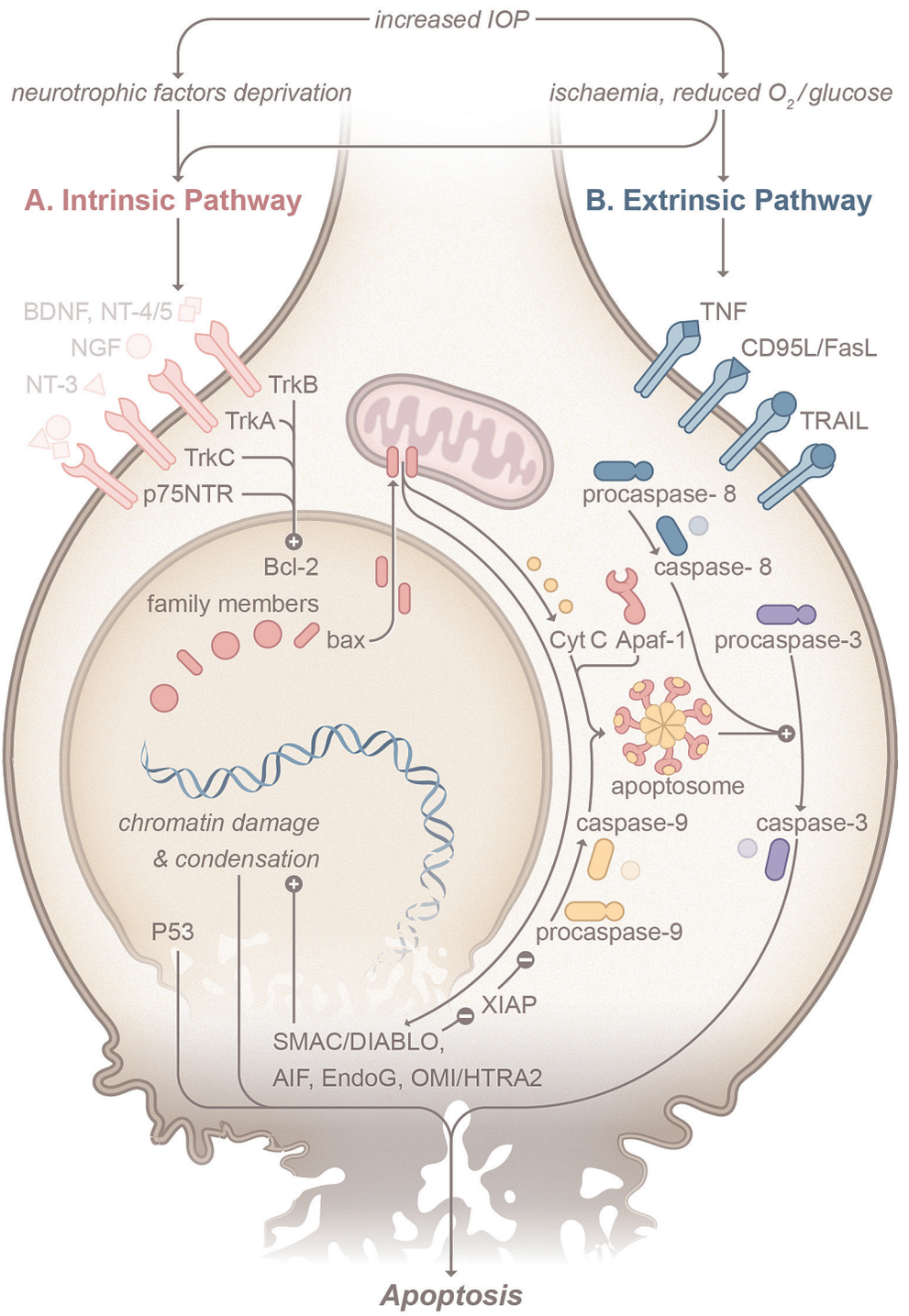


**Figure 17** – *The intrinsic and extrinsic apoptotic pathways. © Diogo Guerra. 2017.*

activator of caspases (SMAC), also known as DIABLO (that binds inhibitor of apoptosis proteins (IAP), thus freeing caspases to activate apoptosis), the apoptosis-inducing factor (AIF), endonuclease G (EndoG) and the high-temperature-requirement protein A2 (OMI/HTRA2) (Saelens *et al.*, 2004). Those proteins may contribute to RGC death by inhibiting

anti-apoptotic molecules including members of the X-linked IAP (XIAP) family or by inducing chromatin damage and condensation (Almasieh *et al.*, 2012). The tumor suppressor and nuclear transcription factor p53 also contribute to RGC death by mediating the apoptosis of post-mitotic cells exposed to a wide range of insults such as DNA damage, neurotrophic factor deprivation, oxidative stress, ischemia and excitotoxicity (Culmsee & Mattson, 2005).

### 1.5.3. Neurotoxicity

**Excitotoxic damage**

Glutamate is the predominant excitatory neurotransmitter in the central nervous system, including the retina. However, the presence of excessive glutamate concentrations for excessive periods of time can induce neurons' death. This phenomenon was first discovered in the retina (Lucas & Newhouse, 1957) and later named ‘‘excitotoxicity’’ (Olney & Ho, 1970). Excitotoxicity glutamate-mediated neurotransmission plays an important role in the relay of visual information from photoreceptors to bipolar cells, then to RGC and into the brain centers (Lukasiewicz, 2005).

Retinal glutamate receptors are located mostly in the inner plexiform layer, which contains most of glutamatergic synapses from bipolar cells to RGC and amacrine cells but also in the outer plexiform layer, where glutamatergic synapses connect photoreceptors to bipolar and horizontal cells. (Lukasiewicz, 2005; Gründer, Kohler, Kaletta & Guenther, 2000; Peng, Blackstone, Huganir & Yau, 1995).

Concerning the region of the retina primary affected in glaucomatous optic neuropathy, it is thought to be the optic nerve head, especially at the lamina cribrosa, where RGC axons or blood vessels are likely to be compressed (Seki & Lipton, 2008). Two theories, mechanical and vascular, have been proposed as pathogenesis of this disease (Fig. 18) and on both, excitotoxicity mediated by NMDA receptors seem to have an important role.

The classical model of excitotoxic damage is based on the premise that excess of glutamate binds to ionotropic glutamate receptors, such as NMDA receptor, triggering calcium influx, organelle stress and activation of pro-apoptotic pathways (Almasieh *et al.*, 2012). During ischemia, when enormous disruption of energy metabolism occurs, glutamate is not cleared properly by glia and can even be inappropriately released (Li, Mealing, Morley & Stys, 1999). As a result, the extracellular glutamate concentration may increase. With the loss of energy due to hypoxia-ischemia, neurons lose their ability to maintain energy-dependent ionic homeostasis, and thus neurons become depolarized. This voltage change removes physiological $Mg^{2+}$ block from NMDA receptor-associated channels (Zeevalk & Nicklas, 1992), which results in $Ca^{2+}$ entry and trigger downstream signalling cascades, leading to cell death (Seki & Lipton, 2008).

Axonal compression, in this case at the level of the lamina cribrosa, may also relieve $Mg^{2+}$ block, and increased IOP may abnormally increase the activity of NMDA receptor-associated channels. Excitotoxicity can play a role in glaucoma even in the absence of elevated extracellular glutamate concentration since $Mg^{2+}$ block of NMDA receptor-associated channels is removed. Glutamate leaking out of dying/dead RGC or compromised glia may contribute to secondary death of neighboring RGC via excessive activation of NMDA receptors (Seki & Lipton, 2008).

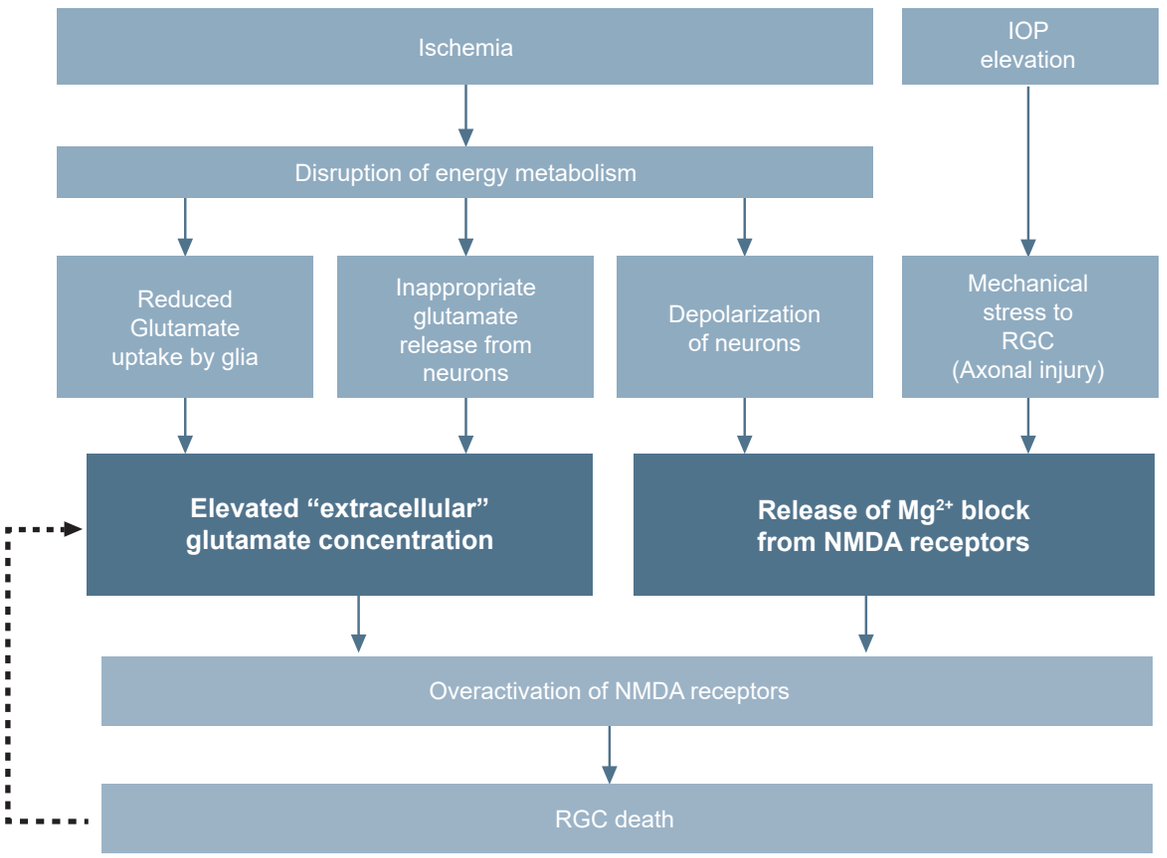


**Figure 18** – *Hypothetical mechanisms leading to excitotoxicity in glaucoma. Adapted from Seki (2008).*

Therefore, experimental data on glutamate excititoxicity has been controversial and other models of excitotoxic damage were proposed. Recently it was proposed that Müller cells exacerbate RGC loss. Those cells are sensitive to glutamate/NMDA and, through a mechanism still unknown, respond rapidly by upregulating nuclear factor Kappa B (NF-kB) activity. Activation of NF-kB in Müller cells leads to production of endogenous glia-derived TNF-α which in turn increases the RGC surface levels of $Ca^{2+}$ permeable AMPA receptors, triggering neuronal death (Almasieh *et al.*, 2012).

**Oxidative stress**

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism, primarily in the mitochondria (Tezel, 2006). Oxidative stress, caused by the imbalance between the production of ROS and their elimination by antioxidants, has been recognized as a central contributor to neuronal injury and death. ROS that is, superoxide anion ($O^{2-}$), hydroxyl radical (OH*), hydrogen peroxide ($H_2O_2$), and singlet oxygen ($^1O_2$), are continuously produced by mitochondria through the electron transport chain, but can also be generated by enzymatic degradation of neurotransmitters, neuroinflammatory mediators, and redox reactions (Halliwell, 2006; Nita & Grzybowski, 2016). Increased levels of ROS, a common feature of neurodegenerative diseases, can originate from mitochondrial dysfunction, abnormal protein folding, and defective ubiquitination and proteasome degradation systems (Andersen, 2004). Patients with glaucoma exhibit delayed choroidal filling, abnormal blood flow velocity in retrobulbar arteries, reduced retinal capillary perfusion and a significantly lower blood flow velocity (Ko, Peng, Ma, Ritch & Chen, 2005). Those vascular perfusion irregularities reduce oxygen supply and are thought to contribute to oxidative damage (Izzotti, Bagnis & Sacca, 2006; Tezel, 2006). During hypoxia, the generation of mitochondrial ROS is necessary for activation of the hypoxia-inducible factor-1 (HIF-1) (Almasieh *et al.*, 2012).

**The Role of Inflammation**

Many aspects of neuroinflammation in glaucoma are similar to what has been shown in other diseases. Generally, an acute and prolonged inflammatory process occurs in response to ischemic injury. This process is characterized by production of pro-inflammatory mediators and infiltration of various types of inflammatory cells into the ischemic tissue (Vohra, Tsai & Kolko, 2013).

Similarly, in glaucoma, a low-grade of inflammation participates in the pathogenesis of the disease. In response to vascular dysfunction, ischemia, hypoxia, and elevated IOP, genes associated with HIF-1α (Ergorul *et al.*, 2010), cytokines (Li *et al.*, 2007), TNF-α in glial cells (Tezel & Wax, 2000) and COX-2/PGE2 (Kawano *et al.*, 2006) are all up-regulated in glaucomatous RGC.

The inflammatory responses in glaucomatous eyes are straight related to the RGC death by inducing pro-apoptotic cascade reactions in those cells (Vohra *et al.*, 2013).

The blood-retina barrier (BRB) plays a fundamental role in retinal function in both health and disease (Cunha-Vaz, 2017). In glaucoma, an impairment of the BRB may increase vascular permeability, causing fluctuation of molecules through the weakened BRB and may further lead to an inflammatory response as a consequence of impaired ability to maintain an adequate blood supply to the optic nerve head (Vohra *et al.*, 2013). The stress caused by the elevated IOP affects the normal mechanisms of autoregulation, thereby compromising the blood supply to the optic nerve head. Disturbed autoregulation results in periods of ischemia leading to reperfusion damage when normal flow is re-established, resulting in an increase in the concentrations of

inflammatory molecules such as nitric oxide (NO) and/or endothelin-1 (ET-1) (Kyhn *et al.*, 2009; Palomba, Cerioni & Cantoni, 2010; Sugiyama, Moriya, Oku & Azuma, 1995).
Several other inflammatory molecules such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), interleukins (IL), C-reactive protein (CRP) and serum amyloid A (SAA) have been shown to be also up-regulated in glaucoma (Leibovitch *et al.*, 2005; Nakazawa *et al.*, 2006; Tezel, Li, Patil & Wax, 2001; Tezel & Wax, 2000; Tezel, Yang, Yang & Wax 2004; Wang *et al.*, 2008).

## 1.6 GLAUCOMA TREATMENT

### 1.6.1 Traditional glaucoma treatment

Commonly, the goal of glaucoma treatment is to decrease the IOP via medical, laser or surgical therapies, both on human and animal patients.
The medical treatments for glaucoma includes topical or oral drugs that decrease aqueous humor production or augment its outflow. Other procedures to decrease IOP include laser and surgical therapies.

**Medical therapies**

The first approach in the glaucoma management is usually through topical medications for lowering the intraocular pressure by decreasing the AH inflow, increasing its outflow, or both.
Pharmacological compounds can be divided into 5 major classes: cholinergic agonists (miotics), drugs acting on adrenoceptors (i.e. alpha agonists and beta-blockers), carbonic anhydrase inhibitors, prostaglandin analogues and osmotic diuretics.

1. Cholinergic agonists, also known as parasympathomimetics or cholinomimetics (due to their similar response to those of acetylcholine), directly or indirectly stimulate the parasympathetic nervous system in the eye (via acetylcholine receptors). Direct-acting parasympathomimetics (i.e. pilocarpine and carbachol) activate acetylcholine receptors, whereas indirect-acting cholinergic parasympathomimetics (i.e. demecarium bromide) inhibit acetylcholinesterase activity, increasing the concentration and exposure time of acetylcholine at its receptor sites. Topical application of these drugs leads to miosis, contraction of the longitudinal fibers of the ciliary muscle and subsequent reduction in IOP. This mechanism is well documented in humans and nonhuman primates: the longitudinal fibers of the ciliary muscle are anchored anteriorly to the scleral spur resulting in widening of the conventional outflow pathway. Since dogs lack a true scleral spur

and have a poorly developed ciliary muscle, the exact mechanism by which IOP is lowered is unknown. However, the attachment of the ciliary muscle tendons to the posterior scleral lamellae of the scleral sulcus might justify their action (Alario, Strong & Pizzirani, 2015; Gelatt *et al.*, 2013). Due to their miotic effect and an increase in blood-aqueous barrier (BAB) permeability, resulting in increase in aqueous humor flare, these drugs are generally contraindicated in animals with pre-existing intraocular inflammation or a tendency to pupil block (Maggio, 2015).

2. Drugs that act on blocking the activity of the ocular sympathetic nervous system may also be used to lower IOP. Epinephrine, or adrenaline, is a nonspecific adrenergic agonist which stimulates α and β membrane receptors. It lowers IOP in humans and canine patients. The main indication for epinephrine use is medical control of POAG since the mydriatic effect can further crowd the iridocorneal angle in PACG (Gelatt *et al.*, 2013). However, epinephrine is infrequently used today for the treatment of glaucoma and has been replaced by the α-2-selective agonists. Different mechanisms contribute to α-2-agonists (i.e. apraclonidine and brimonidine) actions on eye because three different receptors subtypes were described: alpha-2 (A, B, C). Activation of α-2-receptors leads to IOP decrease through reduction of AH production and increased uveoscleral outflow facility (Arthur & Cantor, 2011). Concerning the β-adrenergic antagonists (β-blocker) (i.e. timolol), they have become the most widely used drugs for the control of ocular hypertension in humans. Many investigations have demonstrated that topical β-blockers reduce IOP by decreasing formation of aqueous humor. Other topical β-blockers (i.e. betaxolol, levobunolol, metipranolol and carteolol) have been described as ocular hypotensive compounds in humans (Zimmerman, 1993).
3. As previously described the ciliary body process epithelium contains enzyme systems such as $Na^+$, $K^+$-ATPase and carbonic anhydrase, involved in aqueous humor formation. Carbonic anhydrase inhibitors, that belong to the class of nonbacteriostatic sulfonamide-related compounds, compete with carbonic acid for its site on carbonic anhydrase, to make it inactive. Aqueous humor production can be reduced by both topical (i.e. dorzolamide and brinzolamide) and systemic (i.e. acetazolamide, dichlorphenamide and methazolamide) carbonic anhydrase inhibitors which decrease the production of aqueous humor by the epithelial cells of the ciliary body (Gelatt *et al.*, 2013)
4. Prostaglandin analogues (i.e. latanoprost, bimatoprost, travoprost and unoprostone) primarily reduce IOP via increased uveoscleral outflow but the exact mechanism is still under investigation. Current studies suggest that the activation of matrix metalloproteinases (MMPs) plays a significant role in reducing the density of collagen fibers in the extracellular matrix of the ciliary muscle, thus increasing the facility of uveoscleral outflow and also through the trabecular meshwork. The same way that cholinergic agonists, topical prostaglandins induce miosis and for this reason are contraindicated to treat glaucoma secondary to anteriorly luxated lenses because they may lead to pupillary block and exacerbate existing ocular hypertension. Due to their propensity to disrupt the BAB, topical prostaglandin analogues should be used with caution in cases of glaucoma secondary to uveitis (Alario *et al.*, 2015; Gelatt *et al.*, 2013; Goel *et al.*, 2010).

5. Osmotic diuretics (i.e. Mannitol, saline hypertonic hydroxyethyl starch, glycerol), are typically used in veterinary emergency to treat acute congestive glaucoma but are not indicated for ongoing IOP control. The osmotic agents are administered orally or intravenously and increase the osmolarity of the plasma which creates an osmotic gradient favouring the diffusion of water out of the intraocular tissues (Alario *et al.*, 2015; Gelatt *et al.*, 2013).

**Surgical therapies**

When medical management does not reduce IOP to target levels and the deterioration of the RGC and optic nerve continues, surgical therapies are required. Usually, in veterinary medicine, the surgical techniques have to be used much earlier in the course of a glaucoma disease when compared to human patients. Surgical procedures for treatment of the primary glaucoma both on human and veterinary are divided into two types: 1) techniques that increase outflow of aqueous humor via alternative pathways of drainage within or to the outside of the eye and 2) those that decrease the formation rate of aqueous humor by destroying part of the ciliary body (Gelatt & Gelatt, 2011). In some patients, a combination of two or three techniques can be performed. In cases of pulipary block, iridotomy or iridectomy may be indicated to create a bypass to the AH directly from the posterior to anterior chamber. Table 4 summarize the most common types of surgical procedures described, both in veterinary and in human medicine.

| Mechanism | Type of surgery |
|---|---|
| Increase AH outflow | iridencleisis |
| | corneoscleral trephination |
| | cyclodialysis |
| | posterior sclerectomy |
| | laser trabeculoplasty |
| | trabeculectomy |
| | anterior chamber shunts (i.e. gonioimplants) |
| Reduction AH formation | cyclocryothermy |
| | cyclodiathermy |
| | laser transscleral or endoscopic cyclophotocoagulation |
| Pupil bypass | Iridotomy |
| | iridectomy |

**Table 4** - *Mechanisms of surgical treatments for the glaucomas*

In dogs, the most frequently procedures used are anterior chamber shunts (i.e. gonioimplants) (Fig. 19) and destruction of the ciliary body processes by cryothermy or laser photocoagulation.

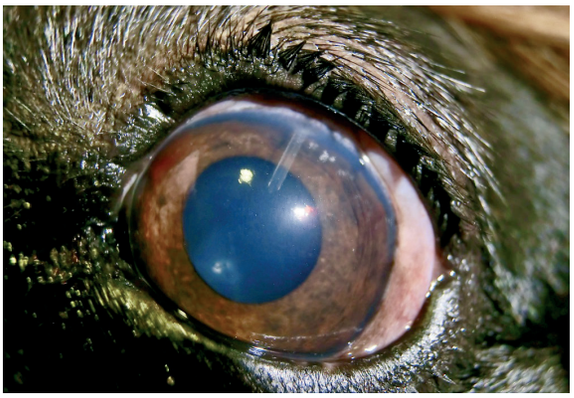

**Figure 19** – *Appearance of the tube of the Ahmed gonioshunt in a dog's anterior chamber (original).*

In cats and horses, when medical therapy fails to control IOP, cyclocryothermy (cat) and cyclophotocoagulation (cat and horse) can be attempted to decrease AH production. Due to the association between chronic uveitis and the development of secondary glaucoma, pre-existing inflammation is common in feline and equine eyes and may contribute to lower success rates in these species. In horses, surgical methods to increase outflow (i.e. gonioimplants, drainage surgeries such as sclerostomies or iridectomies) are usually not successful because of the horse's high inflammatory response to intraocular surgery. The use of anterior chamber shunts has not been reported in cats. In veterinary practice, sometimes due to the rapid disease course or in chronic glaucoma cases, the eyes are painful and permanently blind. In these patients, enucleation (Fig. 20) or evisceration with an intrascleral silicone prosthesis (Fig. 21) is a frequent option (Gelatt *et al.*, 2013; Gilger, 2005; McLellan & Miller, 2011).

**Figure 20** – *In veterinary medicine, enucleation is a frequent option to relieve the pain of blind eyes in medically refractory glaucoma (original).*

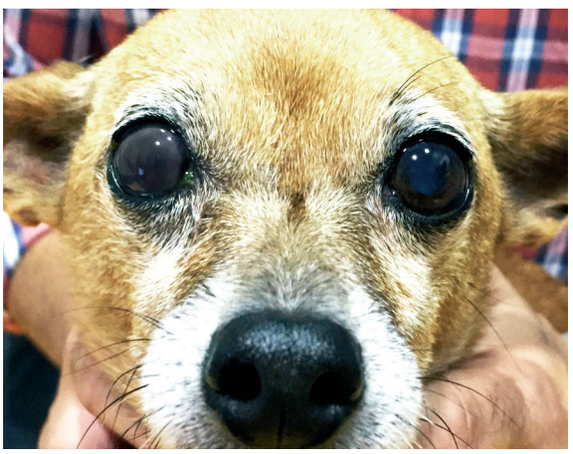

**Figure 21** - *Also evisceration with an intrascleral silicone prosthesis can be an option to relieve the pain of these patients. Appearance of intrascleral silicone prosthesis in a dog's right eye (original).*

In human patients, due to an increase in the availability of pharmacologic treatments to glaucoma and the use of laser trabeculoplasty over the past decade, a decrease in invasive incisional surgery has been observed (Conlon, Saheb & Ahmed, 2017). When the IOP remains too high, despite medical and laser trabeculoplasty treatments, more invasive techniques should be considered. In these cases, trabeculectomy or anterior chamber shunts are the most common choice (Boland *et al.*, 2013; Weinreb, Aung & Medeiros 2014).

### 1.6.2. Neuroprotection in glaucoma treatment

So far, the only treatment proven to be effective for glaucoma is IOP reduction. There is no doubt that IOP is an important risk factor but other factors act in combination to cause loss of RGC. Restoring normal IOP is mandatory in a glaucoma treatment because can itself act as a neuroprotective process by decreasing the oxidative stress, axon transport impairment, and neuroinflammation associated with IOP elevation. However, even with excellent control of IOP, some patients have worsening visual field resulting from progressive RGC loss (Almasieh *et al.*, 2012; Collaborative Normal-Tension Glaucoma Study Group, 1998). It is becoming clear that reduction of IOP in patients is not enough and the focus of research is now the neuroprotection of RGC.

Neuroprotection may be defined as a therapy that prevents the degeneration of the RGC, and necessarily maintains its structural and functional capabilities. Neuroregeneration may be defined as a therapy which reverse the preexisting structural loss of the RGC (Levin, 2016).

Several agents have been tested for neuroprotection in glaucoma. Considering the previous described mechanisms of retinal ganglion cells death in glaucoma, table 5 summarize the main neuroprotective agents (Bagli & Kitsos, 2008; Cheung, Guo & Cordeiro, 2008; Danesh-Meyer, 2011; Levin, 2016; Nucci *et al.*, 2016). Some of these strategies have already been applied in CNS diseases.

The new emerging researches for neuroprotection in glaucoma needs a long-term controlled trials to determine whether or not neuroprotective agents may be beneficial in the management of glaucoma without unwanted side effects (Vasudevan, Gupta & Crowston, 2011). However, it is also evident that the pharmacological approaches for neuroprotection represent an exciting research field in order to prevent RGC apoptosis and contribute to maintenance of vision.

| Mechanism of action | | Agent |
|---|---|---|
| Neurotrophin deprivation | | BDNF |
| | | NGF |
| | | Gene therapy |
| Activation of apoptosis | | BIRC-4 (XIAP) |
| | | Peptide IQACRG |
| Neurotoxicity | Excitotoxic damage | Memantine |
| | | MK801 |
| | | Galantamine |
| | | Cannabidiol |
| | Oxidative Stress | Vitamin E |
| | | Ginkgo biloba |
| | | Melatonin |
| | | Taurine |
| | Mitochondrial dysfunction | Coenzyme Q10 (Ubiquinone) |
| | | Nicotinamide |
| | Inflamation | Copolymer-1 (Cop-1) |
| | | Minocycline |
| | | Anti-inflammatory drugs (target TNF-a) |
| | Protein misfolding | Targeting amyloid-b |
| | | Heat shock proteins (HSPs) |
| Multiple mechanisms of action | | Brimonidine tartrate |
| | | 17b-estradiol |
| | | Erythropoietin |

**Table 5** - *Summary of current research strategies employed to study neuroprotection in glaucoma previously applied in CNS diseases*

## 2. ERITHROPOIETIN

The name erythropoietin (EPO) comes from the Greek words ἐρσθρός (ērythrós) and ποιεῖν (poieîn) that mean 'red' and 'make', respectively. EPO's function was first formulated in 1906 by the french scientists Paul Carnot and DeFlandre (1906) that postulated that a humoral factor, which they called "hemopoietine," regulated red blood cell production. In 1948 the term 'erythropoietin' was introduced by Bonsdorff and Jalavisto (1948) but only in 1977, Miyake *et al.* (1997) succeeded in the purification to homogeneity of human EPO from urine. The cloning of the EPO gene, subsequent production of recombinant protein, and successful introduction into clinical practice for the treatment of the anemia secondary to renal failure, was a milestone in science.

EPO is a 30.4-kDa glycoprotein hormone, produced by peritubular cells (interstitial fibroblasts) in the kidneys of the adult and in hepatocytes of the fetus. Its expression is induced in response to renal hypoxia, via the HIF-1α (Semenza, 2000; Semenza & Wang, 1992). Hypoxia-inducible factor proteins are transcriptional regulators targeting genes involved in angiogenesis, vasomotor control, energy metabolism, apoptosis and erythropoiesis (Marti, 2004).

Once produced, EPO is released into the circulation, targeting hematopoietic cells in the bone marrow. The signalling pathway involves activation of Janus tyrosine kinase 2 (JAK-2), which further propagates the signal by engaging secondary signalling molecules, including signal transducer and activator of transcription (STAT), Ras–mitogen-activated protein kinase (MAPK)

and phosphatidylinositol 3-kinase (PI3K). In erythroid progenitor cells this results in the upregulation of anti-apoptotic proteins of the Bcl-2 family, such as Bcl-xL (Brines & Cerami, 2005). Through induction of members of the Bcl family of antiapoptotic proteins, EPO permits the maturation of red cell precursors that would otherwise undergo apoptosis (Erbayraktar, Yilmaz, Gökmen & Brines, 2003).

Besides the discovery of its presence in the liver and kidney, other tissues were found to secrete EPO, such as brain (Marti, 2004), uterus (Chikuma, Masuda, Kobayashi, Nagao & Sasaki, 2000) and retina. The finding of a hematopoietic factor in these tissues raised the question if erythropoietin was merely a regulator of erythropoiesis. Furthermore, EPO receptors (EPOR) are expressed by nonhematopoietic cells such as neurons, astrocytes and endothelial cells in the brain (Marti, 2004) and retina. This suggests a local nonhormonal role, indicating autocrine or paracrine action rather than telecrine, independent of the endocrine erythropoiesis function.

In recent years, EPO was found to have multiple functions outside of the bone marrow, such as tissue maintenance and function protection (Erbayraktar *et al.*, 2003). EPO has proven to be effective in preventing neuronal apoptosis in a wide-range of neurodegenerative conditions in the brain, retina, and spinal cord including acute, chronic, inherited, and induced degenerations. For example, the neuroprotection role of EPO in the brain has been described in different studies by different ways. On one hand, EPO can act, in an *in vivo* context, to reverse vasospasm (Grasso *et al.*, 2002; Squadrito *et al.*, 1999), to protect vascular endothelial cells (Chong, Kang & Maiese, 2002), to modulate inflammation (Agnello *et al.*, 2002; Brines *et al.*, 2000; Villa *et al.*, 2003) and to recruit stem cells (Shingo, Sorokan, Shimazaki & Weiss, 2001). On the other hand, EPO can act directly on neurons by attenuating the production of damaging molecules such as ROS or glutamate-stimulated excitotoxicity (Calapai *et al.*, 2000; Digicaylioglu & Lipton, 2001; Kawakami, Sekiguchi, Sato, Kozaki & Takahashi, 2001). This likely contributes to lower levels of apoptosis (Celik *et al.*, 2002; Digicaylioglu & Lipton, 2001; Sirén *et al.*, 2001). Therefore, EPO can act at multiple levels to protect neurons.

Another example of the EPO protective action has been described in the heart where EPO exert direct actions on cardiac myocytes after heart post-infarction by decreasing the numbers of apoptotic myocytes, limiting infarct expansion and attenuating the post-infarct deterioration in haemodynamic function (Bogoyevitch, 2004).

These findings (Fig.22) resulted in great interest in the new potential effects of EPO.

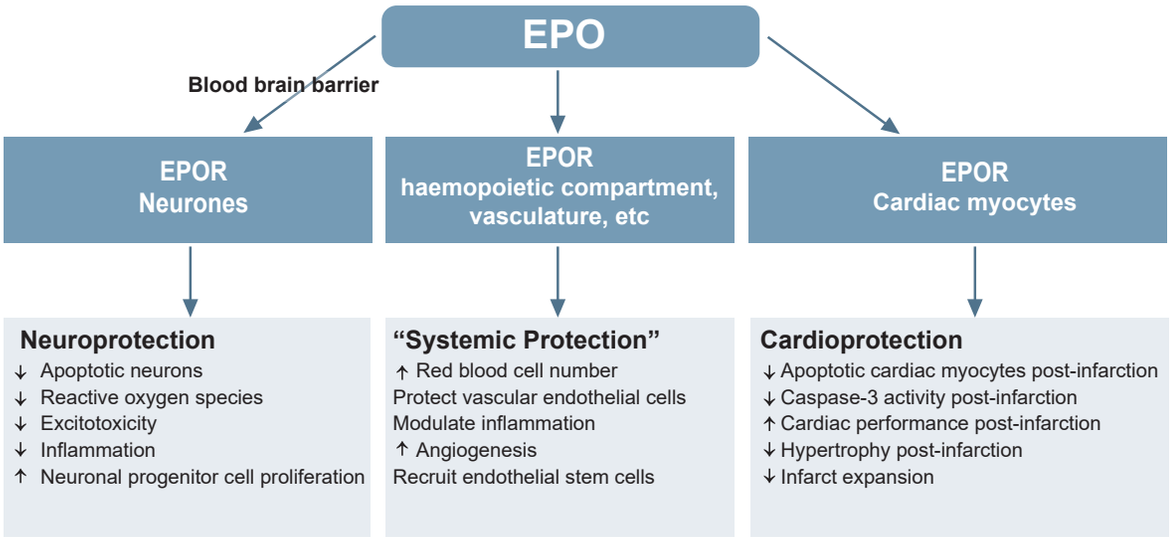


**Figure 22** - *Schematic diagram of the possible actions of EPO in neuroprotection, "systemic protection" and cardioprotection. Adapted from Bogoyevitch (2004).*

## 2.1 THE EXPRESSION OF EPO IN THE RETINA

As mentioned above, EPO and EPOR can be expressed on the human retinal tissue (Garcia-Ramirez, Hernandez & Simo, 2008; Hernandez *et al.*, 2006; Rex *et al.*, 2004; Shah, Tsang & Mahajan, 2009) and also on retinal pigment epithelium (RPE) (Garcia-Ramirez *et al.*, 2008). Moreover, the expression behavior is stronger in RPE cells when compared with neuroretina, which are known to support and act as barriers of the neuroretina (Luo, Hu & Wang, 2015).

EPOR expression was initially found to localize on the rod inner segments and synaptic terminals of the photoreceptors (Grimm *et al.*, 2002). Later, numerous studies showed evidence of EPOR expression in the inner nuclear layer (horizontal, Műller glia, bipolar, and amacrine cells) and the ganglion cell layer (Caprara, Britschgi, Samardzija & Grimm, 2014; Rex, Wong, Kodali & Merry, 2009; Shah *et al.*, 2009).

Szabo *et al.* (2008) also demonstrated that the RGC principally produce and secrete EPO, which will then target and bind to the EPOR present mainly on the amacrine, horizontal, and photoreceptor cells.

The vast presence of EPO and EPOR in the retinal tissue suggests that EPO has important physiological roles in the eye. The basic human eye development requires proper coordination of regulatory genes that determines the fate of cells from different origins. EPO has a significant involvement in the development of visual function before birth (Shirley Ding *et al.*, 2016) by preventing cell apoptosis (Patel, Rowe, Winters & Ohls, 2008; Wu *et al.*, 2008).

Given the link between the protective response of EPO during eye development and the presence of EPOR in the retina, preliminary studies have considered the potential role of EPO in the ocular system. Recent studies evaluated the use of EPO in various types of ocular disorders and have reported its neuroprotective functions such as anti-apoptotic, anti-inflammatory, anti-oxidant properties, besides its role in neovascularization and its contribute to the maintenance of osmotic pressure in the RPE.

New evidence indicates that erythropoietin specifically prevents or retards apoptosis of neurons by signalling through a non-haematopoietic receptor. This modified tissue-protective EPOR, a complex different in structure from the homodimer receptor associated with hematopoiesis, has been reported to possess less binding affinity to EPO and a lower molecular weight than the hematopoietic homodimer EPOR (Brines *et al.*, 2004; Brines & Cerami, 2005; Leist *et al.*, 2004).

## 2.2 THE PROTECTIVE EFFECTS OF EPO ON THE RETINA

### 2.2.1 EPO neuroprotective signaling pathways

EPO is found to have a protective effect in the brain (Mallet & Ryou, 2017; Ponce, Navarro, Ahmed & Robertson, 2013) and a similar action occurs in the retina. The exact mechanism for this neuroprotective effect is not fully elucidated (Tsai, 2008). The tissue-protective molecular pathways that are triggered by EPO have similarities to, as well as differences from, those activated during erythropoiesis.

EPO activates cytoprotective signaling cascades that begins with the interaction with its membrane receptors (Fig. 23). EPO binding provokes EPOR homodimerization and autophosphorylation that activates tyrosine kinase JAK-2. Consequently, JAK-2 activates multiple signaling kinases, such as PI3K-protein kinase B (AKT); STAT5; extracellular signal-related kinase-1/2 (ERK-1/2) and NF-κB each one mediating distinct neuroprotective mechanisms (Digicaylioglu & Lipton, 2001; Kilic *et al.*, 2005a; Maiese, Li & Chong, 2004; Xie *et al.*, 2012).

**PI3K-AKT**

The JAK-2-PI3K pathway is crucial for the EPO neuroprotective role. *In vivo* studies showed that inhibition of either JAK-2 or PI3K abolished the neuroprotective effects of EPO (Zhang *et al.*, 2006; Zhang, Wang, Cao, Gao & Chen, 2007). PI3K activates AKT that is able to modulate

several intracellular signaling routes involved in apoptosis, synaptic signalling, and synthesis of glycogen. The main targets of AKT are tumor suppressor and transcription factor p53, glycogen synthase kinase 3 (GSK-3) and cytochrome c. AKT inhibits p53 activity which acts as pro-apoptotic by stimulating cytochrome c release from mitochondria via Bad/Bax and GSK-3 which is responsible for subsequent caspase formation (Culmsee & Mattson, 2005; Datta, Brunet & Greenberg, 1999).

**STAT5**

The JAK-2-STAT5 pathway is also involved in EPO neuroprotective action. Phosphorylation of STAT5 homodimers induces the transcription of the anti-apoptotic genes Bcl-2 and Bcl-xL (Digicaylioglu & Lipton, 2001) in the nucleous that are capable of preventing the release of cytochrome c from mitochondria. EPO has been found to increase STAT5 and the concentrations of anti-apoptotic genes (Sirén *et al.*, 2001; Sola, Rogido, Lee, Genetta & Wen, 2005; Wei *et al.*, 2006).

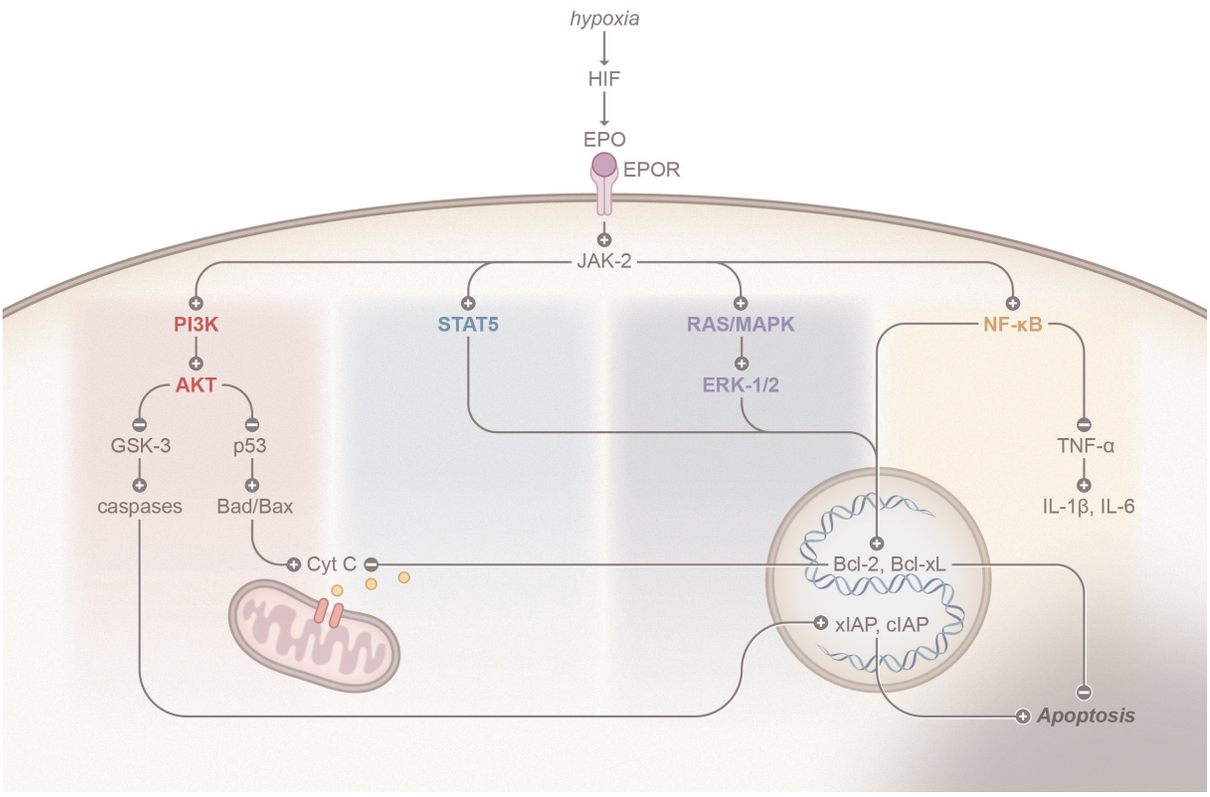


**Figure 23** - *EPO neuroprotective signalling pathways. The production of EPO is mainly evoked by HIF, and this up-regulation results in a sequence of downstream signalling activations, which will protect retinal cells.©Diogo Guerra. 2017.*

**ERK-1/2**

ERK-1/2 pathway also contributes to the beneficial EPO effect, by increasing transcription of the same anti-apoptotic genes (Bcl-2, Bcl-xL) as it occurs in STAT5 pathway. Kilic *et al.* found that EPO dramatically reduced the volume of infarction (after middle cerebral artery occlusion) and protected RGC (after axotomy-induced degeneration) by activating ERK (Kilic *et al.*, 2005a; Kilic *et al.*, 2005b).

**Nuclear Factor-κB**

The NF-κB signaling pathway plays a role in immune and inflammatory responses, cell adhesion, differentiation, oxidative stress responses, and apoptosis in mammals. Moreover, NF-κB activity contributes to the pathology of several human diseases, including many cancers and chronic inflammatory diseases (Gilmore & Garbati, 2010; Gilmore & Wolenski, 2012). As previously mentioned, NF-κB is a nuclear transcription factor that respond directly to oxidative stress such as induced by ROS and $H_2O_2$. Although the primary role for NF-κB in immune cells has been thought to be the activation of defense genes during the inflammatory response, a potential function of NF-κB during cell death has also been suggested. It has been shown that the activation of NF-κB protects cells against TNF-α-induced apoptotic cell death (Beg & Baltimore, 1996). This apparently contradictory actions were recently explained by the fact that acute increases in NF-κB contribute to an apoptotic signaling pathway, whereas preconditioning stimuli that lead to large increases in steady-state NF-kB activity provide neuroprotection (Lezoualc'h, Sagara, Holsboer & Behl, 1998). Accordingly, JAK-2 phosphorylates NF-κB's inhibitory subunit, IκB, releasing the transcription factor, and allowing its translocation to the nucleus to activate expression of neuroprotective genes which in turn induces transcription of p53, XIAP and cellular inhibitors of apoptosis (cIAP), superoxide dismutase and anti-apoptotic genes (Bcl-2 and Bcl-xL) (Ponce *et al.*, 2013).

### 2.2.2 EPO protective effects on the retina

In different diseases, EPO has been proved to exert protective actions on retina such as anti-apoptotic, neurotrophic, anti-Inflammatory actions; helping the survival of cells in hyperoxia and hypoxia; contributing to the maintenance of osmotic pressure in RPE; protecting the retinal vasculature and recruiting endothelial cells. Fig. 24 summarize the main effects and pathways involved in EPO neuroprotection.

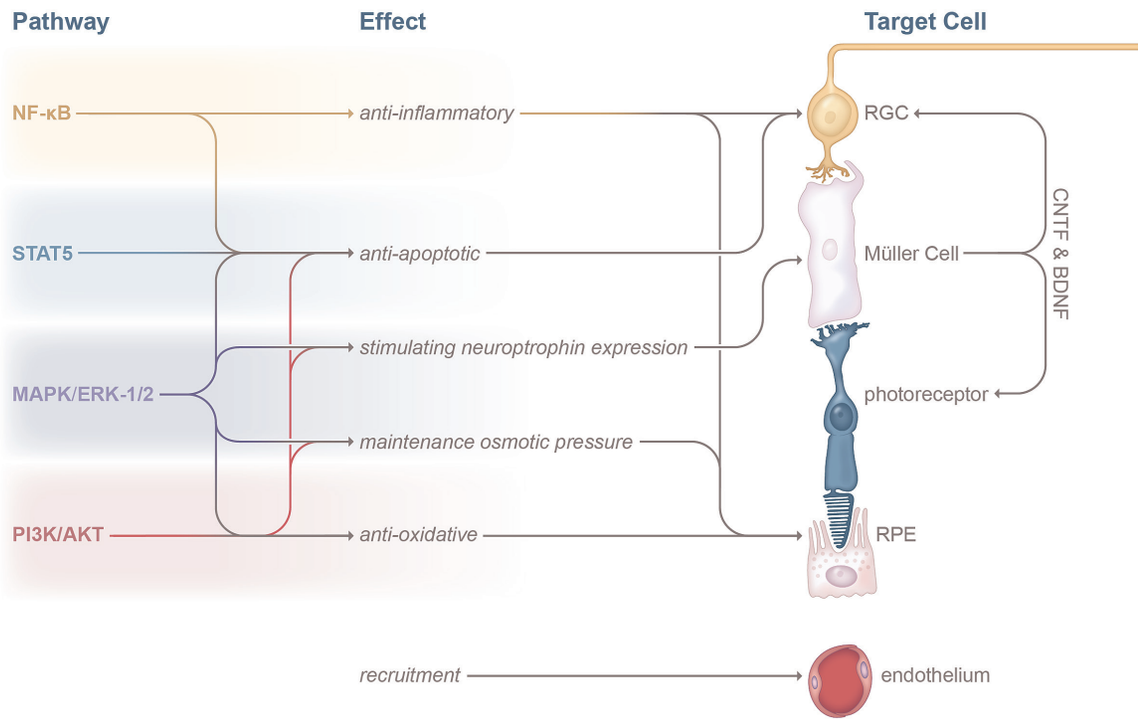


**Figure 24** – *Squematic representation of the EPO protective effects on the retina and its pathways. © Diogo Guerra. 2017.*

### Anti-apoptotic and neurotrophic actions

Degeneration occurs in several chronic retinopathies including glaucoma, diabetic retinopathy, age-related macular degeneration, and retinitis pigmentosa (Athanasiou *et al.*, 2013; He, Li, Chan & Hinton, 2013). As mention above, RGC have been shown to die by apoptosis in glaucoma disease (Garcia-Valenzuela *et al.,* 1995; Quigley *et al.*, 1995; Kerrigan *et al.*, 1997). EPO can activate several neuroprotective signalling mechanisms to prevent the cells apoptosis.

Through the PI3K/AKT pathway, EPO reduces $Ca^{2+}$-induced glutamate release from surrounding cells (Kawakami *et al.*, 2001). EPO can also suppress caspase-3 activation, and enhance Bcl-xL expression (Sullivan, Geisert, Templeton & Rex, 2012), resulting in inhibition of apoptosis and protection of photoreceptor cells by the suppression of the mitochondrial release of cytochrome C and the regulation of intracellular Ca levels (Xie *et al.*, 2012).

Moreover, EPO can provide neuroprotection to RGC and photoreceptors by stimulating neurotrophin expression in Müller cells. Through the ERK-1/-2 and AKT pathways, EPO up-regulated the expression of CNTF and BDNF helping the RGC and photoreceptors. Additionally, EPO can reduce the content of glial fibrillary acidic protein, a protein expressed in reactive Müller cells (Hu *et al.*, 2011).

Finally, some studies also demonstrated that EPO is able to promote neuroregeneration, evidenced by the regeneration of RGC axons after optic nerve transections (Bocker-Meffert *et al.*, 2002; King *et al.*, 2007; Zhong, Yao, Deng, Cheng & Zhou, 2007).

**Anti-Inflammatory mechanisms**

Inflammation is a physiologic response to injury and infection and is necessary for tissue healing. However, when neuroinflammation is severe or chronic, it can produce deleterious effects involving pro-inflammatory signalling pathways, increased oxidative stress, and death of nearby neurons (Bond & Rex, 2014). Neuroinflammation is a common mechanism with influence in the severity and progression of neurodegenerative disease and injury (Frank-Cannon, Alto, McAlpine & Tansey, 2009).

Villa *et al.* (2003) demonstrated that EPO could attenuate inflammatory responses by reducing neuronal apoptosis and increasing resistance to inflammatory injury rather than by a direct inhibition of cytokine release. EPO can reduce the damage in RGC by facilitating their resistance to injury induced by TNF-α (Chang, Yeh, Chiang, Chen & Lu, 2013). It is known that EPO can activate NF-κB (Digicaylioglu & Lipton, 2001), which acts as a survival signal of RGC (Fuchs *et al.*, 2005), and that way forming an antagonizing action on TNF-α-induced injury. Furthermore, EPO also plays a positive role in reducing the expression of TNF-α, as well as IL-6 and IL-1β, two pro-inflammatory cytokines (Liu *et al.*, 2006; McVicar *et al.*, 2011). Wang *et al.* (2009) confirmed this anti-inflammatory effect by attenuation of TNF-α and IL-1β after oxidative damage to RPE cells. Also, the transcription of the anti-inflammatory cytokine IL-10 is significantly increased (Liu *et al.*, 2006).

The decline of pro-inflammatory cytokines is related to the JAK/STAT pathway which also down-regulates the expression of inflammatory factors (Luo *et al.*, 2015; Xie *et al.*, 2012).

**Facilitating the survival of cells in hyperoxia and hypoxia**

Oxidative injury is thought to play an essential role in the degeneration, dysfunction or loss of RPE cells and may contribute to several retinal degenerative diseases. RPE cells form the outer BRB between the photoreceptors and the choriocapillaris. The physiological function of RPE cells, which includes removing photoreceptor turnover products, causes them to be constantly exposed to a number of ROS, including $H_2O_2$ and superoxide anion (Miceli, Liles & Newsome, 1994).

As previously mentioned, EPO has the capacity to reduce oxidative damage to RPE cells by down-regulating inflammatory cytokines and other factors (Wu, Shang, Sun & Liu, 2007). The antioxidant effect of EPO is partly dependent on the activation of AKT1. According to Kilic *et al.* (2005b), EPO protects the brain from cerebral ischemia by activating the ERK-1/-2 and AKT pathways, and a similar mechanism is predicted in signal transduction in the retina (Luo *et al.*, 2015).

Hypoxia is a major stimulus for both systemic and intraocular EPO production. With the decrease in oxygen pressure, the HIF is activated, acting as a cellular oxygen sensor (Jelkmann, 2007). Junk *et al.* (2002) demonstrated that the expression of EPOR in the retina is up-regulated by ischemia. They concluded that the neutralization of endogenous EPO with soluble EPOR exacerbated ischemic injury, which supports a crucial role for an endogenous EPO/EPOR system in the survival and recovery of neurons after an ischemic retina insult. After the systemic administration of exogenous EPO, they observed an histopathological and functional retina recuperation with a diminished level of neurons' apoptosis.

**Contribution to the maintenance of osmotic pressure in RPE**

Studies in different *in vivo* models provided evidence that exogenous EPO provides protection against the permeability of the blood brain barrier (BBB) (Grasso *et al.*, 2007a; Martínez-Estrada *et al.*, 2003; Uzüm, Sarper Diler, Bahçekapili & Ziya Ziylan, 2006). Bahcekapili *et al.* (2007) observed that EPO minimized BBB disruption, brain edema, and lesion volume, and augmented recovery of motor function in a brain ischemic model. Both BRB and BBB share a similar structure so it was expectable that EPO could maintain the osmotic pressure in the BRB. In fact, a research performed by Garcia-Ramirez *et al.* (2011) demonstrated that EPO does benefit RPE cells by preventing the increase of permeability induced by diabetic conditions.

In diabetic retinopathy, hyperglycemia causes damage to the vascular endothelium and disrupts the BRB, leading to the development of diabetic macular edema and hyperosmotic stress. EPO can assist RPE cells against permeability and therefore slow down the subsequent pathological changes (Luo *et al.*, 2015).

One signalling pathway regulated by EPO during neuroprotective events is the MAPK, which is involved in the ERK pathway (Rabie & Marti, 2008). Tang *et al.* (2013) demonstrated that MAPK contributes to astrocytic swelling in the brain through a process involving increased aquaporin4 expression in the plasma membrane that can be blocked by EPOR activation.

Other mechanism involved in maintenance of osmotic pressure in RPE by EPO is the VEGF release, via the activation of JAK-2 and ERK-1/-2 pathways, that result in the opening of potassium and chloride channels and stop cells from swelling (Luo *et al.*, 2015).

**Protection of the retinal vasculature and recruitment of the endothelial cells**

The disturbances of the homeostasis in the retina, including hypoxia, will progressively develop angiogenesis, leading to abnormality in the retinal vasculature, disruption of BRB integrity and finally retinal neovascularization (Arjamaa & Nikinmaa, 2006). In normal conditions, an oxygen detector named as prolyl hydroxylase (PHD) blocks HIF-1α activity. However, during hypoxic conditions, degradation of HIF-1α is inhibited by the downregulation of PHD, leading to

expression of VEGF with consequently angiogenesis mechanisms' activation (Maes, Carmeliet & Schipani, 2012).

However, EPO can inhibit neovascularization by downregulating protein expressions of HIF-1α and VEGF (Zhang *et al.*, 2010). Besides, EPO has a protective effect by preventing apoptosis of the endothelial cells and preserving tight junction proteins in the retinal vasculature. The protection of the retinal vasculature, through JAK-2 and PI3K/AKT pathways, inhibits subsequent blood vessel formation and hypoxia (Shirley Ding *et al.*, 2016).

Chen *et al.* (2008) explain the effect of EPO in maintaining the stability of retinal vessels. They found that exogenous EPO can stimulate recruitment of bone marrow endothelial progenitor cells into the retina, which have been shown to differentiate into endothelial cells and re-vascularize injured retinal vasculature (Heeschen *et al.*, 2003; Hu *et al.*, 2011). Likewise, EPO avoid the next pathological process of further ischemia and neovascularization.

## 3. OCULAR DRUG DELIVERY

In any treatment, the overall goal of drug delivery is to achieve and maintain therapeutic concentrations of the drug at its site of action for sufficient time to produce a beneficial effect. A secondary aim is to avoid exposing any other tissues to the compound that can cause deleterious effects. The intrinsic effects at the target site, distribution and elimination from body determine the efficacy of a compound. Permeation and elimination of a compound from a specific site can radically affect its efficacy. There are specific regions of the body with significant barriers to drug permeation such as the eye and the brain (Jaffe, Ashton & Pearson, 2006).

## 3.1 BLOOD-OCULAR BARRIERS

Concerning ocular drug delivery, several routes are described to reach retinal tissues but its specific anatomical and functional barriers should be considered. In the case of systemic, suprachoroidal and periocular route, the drug must cross the blood-ocular barrier before it can reach its targets in the retina. Therefore, blood-ocular barriers play an important role in defining drug permeation into the eye and from the eye to the blood circulation (Del Amo *et al.*, 2017). The blood-ocular barriers consist of the anterior blood-aqueous barrier and posterior blood-retinal barrier (Fig. 25) (Raviola, 1977).

### Anterior blood-aqueous barrier

The anterior BAB consists of two components: epithelial barriers (posterior iris epithelium and non-pigmented epithelium) that protect the posterior chamber from circulating proteins and other large molecules; and an endothelial barrier (iris and ciliary muscle vasculature), which prevents movement of such molecules from the lumen of the iris vessels into the iridial stroma (Del Amo *et al.*, 2017; Remington, 2012).

### Blood-retinal barrier

The BRB prevents components of blood plasma, that might be obstacles to light pathway to the photoreceptor outer segments, from entering retinal tissue (Remington, 2012) and helps to establish the unique neural environment necessary for proper neural function (Jaffe *et al.*, 2006). The BRB has also two components, the endothelium of retinal capillaries (inner blood retinal barrier) and the pigment epithelium (outer blood retinal barrier) (Raviola, 1977).

The retinal capillaries are not fenestrated and their endothelium contains the zonula occludens that prevent the exit of large molecule from retinal vessels (Remington, 2012). Molecules with a diameter of 2 nm and higher cannot cross this structure, while small molecules are able to permeate across the inner BRB and move into retinal tissue (Cunha-Vaz & Maurice, 1967; Cunha-Vaz & Maurice, 1969; Del Amo *et al.*, 2017; Tachikawa, Takeda, Tomi & Hosoya, 2010). The retinal pigment epithelium is a tight cellular monolayer that is located between the photoreceptors and the choroid. It has important functions in the homeostasis of the neural retina and as the outer part of the BRB. The zonula occludens junctions join the RPE cells preventing movement of large molecules into retinal tissue while the choriocapillaris is fenestrated allowing them to exit into choroidal tissue. Once there, these molecules can usually pass through Bruch's membrane easily (Remington, 2012). The roles of Bruch's membrane and choroidal tissue as barriers to small molecules and neutral macromolecules (up to 500 kDa) are negligible in comparison with the RPE (Del Amo *et al.*, 2017).

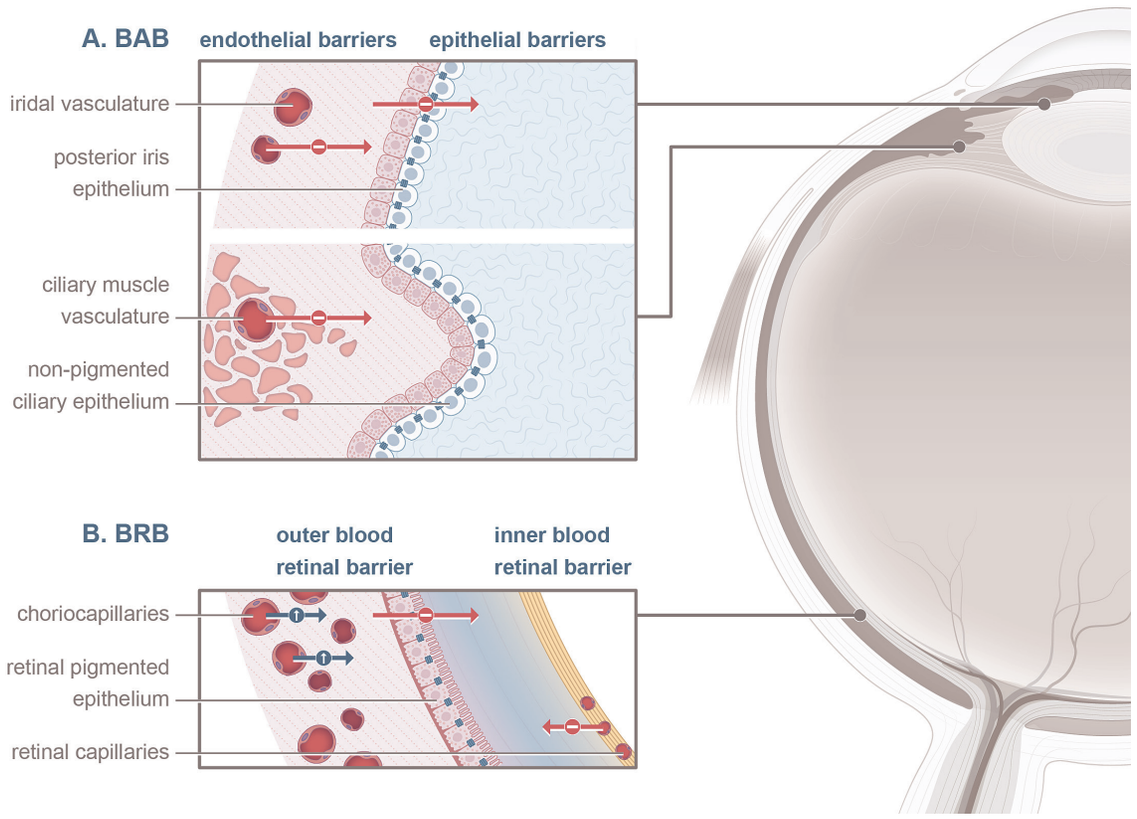


**Figure 25** – *Squematic representation of blood-ocular barriers: anterior blood-aqueous barrier (A) and blood-retinal barrier (B). © Diogo Guerra. 2017.*

### Active transport in blood-ocular barrier

The physical structure of the blood-ocular barrier defines the level of passive drug permeation through the barrier. Obviously, the permeability also depends on the chemical drug properties, however active transporters may affect permeation of drugs that are substrates of transporter proteins (Del Amo *et al.*, 2017).

The pharmacological significance of active transport in the posterior eye segment is still unclear. The passive transport and carrier-mediated transport coexist in biological membrane permeation. However, the importance of transporter activity is relative and dependent on the rate of passive drug diffusion (Sugano *et al.*, 2010). Extensive passive diffusion tends to decrease the relative impact of active transport. Unlike passive diffusion, active transport is saturable and its relative efficacy and importance are marked at low drug concentrations, but decreased at high drug concentrations. Several nutrient transporters, which include peptide, amino acid, folate, monocarboxylic acid transporters and others, have been reported to be expressed on the retina and BRB (Duvvuri, Majumdar & Mitra, 2003). Targeting these transporters can enhance ocular bioavailability of potent, but poorly bioavailable compounds. A small increase in the bioavailability of such drug molecules

could result in a significant increase in therapeutic response. However, not much work has been done on drug delivery to the retina and posterior segment of the eye by using membrane transporters. Further research into this aspect of retinal drug delivery could lead to promising results.

**Loss of the BRB in ocular diseases**

BRB plays a fundamental role in retinal function in both health and disease (Cunha-Vaz, 2017). Several ocular diseases such as glaucoma, diabetic retinopathy, uveoretinitis and age-related macular degeneration may cause pathophysiological alterations in the ocular environment that can lead to a loss of the BRB. An increase in the permeability both on blood vessels and retinal pigment epithelium can change the pharmacokinetics and drug delivery. This is quite an important aspect that should be taken in consideration because the pharmacokinetic outcomes depend on the disease (nature and extent of the changes) and drug properties (Del Amo *et al.*, 2017).

## 3.2 OCULAR DELIVERY ROUTES

Considering drug delivery for ophthalmic conditions, three important aspects should be taken in account: (i) the duration of delivery, (ii) the target tissue, and (iii) owner or patient compliance. The duration of drug delivery can vary from minutes (e.g. topical eye drops), to years (e.g. some ocular implants); the route of drug delivery may determine whether or not the drug can reach the target tissue in therapeutic concentrations without unwanted side effects and finally, the issue of compliance should also be considered in ocular drug delivery. For example, topical administration is the ideal route to allow a drug to reach the cornea and conjunctiva, but is unlikely that it can reach the retina and choroid in therapeutic concentrations. The owner or patient compliance issue is particularly important in veterinary medicine because of the difficulty in consistently treating an animal. Likewise, in human medicine, if a drug must be administered every hour to reach therapeutic concentrations, it is difficult to guarantee adequate therapy, especially in chronic diseases (Weiner & Gilger, 2010).
In this section, our focus will be the drug delivery to the retina since the main targets for glaucoma neuroprotection are the RGC. Several strategies have been used to deliver drugs to the retina. The commonly employed routes for ocular drug delivery include topical, systemic, intravitreal and peri-ocular administration (Fig. 26) being the subconjunctival and the retrobulbar the most routinely peri-ocular routes used in the clinical practice.

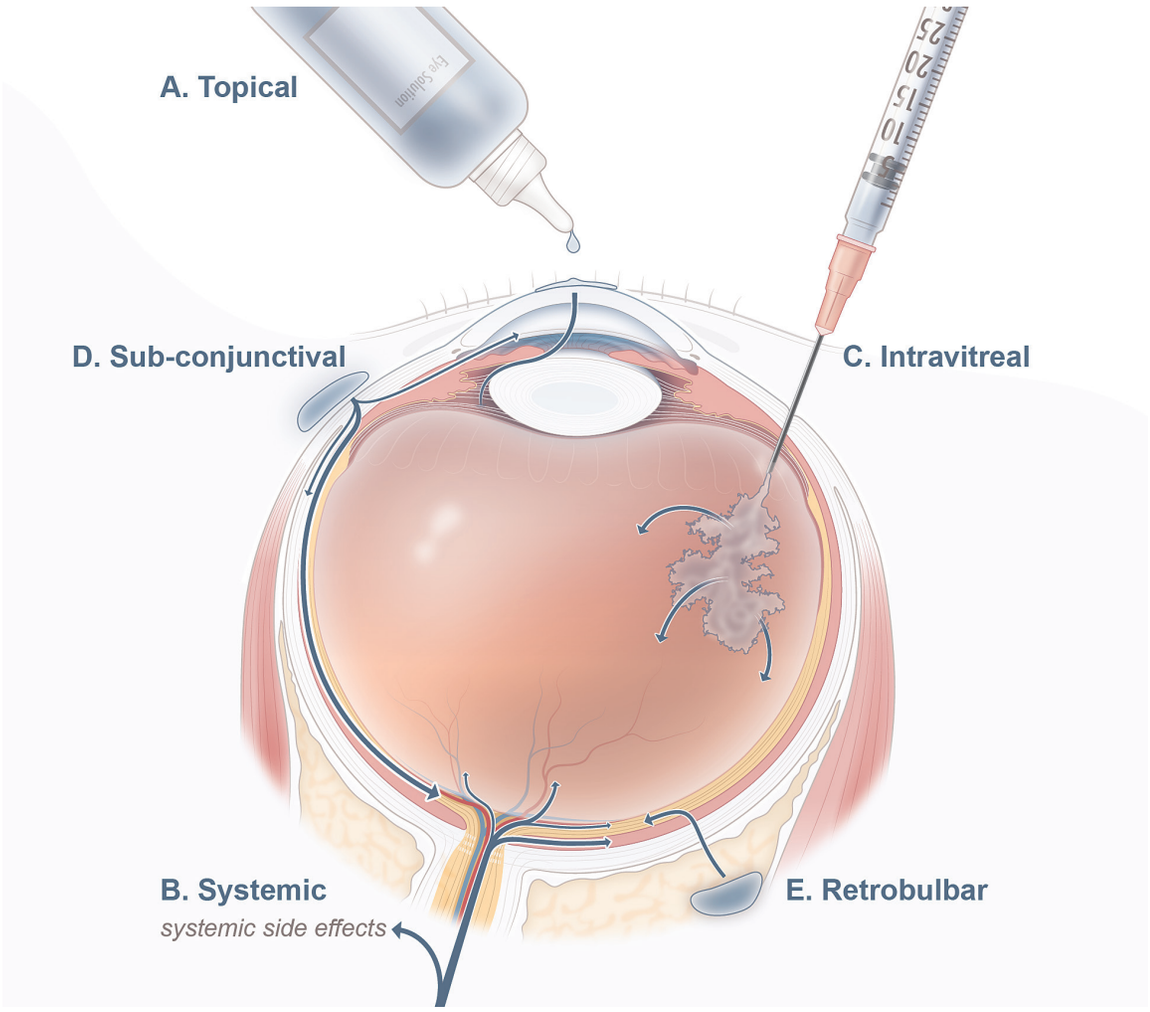


**Figure 26** – *Squematic representation of the ocular routes commonly used for retinal drug delivery: topical (A), systemic (B), intravitreal (C), sub-conjunctival (D) and retrobulbar (E). © Diogo Guerra. 2017.*

### 3.2.1 Topical administration

Conventionally, many ocular diseases are treated with either topical or systemic medications. Topical drug administration, using ophthalmic solutions or ointments, is the most preferred method due to the easy administration and low cost. This route is useful in the treatment of disorders affecting the anterior segment of the eye, which includes the aqueous humor, iris–ciliary body and lens. To reach the posterior segment upon topical administration, the drug has to traverse through the cornea, aqueous humor and lens (Duvvuri *et al.*, 2003). A major fraction of the drug following topical administration is lost by lacrimation, tear dilution, nasolacrimal drainage and tear turnover. Such pre-corneal losses result in very low ocular bioavailability. Typically, less than 5% of the total administered dose reaches the aqueous humor (Gaudana, Jwala, Boddu & Mitra, 2009; Rathore & Nema, 2009). Thus, it is not expected that a drug, upon topical instillation, reaches minimum therapeutic levels on the retinal tissue.

### 3.2.2 Systemic administration

Unlike topical administration, systemic route is commonly used to treat diseases affecting the posterior segment of the eye. However, drug delivery to retinal tissue and vitreous via systemic administration is constrained due to the presence of a BRB which regulates permeation of substances from blood to the retina. The major disadvantage associated with systemic administration is that only 1– 2% of the administered drug reaches the vitreous. Such low levels require frequent administrations of high doses to maintain the drug levels in the ocular tissues above the minimum therapeutic concentrations. This high amounts of drugs can lead to systemic unwanted side effects due to a nonspecific distribution to other tissues, which could result in serious toxicity (Duvvuri *et al.*, 2003; Gaudana *et al.*, 2009). In ocular treatments, the systemic route has been commonly used to deliver antibiotics to treat endophthalmitis, carbonic anhydrase inhibitors to treat elevated intraocular pressure, anti-inflammatory, methotrexate and parenteral antibodies to treat uveitis (Del Amo *et al.*, 2017).

### 3.2.3 Intravitreal drug administration

Intravitreal injection has several advantages over systemic and topical routes for drug delivery to the retina and to the vitreous. The major advantage of intravitreal injections is an immediate increase of the therapeutic effect in the retinal tissue. Moreover, it may result in high drug levels in the posterior segment without causing any systemic unwanted side effects. Since the BRB is avoided through this route, therapeutic levels may be maintained with lower doses (Duvvuri *et al.*, 2003). Some authors considered intravitreal drug administration a safe and effective route (Meyer, Krohne, Charbel Issa, Liu & Holz, 2015). However, intravitreal injections can originate potential side effects, such as elevated IOP, cataract formation, rhegmatogenous retinal detachments, vitreous haemorrhage and endophthalmitis (Duvvuri *et al.*, 2003). Even though the complications are rare, the number of adverse reactions will inevitably increase when millions of patients will be treated using repeated intravitreal drug administrations for years or decades (Ranta *et al.*, 2010). Moreover, in veterinary medicine, the intravitreal injections require patient's general anesthesia, and for that reason repeated administrations in chronic diseases will lead to a probable owner's non-compliance.

### 3.2.4 Periocular drug administration

In periocular administration, the drug is delivered to a region surrounding the eye, for example sub-conjunctival, sub-tenon's, peribulbar, retrobulbar or posterior juxtascleral injections. (Ranta & Urtti, 2006). When compared with topical treatments, periocular routes increase the delivery

of drugs into the posterior eye segment, such as vitreous, neural retina, RPE and choroid. Moreover, these routes are safer and less invasive than intravitreal administration, and the lower doses used to achieve therapeutic concentrations avoid some of the unwanted side effects of the systemic route. For these reasons, the periocular routes are gaining importance for the treatment of posterior segment ocular diseases (Gelatt & Gelatt, 2011). The injections of periocular administration can differ on the location of the injection and in the proximity of the sclera (Del Amo *et al.*, 2017). To potentially increase the drug penetration, the scleral thickness should be taken in account since it diminishes near the equator region (e.g. from 0.53 ± 0.14mm into 0.39 ± 0.17 mm in human) (Olsen, Aaberg, Geroski & Edelhauser, 1998). Among these, the sub-conjunctival and retrobulbar routes are the most commonly used in clinical practice. This work will focus on sub-conjunctival administration since the retrobulbar administration is mostly used for regional anesthesia and it also can be associated with potential complications (e.g. globe perforation, optic nerve injury, extra-ocular muscle injury, and orbital hemorrhage) (Dutton, 2001).

**Sub-conjunctival administration**

Sub-conjunctival administration has been used for a long time in clinical practice to deliver drugs to the anterior part of the eye because, when compared to topical administration, it is possible to achieve higher drug concentrations. On one hand, the corneal barrier is circumvented and on the other hand the retention at the injection site is longer. For these reasons, it is possible to achieve a drug bioavailability of 10% in the aqueous humor after a subconjunctival injection (Del Amo *et al.*, 2017). Moreover, it is possible to decrease the frequency of administration of a drug administered through this route because the drug is released over a prolonged period of time (Duvvuri *et al.*, 2003).

Drug delivery from the sub-conjunctival space to the retina is more challenging than the delivery to the anterior chamber but it has been investigated as an alternative for posterior segment drug delivery. To reach the retina, the drug may permeate from the periocular space via i) anterior chamber, ii) systemic circulation or iii) direct penetration pathway. Through the anterior chamber route, the drug diffuses first into the aqueous humor (both directly across the sclera and ciliary body and indirectly via the tear fluid and cornea after it has refluxed through the conjunctiva) and then to the posterior chamber and vitreous. In the systemic circulation route, the drug is absorbed and distributed to the general circulation (via conjunctival, episcleral, or choroidal vessels) and later returned into the eye through the blood flow. Finally, in the direct penetration pathway, the drug permeates into the vitreous through the underlying tissues. Once the sclera is crossed, there are several pathways to reach the retina: in the anterior eye, the drug may diffuse through the ciliary body, posterior chamber and vitreous, and in the posterior eye, drug has to permeate across the choroid, RPE and neural retina (Ranta & Urtti, 2006). A study

conducted by Lee & Robinson (2001) concludes that the direct penetration is the dominant pathway for a sub-conjunctivally injected compound to enter the vitreous chamber. Moreover, they concluded that the contribution of both anterior chamber and systemic circulation routes is minimal.

Concerning the retinal and vitreal drug bioavailability, the several barriers between the injection site and retina should be considered. They can be divided in two groups: i) flow barriers (elimination to blood flow and lymphatic flow from the sub-conjunctival space and choroid) and ii) penetration barriers (sclera, extravascular choroid, RPE) (Fig. 27).

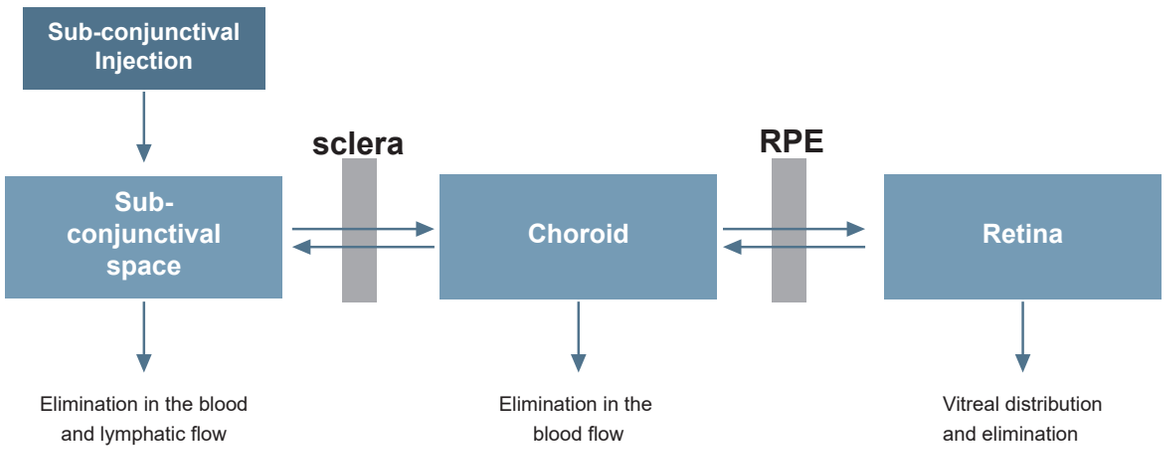


**Figure 27** - *Schematic presentation of the kinetic phases of sub-conjunctival injections for retinal drug delivery. Adapted from Del Amo 2017.*

After sub-conjunctival injection, it is estimated that 80 to 95% of the small molecular drugs enter the systemic circulation. However, the systemic absorption of proteins (e.g. Gd-albumin) can be even 70 times slower than the absorption of small drugs (Del Amo *et al.*, 2017; Kim, Csaky, Wang & Lutz, 2008; Ranta *et al.*, 2010).

Although there are different barriers between the sub-conjunctival space and the retina, Tsuji *et al.* (1988) estimated that 0.2% of prednisolone reached the vitreous after a sub-conjunctival injection in rabbits. After this study, several data suggested that effective intravitreal drug concentrations can be achieved by this route (Escalona-Benz *et al.*, 2005; Hayden *et al.*, 2000; Lee & Robinson, 2001; Suárez *et al.*, 2007; Zhang *et al.*, 2005). A study conducted by Weijtens *et al.* (2000) determined the dexamethasone concentrations in the subretinal fluid following subconjunctival, peribulbar and oral administration in patients suffering from rhegmatogenous retinal detachment. These authors demonstrated that subconjunctival route was the most effective to deliver the drug into the retina with a significantly higher $C_{max}$ value in the vitreous humor, when compared to the peribulbar and oral routes. Moreover, by subconjunctival administration it is possible to achieve a higher retinal drug bioavailability than by intraperitoneal route. Ayalasomayajula & Kompella (2004) concluded that the subconjunctival administration

resulted in a nearly 54-fold higher drug concentration in the ipsilateral eye, compared to the intraperitoneal route.

Finally, a very important aspect related to this route of administration is the possibility to delivery different formulations. The human sub-conjunctival space is highly expandable and through this route, volumes of up to 500 µL can be administered (Srirangam & Majumdar, 2012). Subconjunctival routes can be used for sustained-release delivery because a drug deposit can be formed in this space. Moreover, micro or nano-technology and physical methods (e.g. ultrasound and iontophoresis), can be combined with subconjunctival administration to improve bioavailability (Kim, Chiang, Wu & Prausnitz, 2014; Srirangam & Majumdar, 2012; Weiner & Gilger, 2010).

CHAPTER 2

# RATIONAL, HYPOTHESIS AND STUDY AIMS

## RATIONAL

Glaucoma is an insidious and progressive neurodegenerative eye disease responsible for irreversible blindness both in human and animals worldwide. Presently, the available treatment strategies involve reduction of IOP and there is no effective neuroprotective approach to prevent RGC loss. EPO, a glycoprotein hormone responsible for the production of red blood cells, has revealed neuroprotective properties in several body tissues. Many pre-clinical studies have been conducted in ocular diseases and EPO has proven to prevent RGC apoptosis, preserving visual function in several glaucoma models with very promising results. However, there are a number of risks associated to chronic systemic EPO administration.

## HYPOTHESIS

The study´s **main hypothesis** is: EPO's neuroprotection could be achieved by a non-invasive and safe periocular administration route without adverse effects.

## AIMS

Based on this hypothesis, the study **primary aim** is to evaluate the subconjunctival route as an alternative for EPO administration in glaucoma disease.

**Secondary aims include:**

*a)* to quantify the *in vitro* permeation of EPO across the periocular tissues: conjunctiva, cornea and sclera;

*b)* to test the *in vivo* ocular penetration of EPO after subconjunctival administration and the potential systemic side effects;

*c)* to determine whether the EPO subconjunctival administration reaches the retina in glaucoma conditions;

*d)* to assess functional and structural benefits of EPO subconjunctival administration in the retina of glaucomatous rats.

The whole chapter was published in Drug Delivery and Translational Research
(Drug Delivery and Translational Research. 2017 Oct; 7(5): 625-631.
doi: 10.1007/s13346-017-0399-y)

CHAPTER 3

# *EX VIVO* PERMEATION OF ERYTHROPOIETIN THROUGH PORCINE CONJUNCTIVA, CORNEA AND SCLERA

Ana Paula Resende[1], Beatriz Silva[1], Berta São Braz[1], Telmo Nunes[1], Lídia Gonçalves[2], Esmeralda Delgado[1]

1 Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal.

2 Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, 1649-003 Lisboa, Portugal.

## ABSTRACT

**Purpose:** The aim of this study is to test the permeation of human recombinant erythropoietin (rHuEPO) across conjunctiva, cornea and sclera in an *ex vivo* model.

**Methods:** Thirty fresh pig eyes were collected from a slaughterhouse. Conjunctivas (n=10), corneas (n=10) and scleras (n=10) were surgically dissected from surrounding tissues. Ocular membranes were placed into Franz diffusion cells and rHuEPO was administered into the donor phase of each cell, except for control samples. Samples were collected from the receptor phase at seven time points, from 30 min to 6 h of incubation. Erythropoietin (EPO) was quantified by enzyme-linked immunosorbent assay (ELISA) technique. Ocular membranes immunohistochemistry was also performed at the end of the study.

**Results:** EPO was detected in all test samples. After 6 h of incubation, conjunctiva was the most permeable membrane to rHuEPO (509.3 ± 89.8 mIU/cm$^2$, corresponding to 0.52% of the total rHuEPO administered on the donor phase), followed by sclera (359.1 ± 123.7 mIU/cm$^2$, corresponding to 0.35%) and finally cornea (71.0±31.8 mIU/cm$^2$, corresponding to 0.07%). Differences between ocular membranes' permeation were statistically significant ($p<0.001$). EPO immunostaining signal was positive for the three ocular membranes.

**Conclusion:** We have demonstrated in an *ex vivo* model that porcine conjunctiva, cornea and sclera are permeable to rHuEPO protein. These are promising results concerning ocular EPO administration.

## INTRODUCTION

Erythropoietin (EPO) is a 30.4 kDa hematopoietic glycoprotein produced mostly in the fetal liver and in the adult kidneys that has a key role in stimulating red blood cell production in bone marrow, being routinely used for the treatment of anemia in clinical practice (Abri Aghdam, Soltan Sanjari, & Ghasemi Falavarjani, 2016; Fisher, 2010). EPO has revealed tissue-protective properties in the brain, heart, inner ear and retina (Luo *et al.*, 2015) with significant neuroprotective properties in several animal models of neurodegenerative diseases including optic nerve and retinal diseases, such as glaucoma, optic nerve transection, retinal degeneration and optic neuritis (Bartesaghi, Marinovich, Corsini, Galli, & Viviani, 2005; Bond & Rex, 2014; Grasso *et al.*, 2007b; King *et al.*, 2007; Lagrèze, Feltgen, Bach, & Jehle, 2009; Tsai, Wu, Worgul, Forbes, & Cao, 2005; L. Zhong *et al.*, 2007; Zhong, Liu, Cheng, & Min, 2008; Y. Zhong *et al.*, 2007). In some models, EPO also presented neuroregenerative properties due to anti-apoptotic effects, being considered a valuable drug in the prevention of retinal ganglion cells' (RGC) apoptosis by systemic, intravitreal and retrobulbar routes (King *et al.*, 2007; Luo *et al.*, 2015; Tsai *et al.*, 2005; Zhong *et al.*, 2008). However, systemic administration induces unwanted side-effects related with hematopoiesis stimulation, and intravitreal administration is an invasive procedure that demands general or local anesthesia of the patient and can induce several complications such as endophthalmitis, retinal detachment, vitreitis, retinitis, choroiditis or cataracts (Jordán & Ruíz-Moreno, 2013; Sahoo, Dilnawaz, & Krishnakumar, 2008). Recently it has been demonstrated that EPO reached several retina cell layers after subconjunctival administration, without having detectable systemic side-effects when using a dosage of 1000 IU of human recombinant erythropoietin (rHuEPO) (Resende, São-Braz, & Delgado, 2013; Resende, São Braz, & Delgado, 2016).

Macromolecular drugs such as EPO represent an important therapeutical tool in the treatment of ocular diseases (Bento, Damasceno, & Neto, 2003; Kim *et al.*, 2014; Pescina *et al.*, 2015). Locally applied drugs can reach the intraocular tissues by either corneal and/or the non-corneal (conjunctival-scleral) pathways (Hosoya, Lee, & Kim, 2005). Topical instillation of ophthalmic drops has never been tested so far and due to its noninvasive nature, ease of management, and possibility of a home daily application: it could be a valuable alternative for EPO ocular administration.

In order to evaluate the viability of a noninvasive route of administration of macromolecules, it is extremely important to understand their permeability across the main ocular barriers (Pescina *et al.*, 2015) such as the cornea, the sclera, and the conjunctiva, which was precisely the purpose of this study.

The cornea and the sclera compose the fibrous tunic of the eye and the most anterior sclera is covered by bulbar conjunctiva (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). Cornea has five layers: stratified epithelium, Bowman's membrane (epithelium's basement membrane), stroma

(collagenous), Descemet's membrane (endothelium's basement membrane), and endothelium. Cornea's permeability to large molecules is generally low (Kim *et al.*, 2014), because it is mostly influenced by structural differences between epithelium (lipophilic, hydrophobic), stroma (hydrophilic, lipophobic), and endothelium (lipophilic, hydrophobic) (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). Therefore, moderately charged and moderately lipophilic small molecules are able to penetrate the cornea (Kim *et al.*, 2014).

The sclera is composed of three layers: episclera (outer), stroma and the lamina fusca. The episclera is an extremely vascular fibrous layer, whereas the stroma consists of numerous collagen fibers that differ from each other in diameter, shape, and orientation (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). *In vitro* studies generally demonstrate that scleral permeability decreases with high molecular weight molecules (Pescina *et al.*, 2010; Wen, Hao, & Li, 2013), but large molecules, such as IgG, can diffuse across sclera (Ambati *et al.*, 2000).

The conjunctiva consists of a stroma, and a nonkeratinized columnar epithelium with goblet cells, covering the inner surface of the eyelids and the anterior part of the sclera (bulbar conjunctiva) (Gelatt *et al.*, 2013; Maggs *et al.*, 2008). The epithelial cells at the apical side connect with each other by tight junctions that play a significant role as a permeability barrier (Hosoya *et al.*, 2005). Intercellular spaces of the conjunctival epithelium are wider than cornea's, being more permeable to larger molecules (Kim *et al.*, 2014). Hydrophilic drugs have direct access to the intraocular tissues through conjunctival-scleral pathway (Hosoya *et al.*, 2005). However, lipophilic drugs use transcellular pathway instead and they seem to be better absorbed across cornea and conjunctiva (Hosoya *et al.*, 2005).

To the best of the authors' knowledge, there are no published studies about *ex vivo* ocular membrane's permeability to rHuEPO. Therefore, this study aimed to assess the permeability of porcine conjunctiva, cornea and sclera to rHuEPO in an *ex vivo* model.

## MATERIALS AND METHODS

### Ocular membranes preparation

Thirty fresh pig eyes were collected from a slaughterhouse, kept refrigerated immersed in PBS, and 1 h later conjunctivas (n = 10), corneas (n = 10) and scleras (n = 10) were surgically dissected away from surrounding tissues. Ocular membranes were then placed into unjacketed Franz diffusion cells (receptor volume: 3ml; permeation area: 1cm$^2$) and immediately hydrated with 10mM PBS at pH7.4. Franz cells' mass was measured before and after the addition of PBS to determine the exact volume in the receptor phase. A magnet was placed inside the receptor phase, being the donor phase covered with Parafilm M® to avoid evaporation and dryness of the membrane. Franz cells were immediately placed in an incubator at 37ºC with agitation at 300rpm.

### Permeation assay

NeoRecormon 5000®, (Roche Diagnostics GmbH, Mannheim, Germany) was used in this experiment. Concerning the parameters that affect the diffusion of a protein across ocular tissues, the concentration of epoetin beta in NeoRecormon 5000® was 5000 IU/0.3ml solution, the molecular is globular in shape and its molecular weight is approximately 30kDa.

In a preliminary assay, a total of 1000 IU rHuEPO diluted in 200µL of PBS was added to the donor phase. Despite having observed rHuEPO permeation through the three ocular membranes, the obtained values were out of the range of the calibration curve from the kit used for quantification. Based on these preliminary results, it was decided to dilute rHuEPO ten times. Being so, a solution of 100 IU (6µL) of rHuEPO and 194µL of PBS was administered to the donor phase of each Franz cell, except in the control samples, which received 200µL of PBS. Cells were incubated at 37ºC, constantly agitated at 300rpm.

Samples of 200µL were collected from the receptor phase of each Franz cell at seven time points after the beginning of the incubation period: T1 = 30', T2 = 45', T3 = 60', T4 = 120', T5 = 180', T6 = 240' and T7 = 360'. A total of 210 samples were collected to sterilized vials and stored at 4º C overnight.

### EPO quantification

EPO was quantified using human erythropoietin ELISA kits (Abcam® ab119522, Cambridge, England), following the manufacturer's instructions. Absorbance at 450nm wavelength was measured in a microplate reader (FLUOstar Omega, BMGLabtech, Germany).

The cumulative amount of permeated rHuEPO per membrane surface area (Qt) through different eye membranes was plotted as function of time and determined based on the following equation:

$$Qt = \frac{\mathrm{Vr} \times \mathrm{Ct} + \sum_{t=0}^{t-1} Ci \times Vs}{\mathrm{S}} \quad (1)$$

Where Ct is the EPO concentration of the receptor solution at each sampling time, $\sum_{t=0}^{t-1} Ci$ is the sum of concentration of EPO determined at sampling intervals 1 trough t-1 and Vr and Vs are the volumes of the receptor solution and the sample, respectively. S represents the eye membrane surface area (1cm$^2$).

The transmembrane fluxes of EPO for the three eye membranes (J, mIU/cm$^2$h) were calculated from the slope of the regression line at the time interval used for the calculation corresponded to 0.75 till 3h for conjunctiva and to 0.5 till 4h for scleral tissue and cornea, using the GraphPad PRISM® 5 software (San Diego, CA, USA), and the apparent permeability coefficients (Papp, cm/s) were calculated at the steady state as:

$$Papp = J/CD \quad (2)$$

where CD (mIU/ml) is the concentration of the donor solution.

**Immunohistochemistry**

At the end of the study, ocular membranes samples were stored at the same time in 4% (v/v) paraformaldehyde in PBS (0.1M, pH7.4) overnight for additional processing by paraffin embedding and cut sections (3µm). One cross-section per each ocular membrane was deparaffinized, rehydrated, rinsed in PBS solution for 30min, treated with a 10% Triton X-100 solution for other 30min, and washed twice in PBS solution for 5min. Then, cross-sections were incubated with a blocking solution (10% (v/v) normal donkey serum in PBS) for 30min at room temperature followed by overnight incubation with polyclonal goat anti-EPO antibody (1:100; sc-1310, Santa Cruz Biotechnology) at 4ºC.

A sample without the primary antibody was used as negative control. Bovine fetal kidney cross-section was used as a positive EPO immunostaining control.

The following day, cross-sections were washed in PBS three times during 5min and incubated with rhodamine-conjugated secondary antibody (1:100; sc-2094, Santa Cruz Biotechnology) for 1h, in a dark room. The cross-sections were washed again with PBS and prepared with a mounting medium containing a cell nucleus marker, DAPI (UltraCruzTM Mounting Medium; sc-24941, Santa Cruz Biotechnology). The ocular membranes histological cross-sections, conjunctiva, cornea, and sclera, were blindly analyzed by the same investigator (Resende) using fluorescent microscopy (Leica DM R, Leica Microsystems, Bensheim, Germany).

**Statistical analysis**

Statistical analysis of the differences between ocular membranes permeation was performed with R for Windows (Microsoft®, Redmond, Washington, USA) using Kruskal-Wallis rank sum test and Tukey contrasts for comparisons of means. Data are presented as mean ± standard deviation and percentage.

## RESULTS

### Permeation assay

The results obtained indicated that the tested ocular membranes were permeable to rHuEPO in this *ex vivo* experiment, after rHuEPO instillation in the donor phase and sample collection from the receptor phase.

### EPO quantification

Results, summarized in Table 6, show that after 6h of permeation, conjunctiva was the most permeable ocular membrane to rHuEPO (509.3 ± 89.8 mIU/cm$^2$, corresponding to 0.52% of the total amount of rHuEPO instilled in the donor phase), followed by sclera (359.1 ± 123.7 mIU/cm$^2$, corresponding to 0.35%) and finally cornea (71.0 ± 31.8 mIU/cm$^2$, corresponding to 0.07%) (Fig. 28). Differences between the amount of rHuEPO permeation through the three ocular membranes were statistically significant ($p<0.001$). No endogenous EPO was detected in any of the control samples.

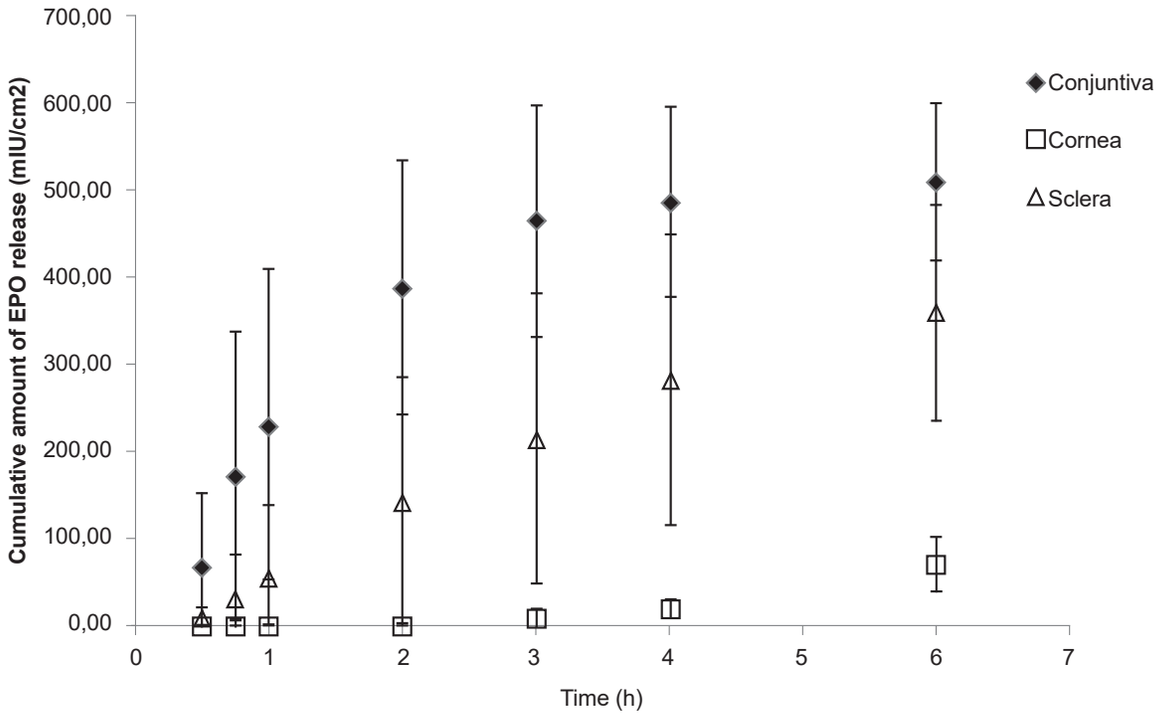


**Figure 28** - *Cumulative amount of EPO permeated (mIU/cm$^2$) of each ocular membrane - conjunctiva (n=10), cornea (n=10) and sclera (n=10) – at each time point.*

Concerning the rHuEPO fluxes through different ocular membranes, the conjunctiva presented a flux of 201 ± 51 mIU cm$^{-2}$ h$^{-1}$, the sclera 93 ± 16mIU cm$^{-2}$ h$^{-1}$ and finally the

cornea 27 ± 8mIU $cm^2$ $h^1$, and the apparent permeability coefficient for conjunctiva was 0.96 ± 0.50 x $10^{-7}$ $cm.s^{-1}$, for sclera was 0.52 ± 0.10 x $10^{-7}$ $cm.s^{-1}$, and for cornea was 0.15 ± 0.05 x $10^{-7}$ $cm.s^{-1}$.

| Time (hours) | EPO | | | | | |
|---|---|---|---|---|---|---|
| | Conjunctiva | | Cornea | | Sclera | |
| | mIU/$cm^2$ | % | mIU/$cm^2$ | % | mIU/$cm^2$ | % |
| 0.5 | 68.4 ± 83.4 | 0.07 | 0.5 ± 0.9 | 0.00 | 10.0 ± 10.8 | 0.01 |
| 0.75 | 171.7 ± 165.1 | 0.17 | 0.1 ± 0.2 | 0.00 | 32.7 ± 49.4 | 0.03 |
| 1 | 230.3 ± 178.5 | 0.23 | 0.5 ± 1.3 | 0.00 | 55.1 ± 82.8 | 0.05 |
| 2 | 388.3 ± 145.6 | 0.39 | 1.5 ± 2.4 | 0.00 | 142.5 ± 142.9 | 0.14 |
| 3 | 464.7 ± 133.6 | 0.47 | 8.4 ± 10.1 | 0.01 | 214.3 ± 166.5 | 0.21 |
| 4 | 486.6 ± 108.8 | 0.49 | 19.7 ± 11.0 | 0.02 | 282.4 ± 166.8 | 0.28 |
| 6 | 509.3 ± 89.8 | 0.52 | 71.0 ± 31.8 | 0.07 | 359.1 ± 123.7 | 0.35 |

**Table 6** - *Cumulative amount of EPO in the receptor phase of each ocular membrane - conjunctiva (n=10), cornea (n=10) and sclera (n=10) – at each time point. Data are presented as mean± standard deviation (mIU/$cm^2$) and percentage (%) of the total amount of rHuEPO instilled in the donor phase.*

**Immunohistochemistry results**

At the end of the study, EPO protein was detected inside all the ocular membranes that received rHuEPO in the donor phase. The presence of EPO protein, detected by immunohistochemistry, was stronger inside the conjunctiva when compared to the sclera, and weaker inside the corneal tissue (Fig. 29).

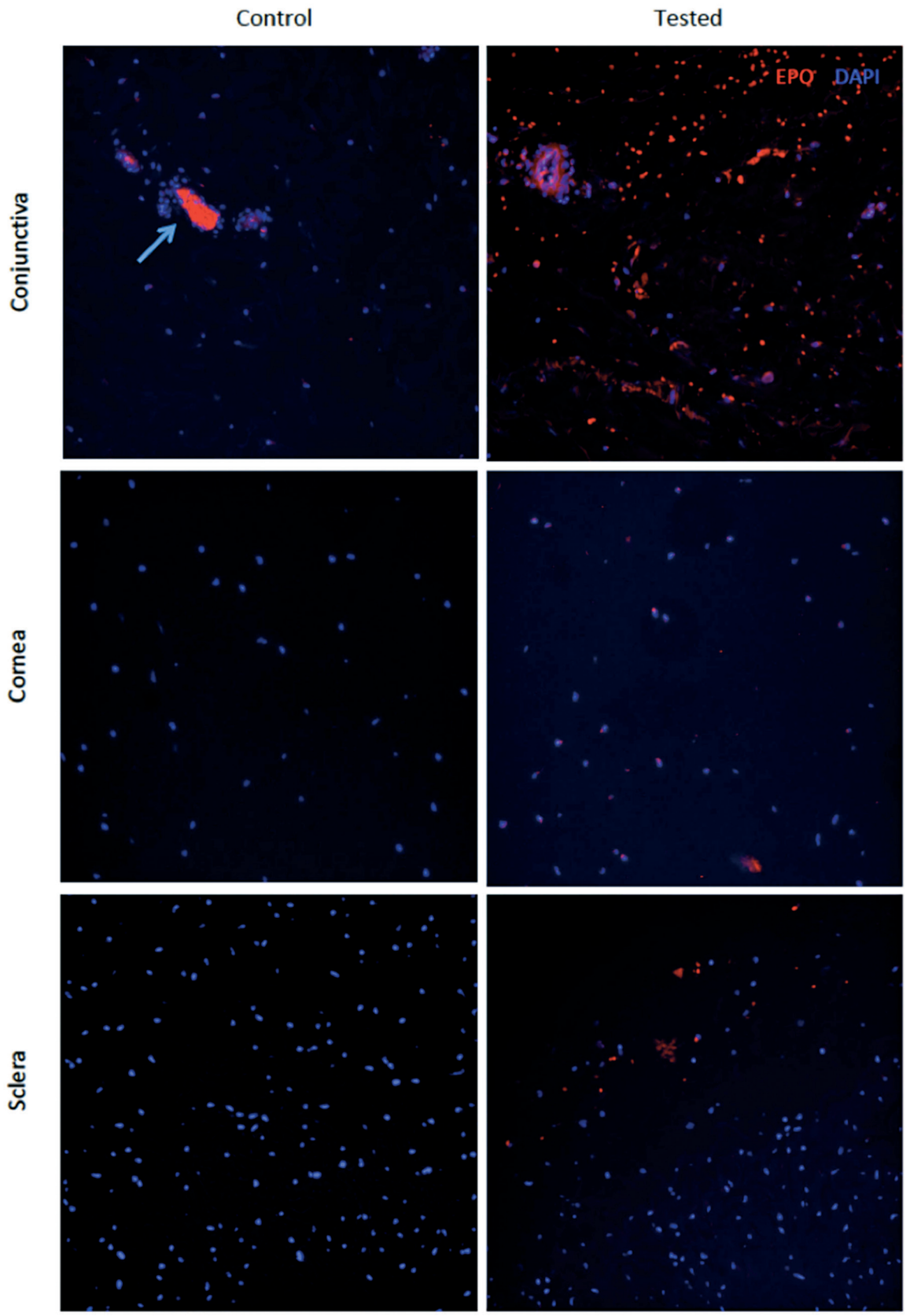


**Figure 29** - *Immunohistochemistry for cell nucleus marker of the conjunctiva, cornea and sclera (DAPI, blue) and EPO antibody (red), six hours after the administration into the donor phase. Note erythrocytes inside a blood vessel in control conjunctiva membrane due to auto-fluorescence staining (arrow). (x 10).*

## DISCUSSION AND CONCLUSIONS

EPO has revealed neuroprotective properties in several animal models of neurodegenerative diseases and also neuroregenerative properties due to its anti-apoptotic effects (Luo *et al.*, 2015; Y. Zhong *et al.*, 2007). Using the subconjunctival route, it was proven that EPO could reach the retina without causing any substantial systemic side-effects (Resende *et al.*, 2013, 2016). Topical instillation of ophthalmic drops containing EPO has never been tested and understanding its permeability across ocular barriers is the first step for the development of a topical drug.

The present study aimed to evaluate the permeability of conjunctiva, cornea and sclera to rHuEPO protein, using a pig eye *ex vivo* model. Permeation studies are generally performed by preparing the tissue *in vitro* or *ex vivo* and placing it in a diffusion cell assembly, such as Franz diffusion cell or Ussing Chamber (Agarwal & Rupenthal, 2016). The membrane separates the donor compartment (donor phase) containing the test product from the receptor compartment (receptor phase) filled with collection medium. Phosphate-buffered saline (PBS) tends to be the first choice among the collection mediums available. The temperature of the entire assembly is maintained at 37ºC and the receptor chamber is sampled at relevant time points to analyze the amount of drug penetrated (Particle Science, 2009). A Franz diffusion cell model was elected because it enables an easy, convenient, and robust experiment, and it is the most commonly used to study drug absorption and penetration across synthetic membranes, tissue constructs, and biological samples (Agarwal & Rupenthal, 2016; Particle Science, 2009). The scleral curvature was not considered in our results since the small size of the Franz cells permeation area enabled the flattening of the tissue and the sclera curvature was not detectable. Porcine ocular membranes were used because human and porcine eyeballs are quite similar in weight, size, corneal thickness, volume of aqueous humor, and vitreous body and both have Bowman's membrane (Remington, 2012). Porcine corneal penetration is expected to be slightly lower than humans' due to its thicker corneal epithelial layer (Agarwal & Rupenthal, 2016).

The dosage of 1000 IU of rHuEPO used in our preliminary assay was chosen based on *in vivo* EPO penetration studies trough the subconjunctival route (Resende *et al.*, 2013, 2016). Once the obtained values were out of the range of the calibration curve from the kit used for quantification, it was decided to dilute rHuEPO ten times. Our goal was to test the permeation of EPO across the three ocular membranes and not to test efficacy, so the 100 IU was chosen to decrease the possibility of additional experimental errors with dilutions.

The obtained results showed that the three tested ocular membranes were permeable to rHuEPO: conjunctiva was the most permeable membrane, followed by sclera, and finally by cornea. The differences between ocular membranes' rHuEPO permeation were statistically significant ($p<0.001$), which is supported by the differences in their histological structure. It is

known that the conjunctiva is more permeable to larger molecules due to the wider intercellular spaces of its epithelium (Kim *et al.*, 2014). The conjunctival-scleral pathway enables the delivery of hydrophilic drugs, permitting direct access to the intraocular tissues and posterior segments (Hosoya *et al.*, 2005). However, apparently, lipophilic drugs are better absorbed through cornea and conjunctiva than hydrophilic drugs because they use the transcellular pathway instead of the paracellular pathway, having a wider absorption area than hydrophilic drugs (Hosoya *et al.*, 2005). On the other hand, scleral permeability decreases considerably with increasing molecular weight (Pescina *et al.*, 2010; Wen *et al.*, 2013) and molecular radius (Ambati *et al.*, 2000), but recent *in vitro* and *in vivo* studies proved that molecules as large as 145-150kDa were able to penetrate the sclera (Ambati *et al.*, 2000; Demetriades *et al.*, 2008; Nomoto *et al.*, 2009) and be detected in the choroid in *in vivo* studies (Demetriades *et al.*, 2008; Nomoto *et al.*, 2009). Considering the cornea, there are structural differences between its epithelium, stroma, and endothelium that affect its permeability: the epithelium and endothelium are moderately lipophilic and hydrophobic and the stroma is relatively hydrophilic and lipophobic (Gelatt *et al.*, 2013; Maggs *et al.*, 2008).

Although there are no previous *ex vivo*, *in vitro*, or *in vivo* published studies concerning topical EPO administration, the existent literature refers that the conjunctiva is the most permeable membrane to large molecules, followed by the sclera and lastly the cornea (Agarwal & Rupenthal, 2016; Ambati *et al.*, 2000; Demetriades *et al.*, 2008; Hosoya *et al.*, 2005; Kim *et al.*, 2014; Koevary, 2003; Lee & Robinson, 2001; Nomoto *et al.*, 2009; Pescina *et al.*, 2010, 2015; Wen *et al.*, 2013), which is consistent with our results. EPO contains a range of peptides, including hydrophilic peptides and glycopeptides (Zhu, Martosella, & Duong, 2013), so it is expected to permeate better through conjunctiva than cornea and sclera, which is consistent with our results.

Ocular drug administration is a difficult task due to the peculiar structure of this organ and the presence of static and dynamic protective barriers (Pescina *et al.*, 2015). A significant reduction in bioavailability of topically applied drugs is observed not only due to nasolacrimal drainage, tear clearance, and lid blinking, but also because of the short residence time of the formulation on the ocular surface and the very low corneal permeability (Ambati *et al.*, 2000; Pescina *et al.*, 2015). Drug bioavailability to the anterior chamber after topical application can be as is lower as 1% or less (Lee & Robinson, 2001), although some studies refer that about 5% (Pescina *et al.*, 2015) or even 10% of the initial dose is absorbed across the cornea (Koevary, 2003). A recent paper evaluated the permeation of macromolecules of different size, conformation, and charge across porcine ocular tissues, in order to collect data of the molecular characteristics impacting their permeation. The authors concluded that it is difficult to predict the behavior of macromolecules based on their physicochemical properties, because the relation between the charge, molecular radius, and conformation all influence the permeation results across the different biological membranes (Pescina *et al.*, 2015).

Considering previous studies, subretinal and intravitreal administration of EPO with dosages as low as 10 IU can lead to anti-apoptotic effects in rat models (Shirley Ding *et al.*, 2016). Taking that into account, we believe that increasing the initial dose to ten times or more would improve the chances of achieving the therapeutic levels on the retina in an *in vivo* study. Moreover, the lower permeability of rHuEPO obtained in this experiment corresponds to the thicker tested membrane (cornea Papp 0.15 ± 0.05 x $10^{-7}$ $cm.s^{-1}$); this is in agreement with other studies where the permeability of other proteins is dependent on the structure of the tissue (Pescina *et al.*, 2015). Also, the EPO's permeability for scleral tissue is in the range of other macromolecules (Pescina *et al.*, 2015).

In a previous published work, we have demonstrated that EPO could reach the retina after subconjunctival administration in a rat animal model (Resende *et al.*, 2013, 2016). Using that route of administration, sclera is the main barrier to be crossed in order to reach intraocular structures. In these experiments, rHuEPO crossed the sclera to reach the retina, which is also consistent with our *ex vivo* findings.

At the end of the permeation study, the three ocular membranes histological cross-sections were stained by immunohistochemistry and assessed by fluorescent microscopy. EPO protein was detected inside all the membranes histological cross-sections that received rHuEPO in the donor phase. A higher EPO immunostaining signal was detected in the conjunctiva, followed by the sclera and lastly the cornea, which is in accordance with our permeation results. Immunohistochemistry of the ocular membranes cross-sections allowed the confirmation that rHuEPO did cross over the ocular membranes and was still present, within them, 6h after the incubation.

It is important to notice that some variability of data between samples was obtained and this can be due to some experimental issues, namely intrinsic variability between different eyes, membrane dissection procedures, and can also relate to Franz diffusion cells characteristics.

Using this *ex vivo* model, we could quantify ocular membrane permeation to EPO, opening the window to its topical or periocular administration, with a possible therapeutic role in several eye diseases. Despite the promising results, the role of the natural tear film clearance, the blinking, the blood flow in the conjunctiva and choriocapillaris, the uveoscleral outflow and the intraocular pressure, the retinal pigment epithelium and the blood-retinal barriers, among other features, are not considered in an *ex vivo* model. Therefore, *in vivo* studies should be performed to access EPO's ocular pharmacodynamics and pharmacokinetics, after instillation of eye drops or subconjunctival administration.

In conclusion, it was demonstrated that rHuEPO can permeate porcine conjunctiva, sclera, and cornea in an *ex vivo* model, which are promising results concerning ocular therapeutics. To find local noninvasive ocular delivery methods that increase drugs' efficacy, safety and bioavailability should be the challenge for the future.

The whole chapter was published in *Graefe's Archive for Clinical and Experimental Ophthalmology*
(Graefes Arch Clin Exp Ophthalmol. 2013, 251:2051–2056 doi: 10.1007/s00417-013-2367-7)

CHAPTER 4

# ALTERNATIVE ROUTE FOR ERYTHROPOIETIN OCULAR ADMINISTRATION

Ana Paula Resende[1], Berta São-Braz[1] & Esmeralda Delgado[1]

1 Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal.

## ABSTRACT

**Purpose:** This study aimed to find an alternative route for erythropoietin (EPO) ocular administration because of its neuroprotective and neuroregenerative known properties. Ocular penetration of EPO after subconjunctival injection was assessed and potential side effects on the haematocrit for a 28-day period were also evaluated.

**Methods:** Wistar Hannover female albino rats (n=42) divided into seven groups of six were used. One group (n=6) served as control. Six groups (n=36) received 1000 IU of EPO through the subconjunctival route in one of the eyes. According to the group, animals were humanely killed at 12 hours (n=6), 24 hours (n=6), 36 hours (n=6), 48 hours (n=6), and 60 hours (n=6), after EPO administration, in a total of 30 animals. Enucleation of both eyes was performed, and EPO protein distribution in the rat's retina was analyzed by immunohistochemistry. Another group of animals (n=6) was used to collect blood samples and perform haematocrit analysis at 0, 7, 14, 21 and 28 days after unilateral EPO subconjunctival administration.

**Results:** The evaluation of EPO expression in the animal's retinas after subconjunctival administration yielded a strong immunostaining signal. Among the retina's layers, EPO expression was more evident in the RGC layer 24 hours after the administration, and was still present on that layer till the end of the study (60 hours). When administered subconjunctivally EPO reached several neuronal cells, in all retinal layers. The subconjunctival EPO administration did not cause significant changes in the haematocrit values over a 28-day period.

**Conclusion:** In this study, it was demonstrated that EPO reached the retinal ganglion cell layers when administered subconjunctivally. EPO reached the retina 24 hours after the subconjunctival administration, and was still present 60 hours after the administration. Furthermore, it was also proved that EPO subconjunctival administration did not cause any haematopoietic significant side-effects. The subconjunctival route was shown to be a promising alternative for EPO ocular delivery.

## INTRODUCTION

Erythropoietin (EPO) is a cytokine produced in the kidney, which has a key role in stimulating red blood cell production in bone marrow (Bartesaghi *et al.*, 2005; Grasso *et al.*, 2007b). Recombinant human erythropoietin has been approved (1989) to treat anaemia in patients suffering from renal failure and/or several tumours. However, in recent years it has been demonstrated that EPO also has significant neuroprotective properties in several animal models of neurodegenerative diseases. Neuroprotective efficacy of EPO treatment has been demonstrated in several models of optic nerve and retinal diseases, including glaucoma, optic nerve transection, retinal degeneration and optic neuritis (King *et al.*, 2007; Tsai, Song, Wu, & Forbes, 2007; Tsai *et al.*, 2005; Zhang *et al.*, 2008; L. Zhong *et al.*, 2007; Y. Zhong *et al.*, 2007). Indeed, in some models, EPO has been shown to have neuroregenerative in addition to neuroprotective properties, due to its anti-apoptotic properties (King *et al.*, 2007).

Glaucoma, one of the leading causes of irreversible blindness in the world, is a progressive neuropathy characterized by retinal degeneration with apoptotic death of retinal ganglion cells (RGC) (Tsai *et al.*, 2005, 2007; L. Zhong *et al.*, 2007). EPO has proved to be a valuable drug in the prevention and in the decrease of apoptosis of retinal ganglion cells, preserving vision in animal models of glaucoma and in human patients (Tsai, 2008).

Recently a randomized, double-blind, phase 2 study used EPO to treat optic neuritis in humans, and concluded that EPO might be neuroprotective in those patients (Sühs *et al.*, 2012).

In an effort to reach therapeutic concentrations at retinal level, systemic or intravitreal EPO administration has been performed in several studies. However unwanted side effects related with haematopoiesis stimulation, such as haematocrit increase and blood hiperviscosity, which enhances the risk of atherosclerosis and stroke, are observed in systemic administration. With regard to intravitreal administration, it is an invasive procedure which demands patient general or local anaesthesia, and can induce several complications such as endophthalmitis, retinal detachment, vitreitis, retinitis, choroiditis or cataracts (Gaudana *et al.*, 2009; Nagarwal, Kant, Singh, Maiti, & Pandit, 2009; Sahoo *et al.*, 2008; Zhong *et al.*, 2008).

At present, the periocular route (namely retrobulbar and subconjunctival) seems to be promising and efficient for drug administration to posterior eye segment. The retrobulbar route was recently used to deliver EPO to the retina with some efficacy (Zhong *et al.*, 2008).

The present study was designed to assess the possibility of using the subconjunctival route for EPO ocular administration. By immunohistochemistry assay, EPO protein distribution in the retina of a rat animal model was studied. The subconjunctival EPO administration potential adverse side-effects on the haematocrit for a 28-day period were also analysed. Preliminary results of this study have been communicated (Resende, A. P., São-Braz, B. & Delgado, E., 2012).

## MATERIALS AND METHODS

### Animals

Animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and respecting the Faculty of Veterinary Medicine – Technical University of Lisbon ethical committee. Wistar Hannover female albino rats (n=42) weighting 307 ± 31 g were included in the study. Animals were housed in boxes type III with 1195 $cm^2$ (groups of three) with water and food ad libitum and maintained in controlled conditions of temperature (20± 2ºC), humidity (≈70%) and cyclic light (12 hours light/12 hours darkness). All the animals included in the study were submitted to a complete ophthalmic examination, including an anterior segment examination performed by slit-lamp biomicroscopy (Kowa SL-15, Tokyo, Japan), intraocular pressure measurement using rebound tonometry (Tonolab, Icare®, Helsinki, Finland), and posterior segment examination with binocular indirect ophthalmoscopy (Heine Omega 500, Herrsching, Germany). All the animals were considered normal in regard to their ophthalmic examination, otherwise they would have been excluded from the study.

Animals were divided into seven groups of six animals each. Six groups (n=36) received EPO through the subconjunctival route in one eye. One of these groups (n=6) was used to perform haematocrit analysis over a 28-da period. One group (n=6) served as control and received only saline through a subconjunctival injection.

### Anaesthesia and subconjunctival injections

Subconjunctival administrations were carried out under general anaesthesia. Anaesthesia was performed by intraperitoneal (i.p.) administration of 75 mg/Kg Ketamine (Imalgene 1000®, Merial Portuguesa, Rio de Mouro, Portugal) and 1 mg/Kg Medetomidine (Domitor®, Orion Corporation, Espoo, Finland).

The subconjunctival route was used for the administration of 1000 IU of EPO (NeoRecormon 5000®, Roche Diagnostics GmbH, Manheim, Germany) in a volume of 60 µl to the right eye of each animal. With the aid of a surgical microscope (Zeiss Opmi Visu Series/S7 Microscope, Munich, Germany), subconjunctival injections were performed using a 0,3 mL pre-filled syringe with a 27-gauge (G) needle in the bulbar conjunctiva.

After the subconjunctival injections in the dorsal bulbar conjunctiva a submucosal bubble was present for a maximum period of 24 hours, during which it disappeared.

Twelve hours after the procedure all the animals submitted to a subconjuctival injection were also submitted to a complete ophthalmic examination, including anterior segment examination performed by slit-lamp biomicroscopy (Kowa SL-15, Tokyo, Japan), intraocular pressure measurement using rebound tonometry (Tonolab, Icare®, Helsinki, Finland), and posterior segment examination with binocular indirect ophthalmoscopy (Heine Omega 500, Herrsching, Germany). In the ones that were

still alive, the exam was repeated 24, 48 and 60 hours after the subconjunctival injection.

**Euthanasia and enucleation**

According to the desired end point at 12, 24, 36, 48 or 60 hours after EPO subconjunctival administration, the rats were euthanized with i.p. injection of 5% pentobarbital sodium (60 mg/kg), following which both eyes were quickly enucleated and stored in 4% paraformaldehyde diluted in 0.1 M phosphate buffer, pH 7,4.

**Immunohistochemistry**

All eyes were processed by paraffin embedding, and longitudinal sequential sections (3 µm) were cut through the globe along the anterior-posterior axis. Retinal sections were deparaffinised and rehydrated. Sections were rinsed in PBS solution for 30 min, treated with a 10% Triton X-100 solution for 30 minutes and washed twice in PBS solution for 5 minutes. Sections incubation with blocking solution (10% normal donkey serum in PBS) was performed for 30 minutes at room temperature followed by overnight incubation with polyclonal goat anti-EPO antibody (1:100; sc-1310, Santa Cruz Biotechnology) and monoclonal mouse anti-PGP9.5 (1:500; ab72911, Abcam) diluted in PBS at 4ºC. A sample without the primary antibody was used as a negative control. The following day, sections were washed three times for 5 minutes with PBS and incubated with rhodamine conjugated secondary antibody (1:100; sc-2094, Santa Cruz Biotechnology), an FITC-conjugated secondary antibody (1:200; ab6724, Abcam), diluted in PSB for 1 hour, at a dark room. The sections were again washed with PBS and prepared with a mounting medium containing DAPI (UltraCruzTM Mounting Medium; sc-24941, Santa Cruz Biotechnology). The retinal sections were analyzed by fluorescent microscopy (Leica DM R, Leica Microsystems, Bensheim, Germany).

**Haematocrit**

*Blood sample collection*

Before EPO subconjunctival administration, a blood sample was collected through caudal vein punch from each animal in the haematocrit group (n=6) corresponding to the basal haematocrit value. Additional blood samples for haematocrit analysis were collected through caudal vein punch at 7, 14, 21 and 28 days after EPO subconjunctival administration. An haematocrit was performed from each sample.

The haematocrit values were determined as a percentage using the micro-haematocrit technique with a microcapillary reader (Orto Alreasa, Mod.Microcen, Madrid, Spain).

### Data analysis

Data were analyzed with R-project (R Foundation for Statistical Computing, Vienna, Austria) and Excel (Microsoft, USA), being reported as mean ± standard error. Haematocrit data were compared between groups using one-way analysis of variance (ANOVA) with a Tukey post-hoc test. A value of $p<0.05$ was considered to be statistically significant. Data were expressed as the mean ± standard deviation (SD).

## RESULTS

### Immunohistochemistry observations

Histological evaluation of EPO expression in the rat's retinas after subconjunctival administration yielded a strong positive immunostaining signal. EPO was already detected in all retinal cell layers, showing a higher concentration in the retinal pigment epithelium 12 hours after the subconjunctival administration. Nevertheless, it also reached the more inner retinal cell layers as well as the ganglion cell layer (Fig. 30 b).

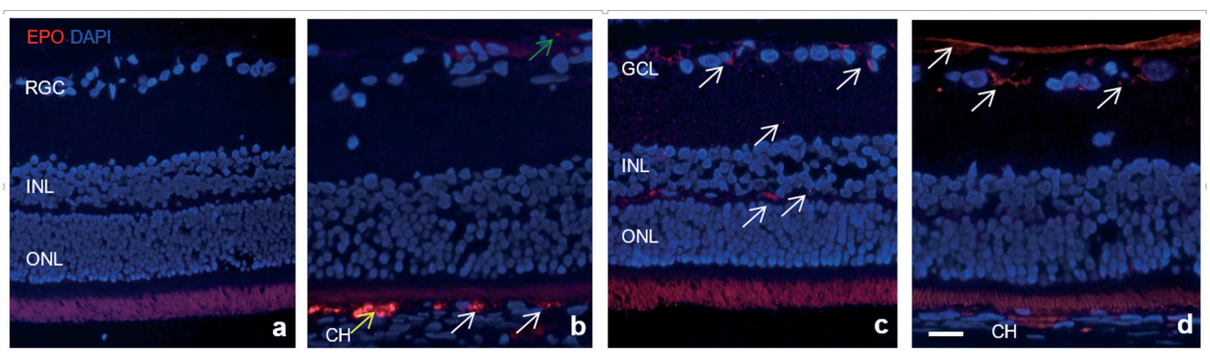


**Figure 30** - *Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; CH, Choroid.* ***a****: control group. In the control group, EPO was not detectable.* ***b****: at 12 hr after EPO administration the protein expression was more evident in the retinal pigment epithelium (white arrows) in spite of starting to appear in the RGC (green arrow). Note a blood vessel with erythrocytes cells (yellow arrow).* ***c****: at 24 hr after EPO administration, the protein was detected in all retinal cell layers.* ***d****: at 36 hr after EPO administration, the signal was more concentrated in the RGC layer (white arrows). Bar 30 µm.*

At 12 hours after EPO administration, the protein expression was more evident in the retinal pigment epithelium in spite of starting to appear in the RGC layer. At 24 hours after EPO administration, the protein was detected in all retinal cell layers. Thirty-six hours after EPO administration and till the

end of the study (60 hours), the signal was concentrated in the RGC layer (Figs. 30 to 33). The use of the anti-PGP9.5 antibody allowed the identification of neuronal cell bodies and processes. The presence of EPO protein, detected by immunohistochemistry, was strongly evident in the neuroretinal cells (Figs. 31 to 33). EPO was not detected in any of the control eyes.

In Fig. 30 b and d, the choroid is present and has been identified with the symbol CH. The choroid is not present in Fig. 30 a and c because during the slide process for immunohistochemistry the retinal detachments are frequent, especially during fixation.

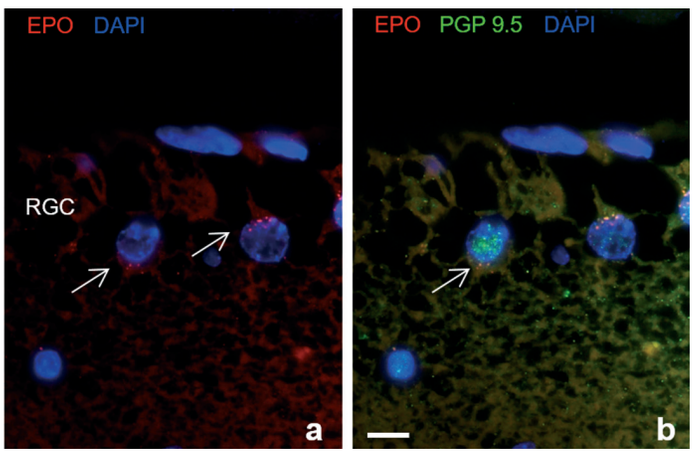


**Figure 31** - ***a****: Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer 60 hr after EPO administration.* ***b****: The same cells are contrasted with PGP 9.5 antibody (green), showing EPO inside a retinal ganglion cell. Bar 12 µm.*

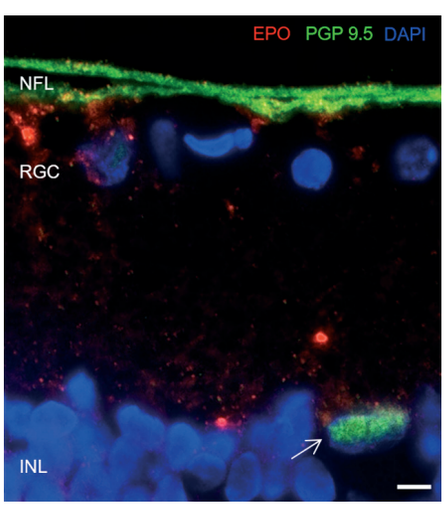


**Figure 32** - *Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and PGP 9.5 antibody (green) showing EPO in others neuroretinal cells, probably a bipolar or amacrine cell once is localized in inner nuclear layer (arrow). Note the strong signal in RGC layer and in nerve fiber layer 60 hr after EPO administration. NFL, nerve fiber layer; RGC, retinal ganglion cell layer; INL, inner nuclear layer. Bar 12 µm.*

### Haematocrit evaluation

Regarding haematocrit changes, the values varied from 54,8±2,9% on day 0 to 55,8±2,1% (p=0,456) on day 7, to 54,0±1,8% on day 14 (p=0,512), to 53,3±2,8% (p=0,246) on day 21 and to 53,2±2,5% on day 28 (p=0,215) showing no significant differences (Fig. 34).

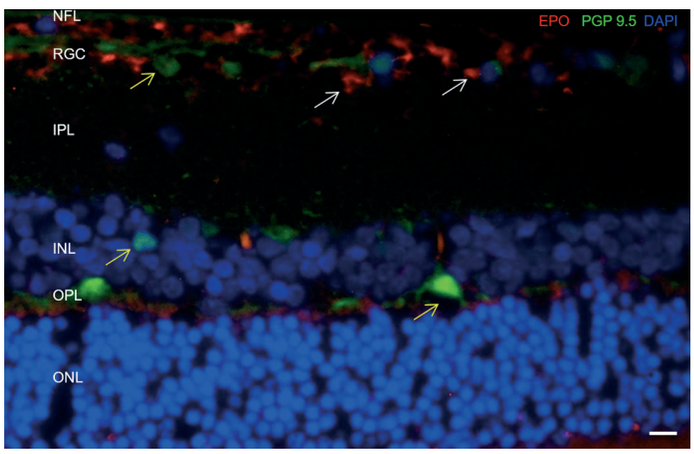


**Figure 33** - *Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and a higher concentration (1/200 ab72911; 1/200 ab6724) of PGP 9.5 antibody (green) 60 hr after EPO administration. PGP9.5 stains neurorretinal cells in the outer plexiform layer, inner nuclear layer and retinal ganglion cell layer (yellow arrows). Note the strong EPO signal in RGC layer, outer plexiform layer and in nerve fiber layer (white arrows). NFL, nerve fiber layer; RGC, retinal ganglion cell layer; INL, inner nuclear layer. Bar 25 µm.*

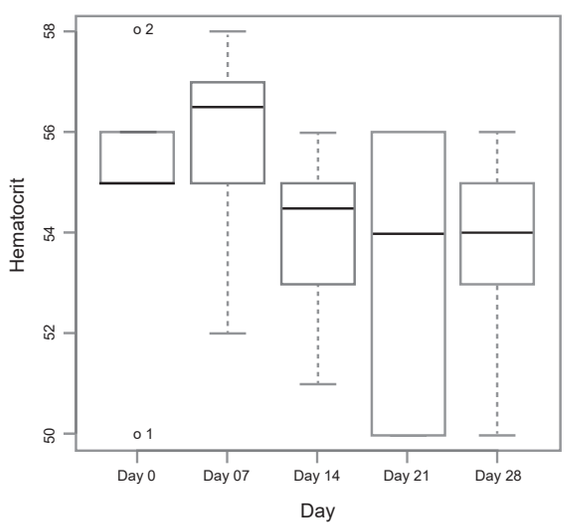


**Figure 34** - *Heamatocrit variation after subconjunctival EPO administration. In the x axis we represent Time in days after subconjunctival administration and in the y axis Heamatocrit levels in percentage. The values changed from 54,8±2,9% on day 0 to 55,8±2,1% (p=0,456) on day 7, to 54,0±1,8% on day 14 (p=0,512), to 53,3±2,8% (p=0,246) on day 21 and to 53,2±2,5% on day 28 (p=0,215) showing no significant differences*

## DISCUSSION AND CONCLUSIONS

Recently, EPO had been shown to have neuroprotective and neuroregenerative effects on retinal ganglion cells, apart from its erythropoietic properties, being a promissory therapeutic alternative on ischemic retinal diseases. *In vitro* studies with retinal pigment epithelium cells cultures (Chung *et al.*, 2009; Wang *et al.*, 2009) and rat retinal ganglion cells cultures (Weishaupt, 2004) incubated with EPO have demonstrated that EPO protected against oxidative damage, increased EPO, and EPO receptors expression, and prevented death of retinal ganglion cells. In the present study, we aimed to evaluate the possibility of using the subconjunctival route for EPO delivery to the eye.

To achieve therapeutic concentrations of EPO on the retina, all the previous studies, namely those on the protective effects of EPO retinal neurons, used systemic or intravitreal administration routes (Song *et al.*, 2008; Zhang *et al.*, 2008), both difficult to use in clinical practice. More recently, the retrobulbar injection was also used in this kind of studies (Zhong *et al.*, 2008). However, all of these EPO administration routes have more potential risks and side effects when compared to the subconjunctival injection (Geroski & Edelhauser, 2000). In humans, subconjunctival administration is a relatively easy, safe and quick procedure performed in ambulatory conditions, during the ophthalmic examination, under topical anaesthesia. There is no need for sedation, and there are very few risks or associated complications.

In this study, it was demonstrated that in the animal model used the subconjunctival administrated EPO histologically reach all retinal layers, specially the RGC layer. The presence of EPO was evident in different neuroretinal cells. Sixty hours after the administration there was still a strong EPO signal present in the retina of the studied animals.

The journey of the injected EPO involves crossing the choroidal blood flow, be transported across the RPE and through the various retinal layers to eventually be taken up by ganglion cells. Some EPO proteins can be inactivated by extracellular enzymes; others will be washed out by the choroidal blood flow, but some EPO definitely reached the neuroretina, as it can be concluded from the immunohistochemistry findings.

With regard to EPO potential systemic side-effects, subconjunctival administration did not cause any significant undesired side-effects on the haematocrit values.

Up to now, authors are not unanimous with regard to the therapeutic concentration of EPO in the retina. A study conducted by L. Zhong *et al.* (2007) concluded that a concentration of 6 IU/ml of EPO decreased apoptosis rates of the cultured retinal neurocytes suffering from glutamate-induced toxicity. Another study in adult rats found that 5-10 IU of EPO administered by intravitreal route protected the RCG and contributed to the partial regeneration of axons after optic nerve transection (King *et al.*, 2007). More recently, 0.6 IU/eye was defined as being the therapeutic dose in the rabbit retina with diabetic retinopathy (Zhang *et al.*, 2008). It is now imperative to carry out further studies to evaluate absorption and distribution of different EPO concentrations, in order to define the kinetics of this substance when administered by subconjunctival route.

In conclusion, after subconjunctival injection, EPO reached all the neuroretinal cell layers in the animal rat model tested. These findings prove that the subconjunctival administration is a possible alternative ocular delivery route for EPO. Further studies are necessary to assess the kinetics of subconjunctival administration of EPO, both in physiological conditions and ischemic ocular disease conditions.

The whole chapter was published in *Ophthalmic Research*
(Ophthalmic Res 2016; 56:104-110. doi: 10.1159/000444327)

CHAPTER 5

# OCULAR ERYTHROPOIETIN PENETRATION AFTER SUBCONJUNCTIVAL ADMINISTRATION IN GLAUCOMATOUS RATS

Ana Paula Resende[1], Berta São Braz[1] & Esmeralda Delgado[1]

1 Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal.

## ABSTRACT

**Purpose:** The present study aimed to determine whether the subconjunctival administration of recombinant erythropoietin (rHuEPO) reaches the retina in glaucoma conditions. After subconjunctival rHuEPO administration, in a rat glaucoma model, erythropoietin (EPO) distribution in the rat's retina was studied by immunohistochemistry.

**Methods:** Female Wistar Hannover albino rats (n=15) were divided into 2 groups, control (n=3) and treated (n=12). The animals' unilateral glaucoma was induced by coagulation of episcleral veins, under general anaesthesia. After vein coagulation, 1000 IU of rHuEPO were administered by the subconjunctival route to the treated group (n=12). The control group (n=3) received only a subconjunctival saline injection. The contralateral eye of each animal remained untouched. Treated group animals were euthanized at different time points, i.e. days 1, 3, 7 and 14. Bilateral enucleation was performed, and EPO distribution in the rat's retina was assessed by immunohistochemistry.

**Results:** Glaucoma was confirmed by results of repeated intraocular pressure measurements over the experimental period. In test group, EPO was identified in different neuroretinal cells, showing a stronger immunostaining signal during the first 2 time points in the retinal ganglion cells (RGC) layer. EPO protein was still present at day 14 after the subconjunctival injection. EPO was not detected in any of the control eyes or in any contralateral eye of the treated group.

**Conclusion:** When administered subconjunctivally in glaucomatous eyes, rHuEPO reached the RGC layer and was still present at least 14 days after administration. The subconjunctival route was shown to be a promising alternative for ocular EPO delivery in glaucomatous conditions in a rat animal model.

## INTRODUCTION

Glaucoma is one of the leading causes of irreversible blindness in the world (Quigley & Broman, 2006). Death of retinal ganglion cells (RGC), which results in the progressive loss of visual function, occurs in glaucoma and other ocular diseases caused by hypoxia and ischemia (Almasieh *et al.*, 2012; Laquis, Chaudhary, & Sharma, 1998). Although glaucoma is a multifactorial neurodegenerative disease, the only currently method of treatment is based on reduction of intraocular pressure (IOP) via medical, physical or surgical therapies (Almasieh, Zhou, Kelly, Casanova, & Di Polo, 2010). Considering the neurodegenerative pattern of the disease, it is of crucial importance to search for new therapeutic approaches that can contribute to maintain vision of patients (Cheung *et al.*, 2008).

Erythropoietin (EPO), commonly used to treat anaemia, is a natural cytokine hormone produced in the kidney that stimulates red blood cell production by inhibiting apoptosis in erythrocyte progenitors. Besides its haematopoietic effect, EPO has revealed neuroprotective properties on the central nervous system (Bartesaghi *et al.*, 2005; Grasso *et al.*, 2007b; Kumral *et al.*, 2006; Sättler *et al.*, 2004). Recent studies involving EPO in glaucoma have yielded very promising results (Song *et al.*, 2008; Tsai *et al.*, 2005, 2007). Due to its neuroprotective and neuroregenerative properties, EPO has proven to be a valuable drug in the prevention of and decrease in RGC apoptosis, preserving visual function in animal models of retinal diseases (Zhang *et al.*, 2008; Luo *et al.*, 2015). The feasibility of intravitreal EPO injection was also assessed in human patients (Lagrèze *et al.*, 2009).

In all these studies, systemic, intravitreal, or retrobulbar EPO administration has been used to reach the desired concentrations in the posterior ocular segment at the retinal level (Zhang *et al.*, 2008b; Zhong *et al.*, 2008). Systemic administration induces unwanted side effects related to haematopoiesis stimulation, and intravitreal or retrobulbar administrations are invasive procedures that demand general or local anaesthesia of the patient and can induce several complications such as endophthalmitis, retinal detachment, vitreitis, retinitis, choroiditis or cataracts (Geroski & Edelhauser, 2000).

In a recent work we have demonstrated that EPO reached RGC layer when administered subconjunctivally in physiological conditions. Furthermore, we have also proven that EPO did not cause any significant haematopoietic side effects when administered by this route (Resende *et al.*, 2013).

In the present study, we intend to assess the possibility of using the subconjunctival route for ocular EPO administration in glaucoma conditions in a rat model.

## MATERIALS AND METHODS

### Animals

Animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and respecting the guidelines of the Faculty of Veterinary Medicine – University of Lisbon Ethical Committee.

Female Wistar Hannover albino rats (n=15) weighting 268,0 ± 20,7g were included in the study. The rats were housed in boxes of type III measuring 1195 $cm^2$, with water and food ad libitum and maintained in controlled conditions of temperature (20± 2ºC), humidity (≈70%) and cyclic light (12 hours light/12 hours darkness). All animals included in the study underwent a complete ophthalmic examination, including an anterior segment examination performed by slit-lamp biomicroscopy (Kowa SL-15, Tokyo, Japan), intraocular pressure measurement using rebound tonometry (Tonolab, Icare®, Helsinki, Finland) and posterior segment examination with binocular indirect ophthalmoscopy (Heine Omega 500, Herrsching, Germany). All animals were considered normal due to their ophthalmic examination.

Unilateral glaucoma was induced in the right eye (operated eye) of each animal. The animals were divided into 2 groups: a control (n=3) and a treated group (n=12). For the control group, the animals received a subconjunctival saline injection. For the treated group, the animals received a subconjunctival injection of 1000 IU of recombinant human EPO (rHuEPO). The contralateral eye (left eye) remained untouched in all animals. Treated group animals were euthanized at different time points after glaucoma induction (day 0), day 1 (n=3), day 3 (n=3), day 7 (n=3) and day 14 (n=3), and the control group remained alive until the end of the study (day 14). Bilateral enucleation was performed in all animals, and EPO distribution in any rat's retina was assessed by immunohistochemistry.

### Intra-ocular pressure measurement

IOP was measured, without mydriasis induction, using a rebound tonometer (Tonolab, Icare®, Helsinki, Finland) on day 0, before and one hour after glaucoma induction, under general anaesthesia. The measurement was repeated under awake conditions on day 1, 3, 7 and 14 for both eyes of the animals.

## Surgical procedures

### *Anaesthesia*

All animals underwent a single general anaesthesia on day 0 to perform: IOP measurement, glaucoma induction, subconjunctival rHuEPO administration (tested group) or subconjunctival saline solution administration (control group) and again IOP measurement (1 hour after surgery). Anaesthesia was performed by intraperitoneal (i.p.) injection of 75 mg ketamine/Kg b.w. (Imalgene 1000®, Merial Portuguesa, Rio de Mouro, Portugal) plus 1 mg medetomidine/Kg b.w. (Domitor®, Orion Corporation, Espoo, Finland).

### *Glaucoma induction and subconjunctival EPO injections*

Under general anaesthesia, glaucoma was induced by coagulation of three episcleral veins to the right eye (operated eye) of each animal, using a surgical technique described by Shareef *et al.* (1995). After veins coagulation, 1000 IU of rHuEPO (NeoRecormon 5000®, Roche Diagnostics GmbH, Manheim, Germany) was administered by a subconjunctival injection to the treated group and an equal volume of saline solution was administered by the same route to the control group. For these procedures a surgical microscope (Zeiss Opmi Visu Series/S7 Microscope, Munich, Germany) for magnification and the coagulation device from the phacoemulsification system (Laureate, Alcon Laboratories, California, U.S.A) for haemostasis were used.

## Euthanasia and enucleation

Treated group animals were euthanized according to the desired end point, i.e. on day 1 (n=3), day 3 (n=3), day 7 (n=3) and day 14 (n=3), and the control group animals were euthanized at the end of the study, on day 14 (n=3).

Euthanasia was performed by administration of an overdose of pentobarbital sodium (60 mg/kg b.w.) by the i.p. route. 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.

## Immunohistochemistry

All eyes were processed by paraffin embedding, and longitudinal sequential sections (3 µm) were cut through the globe along the anterior-posterior axis. Three retinal sections per eye (adjacent to the optic nerve head) were deparaffinised, rehydrated, rinsed in PBS solution for 30 min, treated with a 10% Triton X-100 solution for another 30 minutes and then washed twice in PBS solution for 5 minutes. Then, sections were incubated with a blocking

solution (10% normal donkey serum in PBS) for 30 minutes at room temperature followed by overnight incubation with polyclonal goat anti-EPO antibody (1:100; sc-1310, Santa Cruz Biotechnology) and monoclonal mouse neuron cytoplasmic protein 9.5 antibody (anti-PGP 9.5; 1:500; ab72911, Abcam) at 4ºC. A sample without the primary antibody was used as negative control. A bovine foetal kidney section was used as a positive EPO immunostaining control. The following day, sections were washed in PBS three times for 5 minutes and incubated with rhodamine-conjugated secondary antibody (1:100; sc-2094, Santa Cruz Biotechnology) and a FITC-conjugated secondary antibody (1:200; ab6724, Abcam), for 1 hour, in a dark room. The sections were washed again with PBS and prepared with a mounting medium containing a cell nucleus marker, DAPI (UltraCruzTM Mounting Medium; sc-24941, Santa Cruz Biotechnology). The retinal sections were assessed by fluorescent microscopy (Leica DM R, Leica Microsystems, Bensheim, Germany).

### Statistical data

Data were analysed with GraphPad (InStat version 3.10 for Windows, GraphPad Software, USA) and Excel (Microsoft, USA), results being reported as mean ± standard error of mean. For statistical analysis, Student's t-test for paired comparisons was used with $p<0.05$ testing for significance.

## RESULTS

### IOP measurements

The glaucoma condition was assessed in the right eye in all animals by measurement of IOP in two different conditions: under general anaesthesia on day 0, before and one hour after surgery *(*Fig. 35), and in awake conditions on days 1, 3, 7 and 14 (Fig. 36).

The IOP values (mean ± standard error of mean) are shown in tables 7 and 8. Apart from the first measurement before coagulation of episcleral veins (baseline), IOP measurement values showed a statistically significant increase in the operated eye (right eye) in comparison to the contralateral eye (left eye) for both groups during the entire study. However, this difference between both eyes decreased with time but remained statistically significant on day 14.

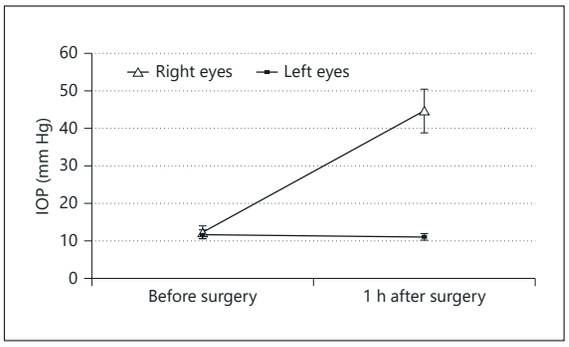


**Figure 35** - *IOP variation under general anaesthesia on day 0, before and one hour after coagulation of episcleral veins. The x-axis represents two time points: A- before and B- one hour after the surgery and in the y-axis we represent IOP values in mm Hg (n=15).*

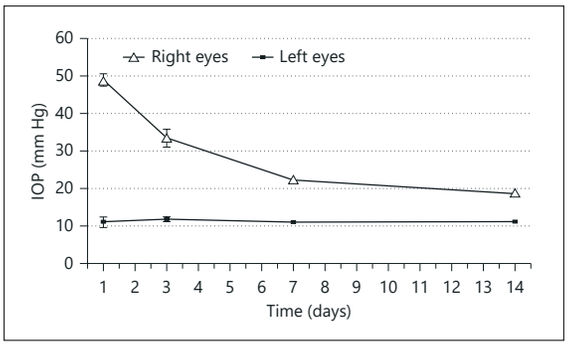


**Figure 36** - *IOP variation during the study. The x-axis represents time in days and the y-axis the IOP values in mm Hg. Day 1 (n=15), day 3 (n=12), day 7 (n=9) and day 14 (n=6) represents the number of days after the glaucoma induction.*

| Table 7<br>IOP under general anaesthesia (mm Hg) | | |
|---|---|---|
| | Baseline<br>n=15 | 1 hour after surgery<br>n=15 |
| Right eyes | 12.3 ± 3.4 | 44.1 ± 11.8 |
| Left eyes | 11.6 ± 2.7 | 11.0 ± 1.8 |
| p | 0.7394 | 0.0049 |

| Table 8<br>IOP in awake condition (mm Hg) | | | | |
|---|---|---|---|---|
| | Day 1<br>n=15 | Day 3<br>n=12 | Day 7<br>n=9 | Day 14<br>n=6 |
| Right eyes | 48.4 ± 4.8 | 33.3 ± 10.8 | 22.2 ± 7.2 | 18.5 ± 2.1 |
| Left eyes | 11.0 ± 1.3 | 11.8 ± 1.6 | 11.2 ± 1.0 | 11.0 ± 0.9 |
| p | 0.0046 | 0.0049 | 0.0046 | 0.0046 |

### Immunohistochemistry observations

The immunostained retinal sections were blindly analysed by the same investigator (Resende) using fluorescent microscopy.

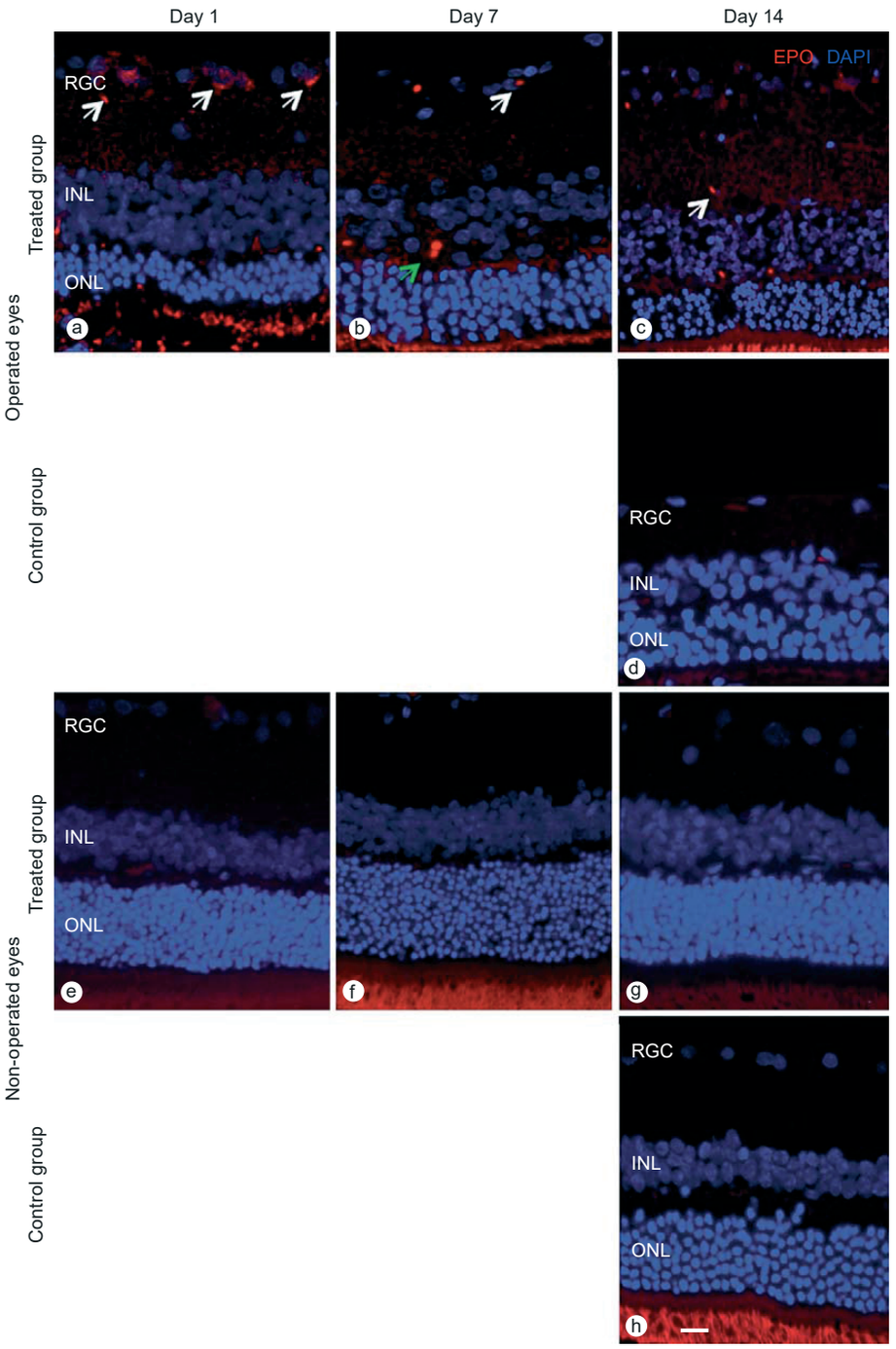


**Figure 37** - *Immunohistochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Operated (right) eyes:* ***a.*** *one day after glaucoma induction and rHuEPO administration, the protein expression was more evident in the RGC layer (white arrows).* ***b.*** *Seven days after glaucoma induction and rHuEPO administration, the protein was still detected in retinal ganglion cells layer (white arrow) and was also detectable in the inner nuclear layer (green arrow).* ***c.*** *Fourteen days after glaucoma induction and rHuEPO administration, the signal was residual but still present in all retinal cell layers.* ***d.*** *Retinal sections fourteen days after glaucoma induction in a non-treated animal (saline solution). Note the significant decrease of retinal thickness. Non-operated (left) eyes:* ***e****,* ***f****,* ***g*** *and* ***h.*** *Bar 25 µm*

All operated eyes (right eye) showed a retinal thickness decrease associated with a lack of several nuclei, especially at the level of the outer nuclear layer during the entire study (Fig. 37 a-d). This finding was more prominent on day 14 in both treated (Fig. 37 c) and control groups (Fig. 37 d) when compared with contralateral eyes (left eye) at the same end points (Fig. 37 e-h). In contralateral eyes and for both groups, retinal thickness and cell density did not yield any difference at each end point (Fig. 37 e-h). However, when we compared the operated eyes according to groups and focused on retinal thickness and cell density, the treated group (Fig. 37 c) showed fewer retinal signs of retinal atrophy than the control group (Fig. 37 d).

EPO protein was detected in all retinal cell layers only in operated eyes that received a 1000 IU of rHuEPO as a subconjunctival injection (Fig. 37 a-c). A higher EPO immunostaining signal was detected on day 1 (Fig. 37 a), and the signal progressively decreased to the end of the study (Fig. 37 b-c). The presence of EPO protein, detected by immunohistochemistry, was strongly evident inside RGC bodies, shown in figures 38 and 39 a, as confirmed by anti-PGP 9.5 antibody, a neuronal cytoplasm marker that allows identification of RGC bodies (Fig. 39 b).

No EPO protein was found in the contralateral eye in any of the experimental groups (Fig. 37 e-h).

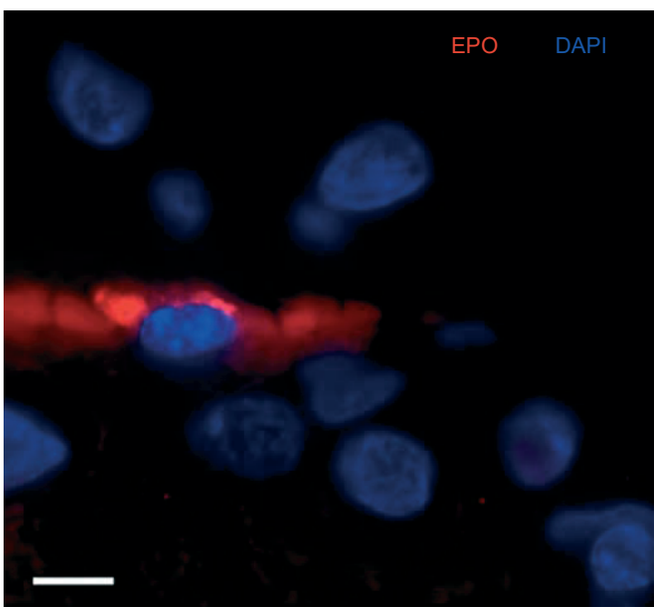


**Figure 38** - *Immunohistochemistry for cell nucleus marker of the retina (DAPI, blue) and EPO antibody (red). Seven days after glaucoma induction and rHuEPO administration a detail of EPO expression located at the RGC layer is shown. Bar 10 µm*

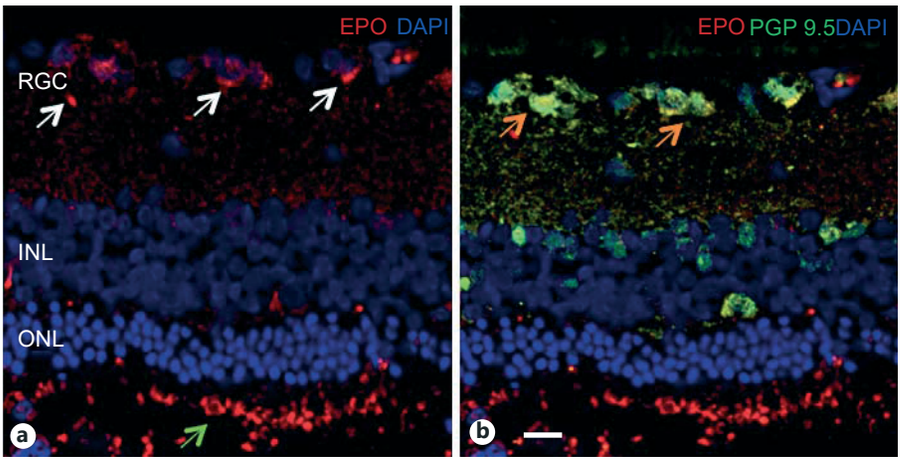


**Figure 39** - *Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and neuronal cell cytoplasm marker (PGP 9.5 antibody, green). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.* ***a.*** *One day after glaucoma induction and rHuEPO administration, EPO protein signal was strongly evident in RGC layer (white arrows). Due to the high intraocular pressure and the hemato-retinal barrier breakdown, erythrocytes are present and overspread on ONL (green arrow).* ***b.*** *The use of the anti-PGP9.5 antibody allowed the identification of neuronal cell bodies (orange arrows). Bar 25 µm.*

## DISCUSSION AND CONCLUSIONS

Recently, EPO has revealed neuroprotective properties concerning the retina, besides its haematopoietic effect (Song *et al.*, 2008; Tsai *et al.*, 2005, 2007; Zhang *et al.*, 2008). EPO acts in the retina in different ways, including increasing resistance to inflammation, oxide-induced damage, ischemia, degeneration and permeability (Bond & Rex, 2014; Luo *et al.*, 2015; Zhang, Wang, Nie, & Wang, 2014). Concerning RGC, especially affected in glaucoma, 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 (Luo *et al.*, 2015). Another study demonstrated that one of the protective mechanisms of EPO for the injured retina caused by chronic intraocular hypertension is via the HIF-1\iNOS signal conduction pathway. EPO inhibits the activation of HIF-1α by negative feedback, then inhibits the transcription of iNOS to avoid neurotoxicity caused by oversynthesis of iNOS (Gui, Yang, Li, & Gao, 2011). For these reasons, EPO is actually considered a promising neuroprotective agent in glaucoma.

The aim of the present study was to evaluate whether subconjunctival rHuEPO administration could reach the retina in glaucomatous conditions in an animal model.

Rodent models of elevated IOP can either occur spontaneously or be produced experimentally. In our study, we used a glaucoma model induced by coagulation of three episcleral veins using a surgical technique previously described by others (Shareef *et al.*, 1995). This experimental model has already been used (Danias *et al.*, 2006; Doh, Kim, Lee, Park, & Park, 2010; Grozdanic *et al.*, 2003) and has been shown to be reliable and reproducible. However, as previously described (Shareef *et al.*, 1995), this model produced a temporary elevation of IOP during some weeks only. Spontaneous glaucoma models, like genetic models, have the disadvantage that pressure elevation is usually bilateral, and, since it is not possible to perform continuous IOP monitoring, the exact level and duration of pressure elevation are unknown (Morrison, Cepurna Ying Guo, & Johnson, 2011). These models are also more expensive.

The IOP raise was confirmed in two different conditions: under general anaesthesia, before and one hour after the surgical procedure, and also without general anaesthesia, during the rest of the study. In both conditions, the differences between IOP values of the operated and the contralateral eyes were statistically significant.

Concerning the dosage of rHuEPO administered in this study, it was chosen according to a previous study showing that 1000 IU rHuEPO administered by retrobulbar injection in rats protected RGC from acute elevated IOP (Zhong *et al.*, 2008).

Using immunohistochemistry, we have demonstrated that rHuEPO reached all retinal layers in glaucoma conditions, including the RGC layer, where it might exert its anti-apoptotic effects. Additionally, the EPO immunostaining signal was stronger in glaucomatous eyes when compared to physiological eyes after subconjunctival rHuEPO administration (Resende *et al.*, 2013). This was probably due to the ocular blood barrier breakdown that followed the rise in IOP, including blood-retinal barrier breakdown, allowing for a more intense crossover of EPO protein.

One day after the surgical procedure, IOPs reached their maximum values, and we believe this is the reason why the photoreceptor layer appears to be the most affected. At the same time, this could explain the maximal EPO immunostaining signal (Fig. 37 a). At this time point, we experienced additional technical problems with the histological sections, probably due to retinal oedema and congestion.

Fourteen days after glaucoma induction, all operated eyes showed retinal atrophy. However, when we compared the treated group (rHuEPO) with the control group (saline solution), the animals that received rHuEPO showed a thickest retina. Since our goal was not to study the effect of EPO as a neuroprotector agent and we had a small number of animals at this time point (n=3), we did not statistically analyse this parameter, but it would be interesting to do it in future studies.

This is the first study that used subconjunctival rHuEPO administration to reach the retina in glaucoma conditions, but other authors have already demonstrated that retrobulbar EPO administration could protect RGC from acute elevated IOP (Zhong *et al.*, 2008), which supports

the idea that EPO protein, when administered locally, can cross the main ocular barriers and reach retinal cells.

At the end of the study (day 14), EPO was still present in the retina, although its immunostaining signal was residual, which demonstrates that this protein stayed in retinal cell layers for a long time. This might be an important factor to consider in treatment of degenerative retinal diseases that uses EPO as an anti-apoptotic agent (King *et al.*, 2007; Y. Zhong *et al.*, 2007).

Apparently glaucoma induction and subsequent blood barrier breakdown did not induce the appearance of endogenous EPO in the retinas of the control group, since the polyclonal rabbit anti-EPO antibody used is recommended for EPO detection of mouse, rat and human origin. For that reason, we used the term “rHuEPO” to describe our administration and “EPO” to describe immunohistochemistry observations, since we were not able to differentiate them in retinal sections.

In conclusion, we have demonstrated that subconjunctival rHuEPO administration reached all retinal layers in glaucomatous animals, which shows that this is a promising alternative route for ocular EPO delivery in this disease. However, more studies should be performed to assess both the kinetics and the morphology of the retina, including quantification of viable RGC, and physiological effects of EPO when administered by subconjunctival route in glaucoma conditions.

The whole chapter was published in *Biomedicine Hub*
(Biomed Hub 2018; 3:488970. doi: 10.1159/000488970)

CHAPTER 6

# FUNCTIONAL AND STRUCTURAL EFFECTS OF ERYTHROPOIETIN SUBCONJUNCTIVAL ADMINISTRATION IN GLAUCOMATOUS ANIMALS

A.P. Resende[1], S. G. Rosolen[2,3], T. Nunes[1], B. São Braz[1], E. Delgado[1]

1 CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa
2 Sorbonne Universités, UPMC Univ Paris 06, UMR_S 968, Institut de la Vision, Paris, France
3 Clinique Veterinaire Voltaire, Asnières sur Seine, France

## 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 in 2 groups of 13 animals each: a treated group (TG) receiving a unique subconjunctival injection of 1000 IU of EPO and a control group (CG) 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 1500 µm (RT2) from the optic nerve (NO).

**Results:** Retinal function evaluation: for both scotopic and photopic conditions, ERG wave amplitudes increased in TG. 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 TG.

**Conclusion:** Subconjunctival 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 (Quigley, 2005). It is considered a progressive neurodegenerative eye disorder characterized in earlier stages by the degeneration and loss of retinal ganglion cells (RGC) and their axons, leading to visual field loss (Cheung *et al.*, 2008; Tian, Shibata-Germanos, Pahlitzsch & Cordeiro, 2015). Presently, the available treatment strategies aim to reduce intraocular pressure (IOP) through medical, laser or surgical methods (Almasieh *et al.*, 2010). Since the loss of function and death of RGC is mainly by programmed cell death (apoptosis) (Garcia-Valenzuela *et al.,* 1995; Guo *et al.*, 2005), it is crucial the development of neuroprotective therapeutic approaches for those cells. Erythropoietin (EPO) is a natural glycoprotein hormone, conventionally thought to be responsible only for producing red blood cells in our body. Additionally to its haematopoietic effect, this cytokine has demonstrated to have neuroprotective and neuroregenerative properties in the central nervous system (Grasso *et al.*, 2007b; Kumral *et al.*, 2006; Maiese, Chong, Shang & Wang, 2012; Sättler *et al.*, 2004). Many pre-clinical 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 (Shirley Ding *et al.*, 2016). EPO has proven to prevent RGC apoptosis, preserving visual function in several glaucoma models (Luo *et al.*, 2015; Shirley Ding *et al.*, 2016; L. Zhong *et al.*, 2007) with very promising results (Song *et al.*, 2008; Tsai *et al.*, 2005).

Previously, different ocular administration routes have been tested to achieve EPO therapeutic effects in the retina, namely systemic, intravitreal and retrobulbar routes (Lagrèze *et al.,* 2009; Zhang *et al.*, 2008; Zhong *et al.*, 2008). The systemic route may cause an undesired secondary effect, the increase in haematopoiesis, while the intravitreous and retrobulbar routes may lead to ocular complications, such as chorioretinitis, retinal detachments, cataracts, vitreitis or even endophthalmitis (Geroski & Edelhauser, 2000; Ranta & Urtti, 2006).

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 (Resende *et al.*, 2013, 2016). The subconjunctival route for EPO ocular administration demonstrated to be a safe and easy procedure with few risks associated (Ambati *et al.*, 2000; Ranta & Urtti, 2006).

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, weighting 247± 28g and 12 males, weighting 378± 34g. Animals were housed in boxes type III (1195 $cm^2$) with water and food ad libitum and maintained in controlled conditions of temperature (20± 2ºC), humidity (≈70%) and cyclic light (12 hours light/12 hours 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 one: 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 recombinant human EPO (rHuEPO) while the control groups received only saline. The contra-lateral eye (left eye) remained untouched in all animals.

### Anaesthesia

Anaesthesia was performed by intraperitoneal (i.p.) administration of 75 mg ketamine / Kg 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 anaesthesia, 1000 IU of rHuEPO (NeoRecormon 5000®, Roche Diagnostics GmbH, Manheim, 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.* (1995). For these procedures a surgical microscope (Zeiss Opmi Visu Series/S7 Microscope, Munich, Germany) and the coagulation device from the phacoemulsification system (Laureate, Alcon Laboratories, California, U.S.A) for haemostasis were used. The two inclusion criteria for the animal in the suty were postexperimental glaucoma induction high IOP and non-measurable ERG traces on day 7 in their right eyes.

### IOP measurements

Intraocular pressure measurement was performed under general anaesthesia to all groups, on both eyes, before and one hour 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 (Rosolen, Rigaudiere, Le Gargasson & Brigell, 2005) for functional evaluation. Under general anaesthesia, binocular full-field (Ganzfeld) ERGs were recorded before, at 7 days and 21 days after glaucoma induction. After 12 hours 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/$m^2$). In photopic settings, cone function was tested by stimulating the retina with flashes (0.98 log cds/$m^2$) and 6.2 Hz flicker. ERG results were acquired for both eyes, at the same time. The left eyes' traces were used as 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, 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 hours 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 analysed and assessed by optical microscopy (Olympus BX51 microscope and a DP21 Digital Camera). Retinal thickness was measured at 500 µm (Fig. 40) and at 1500 µm, dorsally and ventrally from the optic nerve (ON) head in all retinal sections. At 1500 µm from ON the mean of 3 measures were considered for statistical analysis (Fig. 41).

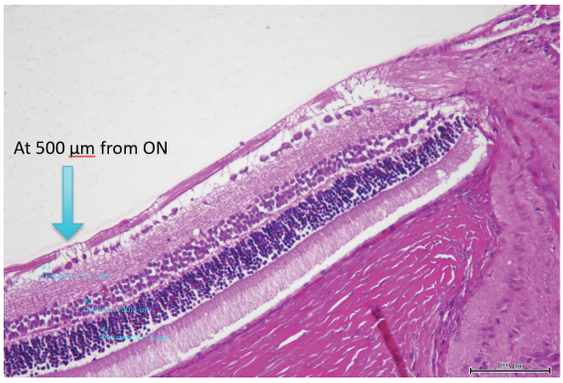


**Figure 40** - *H&E staining of rats' retinas at the optic nerve level. At the distance of 500 µm from the optic nerve (ON) (red trace) ventral and dorsal retinal thickness were measured.*

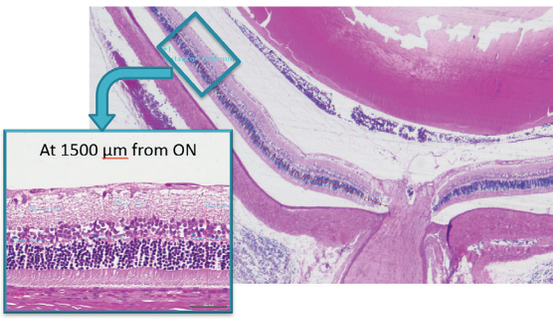


**Figure 41** - *H&E staining of rats' retinas at the optic nerve level. The distance of 1500 µm were measured from the optic nerve (ON) (red trace) and three measurements were done in the same visual field.*

### Data analysis

Data were analysed with GraphPad (InStat version 3.10 for Windows, GraphPad Software, USA), Excel (Microsoft, USA) and R 3.3.2 (R Core, Vienna, Austria) (R Core Team, 2016), results are reported as mean ± standard error for variables with a normal distribution, and as median [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 retinal thickness between test and control groups. Homogeneity of variances was verified by Fisher's F-test.

## RESULTS

### IOP values

Results of EPO measurements are presented on figure 42. Before the coagulation of the episcleral veins (n=26), median IOP values on right eyes and left eyes were 10.27±1.53 mmHg and 10.06±1.09 mmHg, respectively. One hour after coagulation of the episcleral veins (n=26), the median IOP was 56.65±11.21 mmHg on right eyes and 10.52±1.34 mm Hg on left eyes ($p<0.001$). IOP values showed a significant statistical difference between the right eyes and the left eyes after surgery.

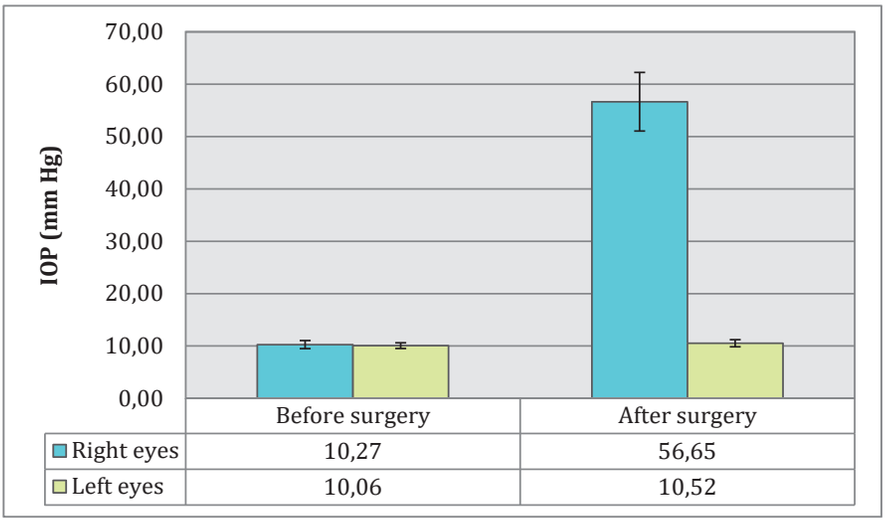


**Figure 42** - *IOP variation before and 1 hour after coagulation of 3 episcleral veins (n=26) with a significant increase on right eyes that underwent surgery.*

### Electroretinography evaluation

The ERG results are presented in table 9. At the end of the study (day 21), ERG wave amplitudes increased in TG in both scotopic and photopic conditions. In scotopic conditions, the b-wave amplitude (median [min;max]) of the TG were 54.3 [21.5; 271.0] µV on day 7 and 189.0 [106.0; 319.0] µV on day 21 and the CG had a median value of 97.4 [7.0; 158.0] µV on day 7 and 182.0 [44.0;259.0] µV on day 21 (p=0,79). In photopic conditions, b-wave amplitude for flashes examination of the TG were 0.0 [0.0; 0.0] µV on day 7 and 73.4 [55.9; 101.0] µV on day 21 and the CG had a median value of 0.0 [0.0; 45.3] µV on day 7 and 47.8 [38.3; 60.1] µV on day 21 (p=0,006). In flicker examination, the TG had a median value of 17.0 [3.1; 49.6] µV on day 7 and 60.9 [30.4; 85.9] µV on day 21 and the CG had a median value of 33.5 [10.0; 82.2] µV on day 7 and 42.4 [26.5;76.5] µV on day 21 (p=0,02).

| Amplitude of photopic flash waves in μV | | | |
|---|---|---|---|
| Group | Day 0 | Day 7 | Day 21 |
| Treated | 171.0 [112.0; 219.0] | 0.0 [0.0; 0.0] | 73.4 [55.9; 101.0]* |
| Control | 149.0 [108.0; 182.0] | 0.0 [0.0; 45.3] | 47.8 [38.3; 60.1] |

| Amplitude of photopic flicker waves in μV | | | |
|---|---|---|---|
| Group | Day 0 | Day 7 | Day 21 |
| Treated | 125.5 [102.0; 172.0] | 17.0 [3.1; 49.6] | 60.9 [30.4; 85.9] * |
| Control | 138.0 [79.8; 207.0] | 33.5 [10.0; 82.2] | 42.4 [26.5; 76.5] |

| Amplitude of scotopic rod peak waves in μV | | | |
|---|---|---|---|
| Group | Day 0 | Day 7 | Day 21 |
| Treated | 478.5 [373.0; 641.0] | 54.3 [21.5; 271.0] | 189.0 [106.0; 319.0] |
| Control | 440.0 [260.0; 836.0] | 97.4 [7.0; 158.0] | 182.0 [44.0; 259.0] |

**Table 9** - *Amplitude of b-waves in photopic flash, photopic flicker and scotopic examinations of the treated and control groups, at day 0, day 7 and day 21. Results are reported as median [min;max] (* p< 0,05).*

These differences were statistically significant in photopic conditions for flash and flicker examination. The presented results concern the right eyes examinations. The results for the left eyes were within normal values. Figure 43 is a representative ERG obtained in photopic luminance conditions: flash (Fig. 43 A) and flicker (Fig. 43 B), and scotopic luminance conditions (Fig. 43 C) in an animal from the treated group and in another animal from the control group.

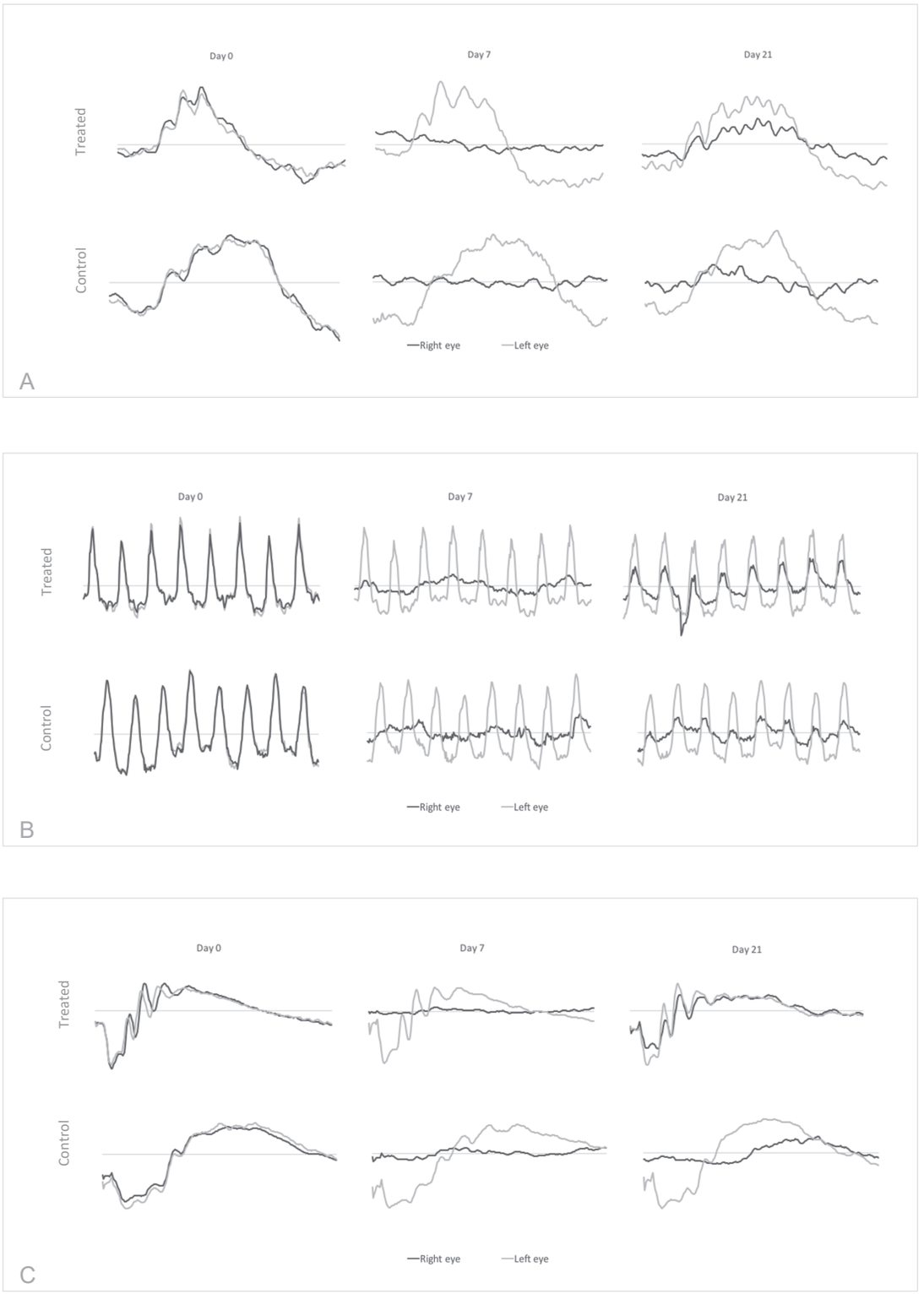


**Figure 43** - *Representative ERG obtained in photopic luminance conditions: flash (A) and flicker (B), and scotopic luminance conditions (C) in an animal from the treated group and in another animal from the control group. In each trace the right eye (operated eye) is represented in black and the left eye (non operated eye) is represented in grey. For each graphic is shown the curves obtained on day 0 (before surgery), day 7 (after surgery) and finally on day 21 (at the end of the study).*

### Retinal thickness evaluation

Retinal thickness results are presented on table 8. At 500 µm from the ON, RT measurements were 206,3±14,8 µm in TG and 176,9±20,1 µm in CG (p=0.004). At 1500 µm from the ON, they corresponded to 175,9±21,2 µm in TG and 154,0±15,4 µm in CG (p=0.02) (Fig. 44). When compared with the CG, the TG presented thicker retinas and these differences were statistically significant (Fig. 45).

| Retinal thickness | 500 µm from the ON | 1500 µm from the ON |
|---|---|---|
| Treated group | 206,3 ± 14,8* | 175,9 ± 21,2* |
| Control group | 176,9 ± 20,1 | 154,0 ± 15,4 |

**Table 10** - *Retinal thickness of the treated and control groups at 500 µm and 1500 µm from the optic nerve (ON). Results are reported in µm as median ± standard error (* p< 0,05).*

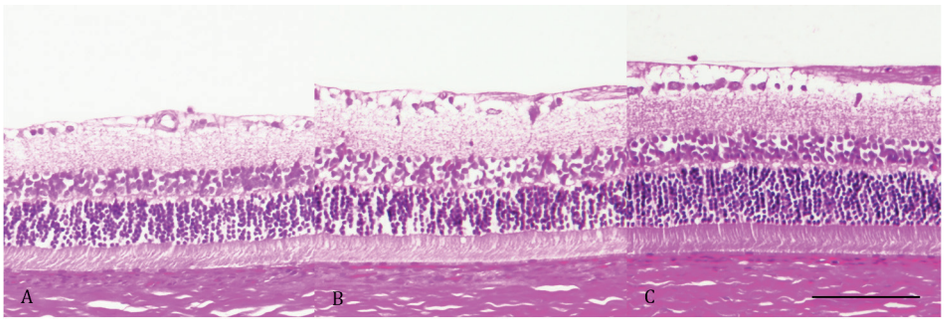


**Figure 44** - *Rats retinas measured at 1500 µm from the optic nerve. Retinas from the glaucomatous eyes (right eye): control group (A) and treated group (B) and a normal retina from a non-glaucomatous eye (left eye) (C) (H&E; Scale bar: 100 µm).*

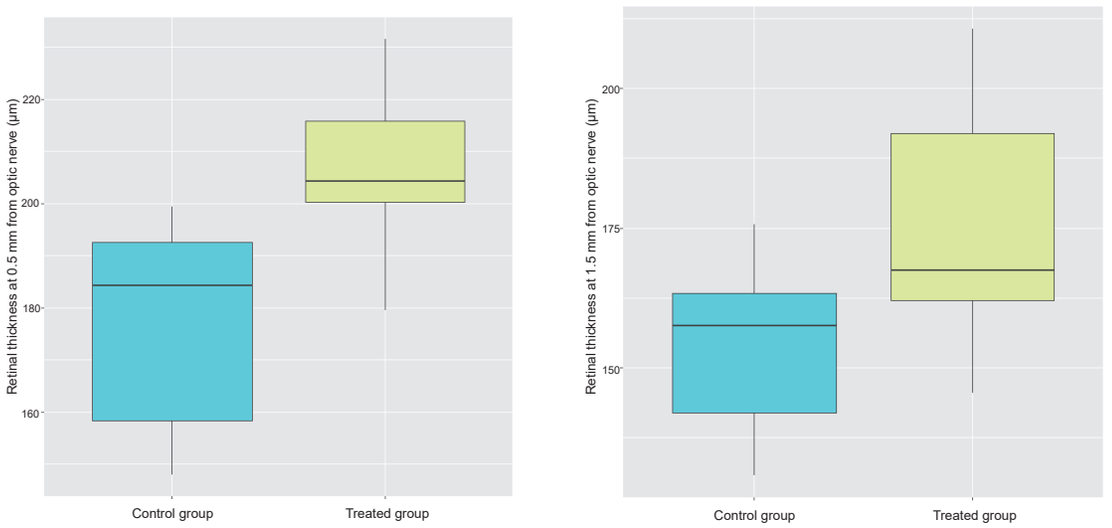


**Figure 45** – *Retinal thickness at 500 µm and 1,500 µm from the optic nerve head, on the control and treated groups. Bars represent median score; boxes include 25th to 75th percentile; whiskers represent ±1.58 Interquartile range/sqrt (n).*

## DISCUSSION AND CONCLUSIONS

Recently, EPO has revealed neuroprotective properties on the retina, in addition to its haematopoietic effect (Aghdam, Sanjari & Falavarjani, 2016; Shirley Ding *et al.*, 2016). Several investigations addressed EPO properties to mediate protection against retinal damage through different pathways, including increasing resistance to inflammation, oxide-induced damage, ischemia, degeneration and permeability (Bond & Rex, 2014; Luo *et al.*, 2015). Concerning RGC, 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 (Luo *et al.*, 2015). Another study demonstrated that one of the protecting mechanisms of EPO to the injuries that retina suffer caused by chronic intraocular hypertension is through HIF-1\iNOS 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 (Dong-Mei, Yang, Li & Gao, 2011). For these reasons, EPO is actually considered a promising neuroprotective agent in glaucoma.

With encouraging results of neuroprotection, 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 (Shirley Ding *et al.*, 2016). Apart from formal clinical trials, EPO therapy using intravitreal injections has shown promise in several other retinal diseases (Shirley Ding *et al.*, 2016).

Drug delivery and pharmacokinetics play important roles in current retinal therapeutics and the development of new medications (Del Amo *et al.*, 2017). The subconjunctival route for EPO ocular administration is an easy and safe procedure with minimal risks associated and without significant unwanted side effects related with haematopoiesis stimulation. The main ocular barrier to subconjuncival administrations are flow barriers (elimination to blood flow and lymphatic flow) and penetration barriers (Del Amo *et al.*, 2017), 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.* (2000) 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 molecular radius. We have previously demonstrated that rHuEPO can permeate porcine conjunctiva, sclera and cornea in an *ex vivo* model (Resende *et al.*, 2017). Since glaucoma is a chronic disease, multiple treatments to protect RGC 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 (Resende *et al.*, 2013, 2016).

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 retinal thickness 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 retinal thickness.

Our glaucoma experimental model has been used before and showed to be reliable and reproducible (Danias *et al.*, 2006; Grozdanic, Matic, Betts, Sakaguchi & Kardon, 2007; Doh *et al.*, 2010). In the present study the IOP rise was confirmed one hour 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. At the end of the study (day 21), ERG results showed a better recovery of the treated group when compared to the non-treated group. In photopic examinations, both flash and flicker b-wave results showed a statistically 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. Retinal thickness measurements at the optic nerve 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 (Danias *et al.*, 2006; Grozdanic *et al.*, 2007; Doh *et al.*, 2010), 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 (Hoon *et al.*, 2014), our findings suggest a neuroprotective effect of subconjunctival rHuEPO injection on the retinas of albino rats with induced glaucoma.

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

CHAPTER 7

# GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

## GENERAL DISCUSSION

Glaucoma is the leading cause of irreversible blindness with approximately 66.8 million people affected worldwide. In 2020 this number is expected to increase to 79.6 million because of both demographic expansion and population aging (Quigley & Broman, 2006). In veterinary medicine, glaucoma has a more rapid progression than in humans and represents a common and frustrating eye disease with a similar outcome, blindness (Maggio, 2015). Both in humans and animals, medical therapy remains the predominant method for managing glaucoma but the failure of this first option leads to surgical and laser treatment approaches, to provide IOP control, by increasing AH outflow and/or decreasing its production. Recent improvements in techniques, materials and post-operative management have resulted in better long-term outcome of IOP control. Despite the advances in the IOP control, patients progress to blindness since there is no effective treatment to prevent retinal neurodegeneration. Being so, glaucoma is considered a multifactorial neurodegenerative disease characterized in earlier stages by the degeneration and loss of RGC and their axons, leading to optic neuropathy and visual field loss.

Notably, besides EPO haematopoietic effect, several investigations have successfully outlined its molecular properties in the context of its neuro- and tissue-protective mechanisms (Aghdam, Sanjari & Falavarjani, 2016; Shirley Ding *et al.*, 2016) through different pathways, including increasing resistance to inflammation, oxide-induced damage, ischemia, degeneration and permeability (Bond & Rex, 2014; Luo *et al.*, 2015). It has been found that EPO offers protection to the optic nerve and retina when they are injured and apoptosis processes starts in retinal ganglion cells. At the present, 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 encouraging results of neuroprotection. For these reasons, EPO is actually considered a promising drug on ischemic retinal diseases, such as glaucoma.

Ocular drug administration is a difficult task due to the peculiar structure of this organ and the presence of static and dynamic protective barriers (Pescina *et al.*, 2015). To achieve therapeutic concentrations of EPO on the retina, all pre-clinical studies used systemic, intravitreal or retrobulbar routes (Song *et al.*, 2008; Zhang *et al.*, 2008; Zhong *et al.*, 2008). These routes of administration have potential risks and side effects (Geroski & Edelhauser, 2000) and are difficult to use in clinical practice. In the clinical trials previously mentioned, patients were treated by i.v. administration, but with a limited number of administrations, for a maximum of three consecutive days. In patients with ocular disease such as glaucoma, an alternative route of administration is mandatory since EPO chronic systemic administration can lead to elevated blood pressure, thrombosis, chronic heart dilatation, ventricular oedema, compromised exercise performance and acute cardiac failure following challenge (Bogoyevitch,

2004). On what concerns intravitreous and retrobulbar administrations routes, both may lead to ocular complications, such as chorioretinitis, retinal detachments, cataracts, vitreitis or even endophthalmitis (Geroski & Edelhauser, 2000; Ranta & Urtti, 2006).
The authors postulated that EPO's neuroprotection could be achieved by a non-invasive and safe periocular administration route without complications associated with repeated systemic injections or intravitreous and retrobulbar routes.

### *In vitro* ocular membrane permeation to EPO protein

Firstly, the authors evaluated the permeation to EPO protein of the different static ocular barriers: conjunctiva, cornea and sclera, using an *ex vivo* model. The authors concluded that the three tested ocular membranes were permeable to EPO, being the conjunctiva the most permeable membrane, followed by sclera and finally by cornea. The lower permeability to the EPO obtained in this experiment corresponds to the thickest tested membrane, the cornea, that is in agreement with other studies where the permeability to other proteins is dependent on the structure of the tissue (Pescina *et al.*, 2015). Being the cornea the thicker membrane, associated to the dynamic protective barriers such as nasolacrimal drainage, tear clearance and lid blinking (Ambati *et al.*, 2000; Pescina *et al.*, 2015), a reduction in bioavailability of topically applied EPO was expected. For this reason, and considering the authors first results, the topical route for EPO administration was not the first choice for the subsequent studies conducted during this thesis but, instead, the authors chose to test the subconjunctival administration.
This route of administration is a relatively easy, safe and quick procedure, performed in ambulatory conditions, under topical anaesthesia both in human and veterinary patients. Also, the subconjunctival route seems to be more feasible for repeated administrations, with very few risks or associated complications, when compared to other routes.
The main ocular barrier to the subconjunctival administration route are flow barriers (elimination to blood and lymphatic flows) and penetration barriers (Del Amo *et al.*, 2017), being the scleral tissue the more important static barrier to be considered. In the *in vitro* study, authors demonstrated that EPO can permeate porcine sclera in accordance to other studies that proved that transscleral delivery of immunoglobulins and other large compounds to the choroid and retina is feasible (Ambati *et al.*, 2000; Pescina *et al.*, 2015). Due to its easy accessibility, large surface area and relatively high permeability to a range of drug molecules, the transscleral route is suitable for delivering a wide range of therapeutic agents, from small molecules to large proteins (Srirangam & Majumdar, 2012). Large molecules, such as IgG, diffuse across sclera in a manner consistent with porous diffusion through a fiber matrix (Ambati *et al.*, 2000). Despite the promising results, barriers such as blood flow in the conjunctiva and choriocapillaris, the uveoscleral outflow and the intraocular pressure, the retinal pigment epithelium and the blood-retinal barriers were not considered in the

*ex vivo* model used. So, to bypass this limitation, it was of the utmost important to evaluate EPO subconjunctival permeation in *in vivo* models, both in physiologic and glaucomatous conditions. Bearing this in mind, the authors designed the protocols of the second and third experiments.

**Subconjunctival administration in physiologic conditions**

To author's knowledge, no studies had been previously performed to assess EPO subconjunctival permeation in *in vivo* models. So, in the second study, authors used a rat animal model to evaluate the EPO´s ocular penetration after subconjunctival administration and the potential hematologic side effects associated to this route in physiologic conditions.

The main limitation in this study was the lack of previous data in regard to EPO's transscleral kinetics which made the choice of EPO dosages a difficult task. The studies that achieved EPO retinal therapeutic concentration by systemic routes used high EPO doses and, on the contrary, those which used intravitreal routes used very small EPO doses because the main ocular barriers were avoided. So, the dosage choice was based on a single previous study conducted by Zhong *et al.* (2008) that administered 1000 IU of rhEPO by the retrobulbar route to evaluate the RGC neuroprotection in an acute elevated IOP model. Like in the subconjunctival route, a drug administered by the retrobulbar route comes across blood flow barriers, lymphatic flow barriers and the scleral tissue as the main penetration static barriers (Del Amo *et al.*, 2017).

Using 1000 IU of rhEPO delivered by the subconjunctival route, authors concluded that EPO crossed the sclera, which was consistent with the *ex vivo* findings, and reached several neuronal cells, in all retinal layers. By immunohistochemistry assay, authors observed that EPO expression was more evident in the RGC layer and sixty hours after the administration there was still a strong EPO signal present in the retina of the studied animals. Regarding EPO potential systemic side-effects, subconjunctival administration did not cause any significant changes in their haematocrit values.

**Subconjunctival administration in glaucomatous conditions**

The journey of the injected EPO in glaucomatous eyes involves crossing the previously mentioned structures but with high values of IOP and, in some cases, with the ocular barriers breakdown. So, after this second study where the authors demonstrated that EPO reached all the neuroretinal cell layers in physiologic conditions, by subconjunctival administration, an important question was postulated: how will IOP affect the diffusion of EPO protein across the periocular tissues? Therefore, the third study aimed to evaluate whether EPO subconjunctival administration could reach the rat's retinas in glaucomatous conditions.

In order to study the underlying mechanisms of glaucoma pathogenesis, several animal models of glaucoma have been developed. Since IOP is regulated by the balance between production and outflow of aqueous humor, experimentally disturbing of this equilibrium can induce elevation of IOP. Impairs in the aqueous humor drainage by obstruction of the aqueous humor outflow pathways can be achieved by several techniques, such as, laser photocoagulation of the trabecular meshwork (Levkovitch-Verbin *et al.*, 2002) or the episcleral and perilimbal veins (Feng, Chen, Suyeoka, & Liu, 2013; Salinas-Navarro *et al.*, 2009); cauterization of episcleral veins (Shareef *et al.*, 1995); injection of hypertonic saline solution into the episcleral veins (Morrison *et al.*, 1997) and injection of polysterene microbeads into the anterior chamber (Cone, Gelman, Son, Pease, & Quigley, 2010). Besides these experimentally induced models based on an increase in IOP, inherited glaucoma models also exist (Johnson & Tomarev, 2010). Nevertheless, there are some disadvantages in genetic models when compared to experimentally-induced models, mainly based on the high variability and the rather slow disease progression that makes studies using these animals very expensive and time consuming. Therefore, the first part of this study was dedicated to the introduction and optimization of the experimental glaucoma rat model through cauterization of three episcleral veins (Shareef *et al.*, 1995).

The authors followed the same methodology applied on the previous study, on what concerns EPO dosage. However, in the previous study, sixty hours after the administration there was still a strong EPO signal, so authors extended the length of the experimental design to 14 days. Using the same immunohistochemistry technique, authors have demonstrated that EPO reached all retinal layers, including the RGC layer. Surprisingly, EPO immunostaining signal was stronger in glaucomatous eyes when compared to physiological eyes. This was probably due to the ocular blood barrier breakdown that followed the rise in IOP, including blood-retinal barrier breakdown, allowing for a more intense crossover of EPO protein. At the end of the study (day 14), EPO was still present in the retina, although its immunostaining signal was residual, which demonstrates that this protein stayed in retinal cell layers for a long time.

**Assessment of functional and structural benefits of subconjunctival EPO**

To conclude, and since the authors had demonstrated that EPO can reach the retinal by subconjunctival administration both in physiologic and glaucomatous conditions, the authors aimed to assess both functional and structural benefits of the subconjunctival EPO administration in the retina of glaucomatous rats. So, in this fourth study, it was demonstrated that treated glaucomatous animals showed a better recovery on the ERG examination and showed thicker retinas when compared to the non-treated group. Since structure and function are highly correlated in the vertebrate retina (Hoon *et al.*, 2014), the authors findings suggest a neuroprotective effect of subconjunctival EPO injection on the retinas of the rats with induced glaucoma.

**Preliminary clinical case**

Doing the link between research and veterinary clinical applications and considering that EPO´s neuroprotective effect should be tested in other animal species, the authors had the opportunity to administer a single EPO subconjunctival injection in a sudden acute glaucoma case. A 6-year-old female French bulldog dog was referred with an acute unilateral glaucoma and, after a complete ophthalmic examination, a diagnosis of a PACG was done. A blind eye was presented with negative menace response, negative direct and indirect pupillary light reflexes, negative dazzle reflex and a flat ERG examination on the affected eye. The initial IOP (88 mmHg) was only controlled by an Ahmed drainage valve implantation (Caravalve, Model VfP8, New World Medical, USA) and with previous owner's agreement, 1000 IU of rHuEPO was administered subconjunctivally in the glaucomatous eye at the end of surgery. IOP regained normal values after surgery and, surprisingly, three days later, the patient recovered menace response. Two weeks after EPO injection, dazzle reflex was positive, PLR were present but incomplete and ERG traces showed a recovery when compared with the initial presentation. Furthermore, any significant haematopoietic side-effects were not observed on the complete blood count. Of course, this is a single case of rHuEPO subconjunctival administration in an acute glaucomatous dog and caution in conclusions should be taken, since no control animal was tested. However, rHuEPO subconjunctival administration seems to have had a beneficial effect on vision recovery in this dog. The authors considered that a large multicentre double-arm study with a control group in PACG dogs could give precious information about the use of subconjunctival EPO as a neuroprotective therapy.

**Limitations of the study**

Recognizing that the main focus of the authors during the works conducted in this thesis was to prove that the subconjunctival route was an alternative route for ocular EPO delivery, several limitations must be considered on what concerns the neuroprotective effect of EPO achieved by this route.

The main limitation is the low number of animals per group for a neuroprotection study, which consequently influences the statistical results. To reduce this problem in this last study the authors separated animals by gender in the two different experiments, the group of female rats was used to test visual function and the group of male rats was used to evaluate changes in retinal thickness. Nonetheless, in the visual function experiment, the authors did not obtain statistical significant differences in the scotopic ERG examination, due to the large individual variability in this exam results.

Another limitation that should be considered is the use of single EPO injections in these studies. Repeated EPO subconjunctival injections should be administered to evaluate both local and systemic potential side effects. However, even considering some systemic absorption resulting

from repeated subconjunctival EPO administrations, systemic adverse side effects are not expected due to the low dosage necessary by this route of administration.

The glaucoma model used is another limitation recognized by the authors. Although this glaucoma experimental model has already been used before and showed to be reliable and reproducible (Danias *et al.*, 2006; Doh *et al.*, 2010; Grozdanic *et al.*, 2007), the glaucoma is a multifactorial and very complex disease. So, combining data from studies using several different glaucoma models is important in order to assess and try to understand the complete picture of EPO ocular neuroprotection in glaucoma disease. Although animal studies were useful in advancing human clinical, they also have limitation because do not predict the exact clinical responses in humans.

In spite of the limitations stated, these studies opened new perspectives concerning EPO administration in future works that target ocular neuroprotection, both in pre-clinical and clinical scenarios.

## CONCLUSIONS

In conclusion:

- It was demonstrated that EPO protein can permeate porcine conjunctiva, sclera and cornea in an *ex vivo* model.
- In physiologic conditions, EPO reached all neuroretinal cell layers in an animal rat model, after subconjunctival injection, without significant adverse side effects.
- In experimentally induced glaucomatous rats, subconjunctival EPO administration also reached all retinal layers, which shows that this is a promising alternative route for EPO delivery in these conditions.
- EPO administered by subconjunctival route after glaucoma induction showed beneficial effects both on cones, rods and their outputs and also on retinal thickness.
- The results of the present study are of great interest to scientists and researchers because they constitute a basis for examining the effectiveness of EPO subconjunctival administration in the treatment of glaucoma neuropathy.

## FUTURE DIRECTIONS

Currently, EPO is used in clinical trials for ocular disorders. EPO supports cell proliferation and differentiation into neurons to maintain optimal tissue function. Based on the positive pre-clinical outcomes with various animal models, EPO may offer a promising future therapy for the treatment of many common ocular disorders, not only glaucoma, but also diabetic retinopathy, retinal detachment, retinopathy of prematurity, age-related macular degeneration, and optic neuritis.

Refining the preparation and administration of EPO for this purpose should be the major focus of scientists in the near future. To find local non-invasive ocular delivery methods that increase drugs' efficacy, safety and bioavailability should be the challenge for the future. The sustained-release systems could provide controlled, long-term drug release. This would permit improved drug flux through thinner areas of the tissue and minimize systemic drug absorption by the conjunctival vasculature.

The results obtained in this work are likely to be of great interest to the scientists and researchers both in pre-clinic and clinical trials. In accordance with other experimental evidences, transscleral delivery of drugs can be accomplished and constitutes a great promise in new therapeutic approaches for treating visually devastating diseases of the posterior segment of the eye. However, before considering EPO subconjunctival administration for retinal diseases in clinical patients, further studies are necessary to evaluate absorption and distribution of EPO in different concentrations, in order to characterize the kinetics of this substance when administered by this route.

Another important topic to take in account for future research works is the efficiency of EPO neuronal tissue repair. There is a change of molecule interaction in response to EPO administration in the damaged retina microenvironment. Understanding the mechanism of EPO's action could lead to novel therapeutic strategies for the treatment of neurodegenerative diseases and injuries. Thus, researchers could center their efforts on the study of specific mechanisms of tissue repair in the eye.

# REFERENCES

# References


Agarwal, P. & Rupenthal, I. D. (2016). *In vitro* and ex vivo corneal penetration and absorption models. *Drug Delivery and Translational Research*, *6*(6), 634–647.

Aghdam, K. A., Sanjari, M. S. & Falavarjani, K. G. (2016). Erythropoietin in ophthalmology: A literature review. *Journal of Current Ophthalmology*, *28*(1), 5–11.

Agis, I. (2000). The Advanced Glaucoma Intervention Study (AGIS): The relationship between control of intraocular pressure and visual field deterioration. *American Journal of Ophthalmology*, *130*(4), 429–40.

Agnello, D., Bigini, P., Villa, P., Mennini, T., Cerami, A., Brines, M. L. & Ghezzi, P. (2002). Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Research*, *952*(1), 128–34.

Alario, A. F., Strong, T. D. & Pizzirani, S. (2015). Medical treatment of primary canine glaucoma. *Veterinary Clinics of North America: Small Animal Practice*, *45*(6), 1235–1259.

Almasieh, M., Zhou, Y., Kelly, M. E., Casanova, C. & Di Polo, A. (2010). Structural and functional neuroprotection in glaucoma: role of galantamine-mediated activation of muscarinic acetylcholine receptors. *Cell Death & Disease*, *1*(2), e27.

Almasieh, M., Wilson, A. M., Morquette, B., Cueva Vargas, J. L. & Di Polo, A. (2012). The molecular basis of retinal ganglion cell death in glaucoma. *Progress in Retinal and Eye Research*, *31*(2), 152–181.

Ambati, J., Canakis, C. S., Miller, J. W., Gragoudas, E. S., Weissgold, D. J., Kim, I. & Adamis, A. P. (2000). Diffusion of high molecular weight compounds through sclera. *Investigative Ophthalmology & Visual Science*, *41*(5), 1181–1185.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: cause or consequence? *Nature Reviews Neuroscience*, *10*(7), S18–S25.

Arjamaa, O. & Nikinmaa, M. (2006). Oxygen-dependent diseases in the retina: Role of hypoxia-inducible factors. *Experimental Eye Research*, *83*(3), 473–483.

Arthur, S. & Cantor, L. B. (2011). Update on the role of alpha-agonists in glaucoma management. *Experimental Eye Research*, *93*(3), 271–83.

Athanasiou, D., Aguilà, M., Bevilacqua, D., Novoselov, S. S., Parfitt, D. A. & Cheetham, M. E. (2013). The cell stress machinery and retinal degeneration. *FEBS Letters*, *587*(13), 2008–2017.

Ayalasomayajula, S. P. & Kompella, U. B. (2004). Retinal delivery of celecoxib is several-fold higher following subconjunctival administration compared to systemic administration. *Pharmaceutical Research*, *21*(10), 1797–804.

Bagli, E. & Kitsos, G. (2011). *The mystery of glaucoma*. Chapter 6 - Neuroprotective agents in glaucoma (T. Kubena, Ed.) (Open Acces). InTech.

Bahcekapili, N., Üzüm, G., Gökkusu, C., Kuru, A. & Ziylan, Y. Z. (2007). The relationship between erythropoietin pretreatment with blood–brain barrier and lipid peroxidation after ischemia/reperfusion in rats. *Life Sciences*, *80*(14), 1245–1251.

Bartesaghi, S., Marinovich, M., Corsini, E., Galli, C. L. & Viviani, B. (2005). Erythropoietin: a novel neuroprotective cytokine. *Neurotoxicology*, *26*(5), 923–8.

Bear, M. F., Connors, B. W. & Paradiso, M. A. (2007). *Neuroscience: exploring the brain.* (3rd ed.). Philadelphia: Lippincott Williams and Wilkins.

Bedford, P. G. C. (2016). Open-angle glaucoma in the Petit Basset Griffon Vendeen. *Veterinary Ophthalmology*, *20*(2), 98–102.

Beg, A. A. & Baltimore, D. (1996). An essential role for NF-κB in preventing TNF-α-induced cell death. *Science*, *274*(5288), 782–4.

Bento, R. M. D. A., Damasceno, L. M. P. & Neto, F. R. A. (2003). Recombinant human erythropoietin in sports: a review. *Revista Brasileira de Medicina Do Esporte*, *9*(3), 181–190.

Bocker-Meffert, S., Rosenstiel, P., Röhl, C., Warneke, N., Held-Feindt, J., Sievers, J. & Lucius, R. (2002). Erythropoietin and VEGF promote neural outgrowth from retinal explants in postnatal rats. *Investigative ophthalmology & visual science, 43*(6), 2021–2026.

Boevé, M. H. & Stades, F. C. (1985a). Glaucoma in dogs and cats. Review and retrospective evaluation of 421 patients. I. Pathobiological background, classification and breed predisposition [Article in Dutch]. *Tijdschrift Voor Diergeneeskunde*, *110*(6), 219–27.

Boevé, M. H. & Stades, F. C. (1985b). Glaucoma in dogs and cats. Review and retrospective evaluation of 421 patients. II. Clinical aspects [Article in Dutch]. *Tijdschrift Voor Diergeneeskunde*, *110*(6), 228–36.

Bogoyevitch, M. (2004). An update on the cardiac effects of erythropoietin cardioprotection by erythropoietin and the lessons learnt from studies in neuroprotection. *Cardiovascular Research*, *63*(2), 208–216.

Boland, M. V., Ervin, A. M., Friedman, D. S., Jampel, H. D., Hawkins, B., Vollenweider, D., Chelladurai, Y., Ward, D., Suarez-Cuervo, C. & Robinson, K. A. (2013). Comparative effectiveness of treatments for open-angle glaucoma : A systematic review for the U.S. preventive services task force. *Annals of Internal Medicine*, *158*(4), 271–279.

Bond, W. S. & Rex, T. S. (2014). Evidence that erythropoietin modulates neuroinflammation through differential action on neurons, astrocytes, and microglia. *Frontiers in Immunology*, *5*, 1–8.

Bonsdorff, E. & Jalavisto, E. (1948). A humoral mechanism in anoxic erythrocytosis. *Acta Physiologica Scandinavica*, *16*(2–3), 150–170.

Brines, M. L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N. C., Cerami, C., Itri, L. M. & Cerami, A. (2000). Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(19), 10526–31.

Brines, M., Grasso, G., Fiordaliso, F., Sfacteria, A., Ghezzi, P., Fratelli, M., Latini, R., Xie, Q.

W., Smart, J., Su-Rick, C. J., Pobre, E., Diaz, D., Gomez, D., Hand, C., Coleman, T. & Cerami, A. (2004). Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14907–12.

Brines, M. & Cerami, A. (2005). Emerging biological roles for erythropoietin in the nervous system. *Nature Reviews Neuroscience*, *6*(6), 484–494.

Burgoyne, C. F., Downs, J. C., Bellezza, A. J., Francis Suh, J. K. & Hart, R. T. (2005). The optic nerve head as a biomechanical structure: A new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Progress in Retinal and Eye Research*, *24*(1), 39–73.

Burgoyne, C. F. & Downs, J. C. (2008). Premise and prediction – How optic nerve head biomechanics underlies the susceptibility and clinical behavior of the aged optic nerve head. *Journal of Glaucoma*, *17*(4), 318–328.

Calapai, G., Marciano, M. C., Corica, F., Allegra, A., Parisi, A., Frisina, N., Caputi A. P. & Buemi, M. (2000). Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *European Journal of Pharmacology*, *401*(3), 349–356.

Calkins, D. J. (2012). Critical pathogenic events underlying progression of neurodegeneration in glaucoma. *Progress in Retinal and Eye Research*, *31*(6), 702–719.

Caprara, C., Britschgi, C., Samardzija, M. & Grimm, C. (2014). The erythropoietin receptor is not required for the development, function, and aging of rods and cells in the retinal periphery. *Molecular Vision*, *20*, 307–24.

Carnot, P. & DeFlandre, C. (1906). Sur l'activite hemopoietique de serum au cours de la regeneration du sang. *Comptes Rendus de l'Académie des Sciences (Paris)*, *143*, 384–386.

Celik, M., Gökmen, N., Erbayraktar, S., Akhisaroglu, M., Konakc, S., Ulukus, C., Genc, S., Genc, K., Sagiroglu, E., Cerami, A. & Brines, M. (2002). Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(4), 2258–63.

Chang, Z. Y., Yeh, M. K., Chiang, C. H., Chen, Y. H. & Lu, D. W. (2013). Erythropoietin protects adult retinal ganglion cells against NMDA-, trophic factor withdrawal-, and TNF-α-induced damage. *PloS One*, *8*(1), e55291.

Chen, J., Connor, K. M., Aderman, C. M. & Smith, L. E. H. (2008). Erythropoietin deficiency decreases vascular stability in mice. *The Journal of Clinical Investigation*, *118*(2), 526–33.

Cheung, W., Guo, L. & Cordeiro, M. F. (2008). Neuroprotection in glaucoma: drug-based approaches. *Optometry and Vision Science, 85*(6), 406–416.

Chikuma, M., Masuda, S., Kobayashi, T., Nagao, M. & Sasaki, R. (2000). Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus. *American Journal of Physiology - Endocrinology and Metabolism*, *279*(6), 1242-8.

Chong, Z. Z., Kang, J. Q. & Maiese, K. (2002). Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation*, *106*(23), 2973–9.

Chung, H., Lee, H., Lamoke, F., Hrushesky, W. J. M., Wood, P. A. & Jahng, W. J. (2009). Neuroprotective role of erythropoietin by antiapoptosis in the retina. *Journal of Neuroscience Research*, *87*(10), 2365–74.

Civan, M. M. & Macknight, A. D. C. (2004). The ins and outs of aqueous humour secretion. *Experimental Eye Research*, *78*(3), 625–631.

Cohen-Cory, S. & Fraser, S. E. (1994). BDNF in the development of the visual system of Xenopus. *Neuron*, *12*(4), 747–761.

Collaborative Normal-Tension Glaucoma Study Group (1998). Comparison of glaucomatous progression between untreated patients with normal-tension glaucoma and patients with therapeutically reduced intraocular pressures. *American Journal of Ophthalmology*, *126*(4), 487–97.

Cone, F. E., Gelman, S. E., Son, J. L., Pease, M. E. & Quigley, H. A. (2010). Differential susceptibility to experimental glaucoma among 3 mouse strains using bead and viscoelastic injection. *Experimental Eye Research*, *91*(3), 415–424.

Conlon, R., Saheb, H. & Ahmed, I. I. K. (2017). Glaucoma treatment trends: a review. *Canadian Journal of Ophthalmology / Journal Canadien d'Ophtalmologie*, *52*(1), 114–124.

Cook, C. S. (1997). Surgery for glaucoma. *Veterinary Clinics of North America: Small Animal Practice*, *27*(5), 1109–1129.

Cordeiro, M. F., Guo, L., Luong, V., Harding, G., Wang, W., Jones, H. E., Moss, S. E., Sillito, A. M. & Fitzke, F. W. (2004). Real-time imaging of single nerve cell apoptosis in retinal neurodegeneration. *Proceedings of the National Academy of Sciences*, *101*(36), 13352–13356.

Culmsee, C. & Mattson, M. P. (2005). p53 in neuronal apoptosis. *Biochemical and Biophysical Research Communications*, *331*(3), 761–777.

Cunha-Vaz, J. & Maurice, D. M. (1967). The active transport of fluorescein by the retinal vessels and the retina. *The Journal of Physiology*, *191*(3), 467–86.

Cunha-Vaz, J. & Maurice, D. (1969). Fluorescein dynamics in the eye. *Documenta Ophthalmologica*, *26*(1), 61–72.

Cunha-Vaz, J. (2017). The blood-retinal barrier in the management of retinal disease: EURETINA Award Lecture. *Ophthalmologica*, *237*, 1–10.

Danesh-Meyer, H. V. (2011). Neuroprotection in glaucoma: recent and future directions. *Current Opinion in Ophthalmology*, *22*(2), 78–86.

Danias, J., Shen, F., Kavalarakis, M., Chen, B., Goldblum, D., Lee, K., Zamora, M. F., Su, Y., Brodie, S. E., Podos, S. M., & Mittag, T. (2006). Characterization of retinal damage in the episcleral vein cauterization rat glaucoma model. *Experimental Eye Research*, *82*(2), 219–228.

Datta, S. R., Brunet, A. & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes & Development*, *13*(22), 2905–27.

De Marco, N., Buono, M., Troise, F. & Diez-Roux, G. (2006). Optineurin increases cell survival and translocates to the nucleus in a Rab8-dependent manner upon an apoptotic stimulus. *The Journal of Biological Chemistry*, *281*(23), 16147–56.

Del Amo, E. M., Rimpelä, A.-K., Heikkinen, E., Kari, O. K., Ramsay, E., Lajunen, T., Schmitt, M., Pelkonen, L., Bhattacharya, M., Richardson, D., Subrizi, A., Turunen, T., Reinisalo, M., Itkonen, J., Toropainen, E., Casteleijn, M., Kidron, H., Antopolsky, M., Vellonen, K. S., Ruponen, M. & Urtti, A. (2017). Pharmacokinetic aspects of retinal drug delivery. *Progress in Retinal and Eye Research*, *57*, 134–185.

Del Sole, M. J., Sande, P. H., Bernades, J. M., Aba, M. A. & Rosenstein, R. E. (2007). Circadian rhythm of intraocular pressure in cats. *Veterinary Ophthalmology*, *10*(3), 155–161.

Demetriades, A. M., Deering, T., Liu, H., Lu, L., Gehlbach, P., Packer, J. D., Mac Gabhann, F., Popel, A. S., Wei, L. L. & Campochiaro, P. A. (2008). Transscleral delivery of antiangiogenic proteins. *Journal of Ocular Pharmacology and Therapeutics*, *24*(1), 70–79.

Digicaylioglu, M. & Lipton, S. A. (2001). Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature*, *412*(6847), 641–647.

Doh, S. H., Kim, J. H., Lee, K. M., Park, H. Y. & Park, C. K. (2010). Retinal ganglion cell death induced by endoplasmic reticulum stress in a chronic glaucoma model. *Brain Research*, *1308*, 158–66.

Dutton, J. J. (2001). Editorial: Anatomic considerations in ophthalmic anesthesia. *Survey of Ophthalmology*, *46*(2), 172–84.

Duvvuri, S., Majumdar, S. & Mitra, A. K. (2003). Drug delivery to the retina: challenges and opportunities. *Expert Opinion on Biological Therapy*, *3*(1), 45–56.

Erbayraktar, S., Yilmaz, O., Gökmen, N. & Brines, M. (2003). Erythropoietin is a multifunctional tissue-protective cytokine. *Current Hematology Reports*, *2*, 465–470.

Ergorul, C., Ray, A., Huang, W., Wang, D. Y., Ben, Y., Cantuti-Castelvetri, I. & Grosskreutz, C. L. (2010). Hypoxia inducible factor-1α (HIF-1α) and some HIF-1 target genes are elevated in experimental glaucoma. *Journal of Molecular Neuroscience*, *42*(2), 183–191.

Escalona-Benz, E., Jockovich, M. E., Murray, T. G., Hayden, B., Hernandez, E., Feuer, W. & Windle, J. J. (2005). Combretastatin A-4 prodrug in the treatment of a murine model of retinoblastoma. *Investigative Ophthalmology and Visual Science*, *46*(1), 8–11.

European Glaucoma Society (2008). *Terminology and guidelines for glaucoma* (3rd ed.). Savona, Italy.

Feng, L., Chen, H., Suyeoka, G. & Liu, X. (2013). A laser-induced mouse model of chronic ocular hypertension to characterize visual defects. *Journal of Visualized Experiments*, 14(78), e50440.

Fingert, J. H. (2011). Primary open-angle glaucoma genes. *Eye*, *25*(5), 587–595.

Fisher, J. W. (2010). Landmark advances in the development of erythropoietin. *Experimental Biology and Medicine (Maywood)*, *235*(12), 1398–1411.

Frank-Cannon, T. C., Alto, L. T., McAlpine, F. E. & Tansey, M. G. (2009). Does neuroinflammation fan the flame in neurodegenerative diseases? *Molecular Neurodegeneration*, *4*(1), 47.

Fuchs, C., Forster, V., Balse, E., Sahel, J. A., Picaud, S. & Tessier, L. H. (2005). Retinal-cell–conditioned medium prevents TNF-α-induced apoptosis of purified ganglion cells. *Investigative Opthalmology & Visual Science*, *46*(8), 2983.

Gabelt, B. T. & Kaufman, P. L. (2005). Changes in aqueous humor dynamics with age and glaucoma. *Progress in Retinal and Eye Research*, *24*(5), 612–637.

Gallenberger, M., Meinel, D. M., Kroeber, M., Wegner, M., Milkereit, P., Bösl, M. R. & Tamm, E. R. (2011). Lack of WDR36 leads to preimplantation embryonic lethality in mice and delays the formation of small subunit ribosomal RNA in human cells *in vitro*. *Human Molecular Genetics*, *20*(3), 422–35.

Garcia-Ramirez, M., Hernandez, C. & Simo, R. (2008). Expression of Erythropoietin and Its Receptor in the Human Retina: A comparative study of diabetic and nondiabetic subjects. *Diabetes Care*, *31*(6), 1189–1194.

Garcia-Ramirez, M., Hernández, C., Ruiz-Meana, M., Villarroel, M., Corraliza, L., García-Dorado, D. & Simó, R. (2011). Erythropoietin protects retinal pigment epithelial cells against the increase of permeability induced by diabetic conditions: essential role of JAK2/ PI3K signaling. *Cellular Signalling*, *23*(10), 1596–1602.

Garcia-Valenzuela, E., Shareef, S., Walsh, J. & Sharma, S. (1995). Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp. Eye Res.*, *61*, 33–44.

Gaudana, R., Jwala, J., Boddu, S. H. S. & Mitra, A. K. (2009). Recent perspectives in ocular drug delivery. *Pharmaceutical Research*, *26*(5), 1197–216.

Gelatt, K. N. & Gum, G. G. (1981). Inheritance of primary glaucoma in the beagle. *American Journal of Veterinary Research*, *42*(10), 1691–3.

Gelatt, K. N. & MacKay, E. O. (1998). Distribution of intraocular pressure in dogs. *Veterinary Ophthalmology*, *1*(2–3), 109–114.

Gelatt, K. N. & Gelatt, J. P. (2011). *Veterinary Ophtalmic Surgery*. Elsevier Saunders.

Gelatt, K., Gilger, B. & Kern, T. (2013). *Veterinary Ophthalmology*. (5th ed.). Wiley-Blackwell.

Geroski, D. H. & Edelhauser, H. F. (2000). Drug delivery for posterior segment eye disease. *Investigative Ophthalmology & Visual Science*, *41*(5), 961–4.

Gilger, B. C. (2005). *Equine Ophthalmology*. St. Louis, Missouri: Elsevier Saunders.

Gilmore, T. D. & Garbati, M. R. (2010). Inhibition of NF-κB signaling as a strategy in disease therapy. Current Topics Microbiology, 349, 245-263.

Gilmore, T. D. & Wolenski, F. S. (2012). NF-κB: where did it come from and why? *Immunological Reviews*, *246*(1), 14–35.

Goel, M., Picciani, R. G., Lee, R. K. & Bhattacharya, S. K. (2010). Aqueous humor dynamics: a review. *The Open Ophthalmology Journal*, *4*, 52–59.

Gordon, M. O., Beiser, J. A., Brandt, J. D., Heuer, D. K., Higginbotham, E. J., Johnson, C. A., Keltner, J. L., Miller, J. P., Parrish II, R. K., Wilson, M. R. & Kass, M. A. (2002). The ocular hypertension treatment study. *Archives of Ophthalmology*, *120*(6), 714.

Gottanka, J., Johnson, D., Martus, P. & Lutjen-Drecoll, E. (1997). Severity of optic nerve damage in eyes with POAG is correlated with changes in the trabecular meshwork. *J Glaucoma, 6*(2), 123–132.

Grasso, G., Buemi, M., Alafaci, C., Sfacteria, A., Passalacqua, M., Sturiale, A., Calapai, G., De Vico, G., Piedimonte, G., Salpietro, F. M., & Tomasello, F. (2002). Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5627–31.

Grasso, G., Sfacteria, A., Meli, F., Fodale, V., Buemi, M. & Iacopino, D. G. (2007a). Neuroprotection by erythropoietin administration after experimental traumatic brain injury. *Brain Research*, *1182*, 99–105.

Grasso, G., Sfacteria, A., Meli, F., Passalacqua, M., Fodale, V., Buemi, M., Giambartino, F., Iacopino, D. G., & Tomasello, F. (2007b). The role of erythropoietin in neuroprotection:

therapeutic perspectives. *Drug News & Perspectives*, *20*(5), 315–20.

Grimm, C., Wenzel, A., Groszer, M., Mayser, H., Seeliger, M., Samardzija, M., Bauer, C., Gassmann, M., & Remé, C. E. (2002). HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nature Medicine*, *8*(7), 718–724.

Grozdanic, S. D., Betts, D. M., Sakaguchi, D. S., Kwon, Y. H., Kardon, R. H. & Sonea, I. M. (2003). Temporary elevation of the intraocular pressure by cauterization of vortex and episcleral veins in rats causes functional deficits in the retina and optic nerve. *Experimental Eye Research*, *77*(1), 27–33.

Grozdanic, S. D., Matic, M., Betts, D. M., Sakaguchi, D. S. & Kardon, R. H. (2007). Recovery of canine retina and optic nerve function after acute elevation of intraocular pressure: implications for canine glaucoma treatment. *Veterinary Ophthalmology*, *10 Suppl 1*, 101–7.

Gründer, T., Kohler, K., Kaletta, A. & Guenther, E. (2000). The distribution and developmental regulation of NMDA receptor subunit proteins in the outer and inner retina of the rat. *Journal of Neurobiology*, *44*(3), 333–42.

Gui, D. M., Yang, Y., Li, X. & Gao, D. W. (2011). Effect of erythropoietin on the expression of HIF-1 and iNOS in retina in chronic ocular hypertension rats. *International Journal of Ophthalmology*, *4*(1), 40–43.

Guo, L., Moss, S. E., Alexander, R. A., Ali, R. R., Fitzke, F. W. & Cordeiro, M. F. (2005). Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix. *Investigative Ophthalmology & Visual Science*, *46*(1), 175–82.

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry*, *97*(6), 1634–1658.

Hayden, B. H., Murray, T. G., Scott, I. U., Cicciarelli, N., Hernandez, E., Feuer, W., Feuer, W. & O'Brien, J. M. (2000). Subconjunctival carboplatin in retinoblastoma: impact of tumor burden and dose schedule. *Archives of Ophthalmology*, *118*(11), 1549–54.

He, S., Li, X., Chan, N. & Hinton, D. R. (2013). Review: Epigenetic mechanisms in ocular disease. *Molecular Vision*, *19*, 665–74.

Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., Mildner-Rihm, C., Martin, H., Zeiher, A. M. & Dimmeler, S. (2003). Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood*, *102*(4), 1340–6.

Heijl, A., Leske, M. C., Bengtsson, B., Hyman, L., Bengtsson, B. & Hussein, M. (2002). Reduction of intraocular pressure and glaucoma progression. *Arch Ophthalmol.*, *120*, 1268–1279.

Hernandez, C., Fonollosa, A., Garcia-Ramirez, M., Higuera, M., Catalan, R., Miralles, A., García-Arumí, J. & Simo, R. (2006). Erythropoietin is expressed in the human retina and it is highly elevated in the vitreous fluid of patients with diabetic macular edema. *Diabetes Care*, *29*(9), 2028–2033.

Hernandez, M. R. & Pena, J. (1997). The optic nerve head in glaucomatous optic neuropathy. *Arch Ophthalmol.*, *115*(3), 389–395.

Hoon, M., Okawa, H., Della Santina, L. & Wong, R. O. L. (2014). Functional architecture of the retina: development and disease. *Progress in Retinal and Eye Research*, *42*(i), 44–84.

Hosoya, K., Lee, V. H. L. & Kim, K. J. (2005). Roles of the conjunctiva in ocular drug delivery: a review of conjunctival transport mechanisms and their regulation. *European Journal of Pharmaceutics and Biopharmaceutics*, *60*(2), 227–240.

Hu, L. M., Luo, Y., Zhang, J., Lei, X., Shen, J., Wu, Y., Qin, M., Unver, Y. B., Zhong, Y., Xu, G. T. & Li, W. (2011). EPO reduces reactive gliosis and stimulates neurotrophin expression in Muller cells. *Frontiers in Bioscience (Elite Edition)*, *3*, 1541–55.

Hu, L. M., Lei, X., Ma, B., Zhang, Y., Yan, Y., Wu, Y., Xu, G. Z., Ye, W., Wang, L., Xu, G. X., Xu, G.T. & Wei-Ye, L. (2011). Erythropoietin receptor positive circulating progenitor cells and endothelial progenitor cells in patients with different stages of diabetic retinopathy. *Chinese Medical Sciences Journal*, *26*(2), 69–76.

Huang, E. J. & Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annual Review of Neuroscience*, *24*(1), 677–736.

Huang, E. J. & Reichardt, L. F. (2003). Trk receptors: roles in neuronal signal transduction. *Annual Review of Biochemistry*, *72*(1), 609–642.

Izzotti, A., Bagnis, A. & Sacca, S. (2006). The role of oxidative stress in glaucoma. *Mutation Research/Reviews in Mutation Research*, *612*(2), 105–114.

Jaffe, G. J., Ashton, P. & Pearson, P. A. (2006). *Intraocular drug delivery*. New york: Taylor and Francis Group.

Jelkmann, W. (2007). Erythropoietin after a century of research: younger than ever. *European Journal of Haematology*, *78*(3), 183–205.

Johnson, T. V. & Tomarev, S. I. (2010). Rodent models of glaucoma. *Brain Research Bulletin*, *81*(301), 349–358.

Jordán, J. & Ruíz-Moreno, J. M. (2013). Advances in the understanding of retinal drug disposition and the role of blood-ocular barrier transporters. *Expert Opinion on Drug Metabolism & Toxicology*, *9*(9), 1181–92.

Junk, A. K., Mammis, A., Savitz, S. I., Singh, M., Roth, S., Malhotra, S., Rosenbaum, P. S., Cerami, A., Brines, M. & Rosenbaum, D. M. (2002). Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10659–64.

Kanemaki, N., Tchedre, K. T., Imayasu, M., Kawarai, S., Sakaguchi, M., Yoshino, A., Itoh, N., Meguro, A. & Mizuki, N. (2013). Dogs and humans share a common susceptibility gene SRBD1 for glaucoma risk. *PloS One*, *8*(9), e74372.

Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V & Parada, L. F. (1991). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science (New York)*, *252*(5005), 554–8.

Kawakami, M., Sekiguchi, M., Sato, K., Kozaki, S. & Takahashi, M. (2001). Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *The Journal of Biological Chemistry*, *276*(42), 39469–75.

Kawano, T., Anrather, J., Zhou, P., Park, L., Wang, G., Frys, K. A., Kunz, A., Cho, S., Orio, M. & Iadecola, C. (2006). Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nature Medicine*, *12*(2), 225–229.

Kerrigan, L. A., Zack, D. J., Quigley, H. A., Smith, S. D. & Pease, M. E. (1997). TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Archives of Ophthalmology*, *115*(8), 1031–5.

Kiel, J. W., Hollingsworth, M., Rao, R., Chen, M. & Reitsamer, H. A. (2011). Ciliary blood flow and aqueous humor production. *Progress in Retinal and Eye Research 30*(1), 1–17.

Kilic, E., Kilic, U., Soliz, J., Bassetti, C. L., Gassmann, M. & Hermann, D. M. (2005a). Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *19*(14), 2026–8.

Kilic, U., Kilic, E., Soliz, J., Bassetti, C. I., Gassmann, M. & Hermann, D. M. (2005b). Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK-1/-2. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *19*(2), 249–51.

Kim, S. H., Csaky, K. G., Wang, N. S. & Lutz, R. J. (2008). Drug elimination kinetics following subconjunctival injection using dynamic contrast-enhanced magnetic resonance imaging. *Pharmaceutical Research*, *25*(3), 512–520.

Kim, Y. C., Chiang, B., Wu, X. & Prausnitz, M. R. (2014). Ocular delivery of macromolecules. *Journal of Controlled Release : Official Journal of the Controlled Release Society*, *190*, 172–81.

King, C. E., Rodger, J., Bartlett, C., Esmaili, T., Dunlop, S. A. & Beazley, L. D. (2007). Erythropoietin is both neuroprotective and neuroregenerative following optic nerve transection. *Experimental Neurology*, *205*(1), 48–55.

Klein, R., Nanduri, V., Jing, S. A., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K. R., Reichardt, L. F. & Barbacid, M. (1991). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell*, *66*(2), 395–403.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R. & Newmeyer, D. D. (1997). The release of cytochrome C from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science (New York)*, *275*(5303), 1132–6.

Knollinger, A. M., La Croix, N. C., Barrett, P. M. & Miller, P. E. (2005). Evaluation of a rebound tonometer for measuring intraocular pressure in dogs and horses. *Journal of the American Veterinary Medical Association*, *227*(2), 244–8.

Ko, M. L., Peng, P. H., Ma, M. C., Ritch, R. & Chen, C. F. (2005). Dynamic changes in reactive oxygen species and antioxidant levels in retinas in experimental glaucoma. *Free Radical Biology and Medicine*, *39*(3), 365–373.

Koeberle, P. D. & Ball, A. K. (2002). Neurturin enhances the survival of axotomized retinal ganglion cells *in vivo*: combined effects with glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor. *Neuroscience*, *110*(3), 555–567.

Koevary, S. B. (2003). Pharmacokinetics of topical ocular drug delivery: potential uses for the treatment of diseases of the posterior segment and beyond. *Current Drug Metabolism*, *4*(3), 213–22.

Kroeber, M., Davis, N., Holzmann, S., Kritzenberger, M., Shelah-Goraly, M., Ofri, R., Ashery-Padan, R. & Tamm, E. R. (2010). Reduced expression of Pax6 in lens and cornea of mutant mice leads to failure of chamber angle development and juvenile glaucoma. *Human Molecular Genetics*, *19*(17), 3332–3342.

Kroemer, G., Galluzzi, L. & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological Reviews*, *87*, 99–163.

Kroll, M. M., Miller, P. E. & Rodan, I. (2001). Intraocular pressure measurements obtained as part of a comprehensive geriatric health examination from cats seven years of age or older. *Journal of the American Veterinary Medical Association*, *219*(10), 1406–10.

Kuchtey, J., Olson, L. M., Rinkoski, T., Mackay, E. O., Iverson, T. M., Gelatt, K. N., Haines, J. L. & Kuchtey, R. W. (2011). Mapping of the disease locus and identification of ADAMTS10

as a candidate gene in a canine model of primary open angle glaucoma. *PLoS Genetics*, *7*(2), e1001306.

Kumral, A., Genc, S., Ozer, E., Yilmaz, O., Gokmen, N., Koroglu, T. F., Duman, N., Genc, K., & Ozkan, H. (2006). Erythropoietin downregulates bax and DP5 proapoptotic gene expression in neonatal hypoxic-ischemic brain injury. *Biology of the Neonate*, *89*(3), 205–10.

Kyhn, M. V., Klassen, H., Johansson, U. E., Warfvinge, K., Lavik, E., Kiilgaard, J. F., Prause, J. U., Scherfig, E., Young, M. & la Cour, M. (2009). Delayed administration of glial cell line-derived neurotrophic factor (GDNF) protects retinal ganglion cells in a pig model of acute retinal ischemia. *Experimental Eye Research*, *89*(6), 1012–1020.

Lagrèze, W. A., Feltgen, N., Bach, M. & Jehle, T. (2009). Feasibility of intravitreal erythropoietin injections in humans. *The British Journal of Ophthalmology*, *93*(12), 1667–1671.

Lamballe, F., Klein, R. & Barbacid, M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell*, *66*(5), 967–79.

Laquis, S., Chaudhary, P. & Sharma, S. C. (1998). The patterns of retinal ganglion cell death in hypertensive eyes. *Brain Research*, *784*(1–2), 100–4.

Lee, T. W. & Robinson, J. R. (2001). Drug delivery to the posterior segment of the eye: some insights on the penetration pathways after subconjunctival injection. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *17*(6), 565–572.

Leibovitch, I., Kurtz, S., Kesler, A., Feithliher, N., Shemesh, G. & Sela, B.-A. (2005). C-reactive protein levels in normal tension glaucoma. *Journal of Glaucoma*, *14*(5), 384–6.

Leist, M., Ghezzi, P., Grasso, G., Bianchi, R., Villa, P., Fratelli, M., Savino, C., Bianchi, M., Nielsen, J., Gerwien, J., Kallunki, P., Larsen, A. K., Helboe, L., Christensen, S., Pedersen, L. O., Nielsen, M., Torup, L., Sager, T., Sfacteria, A., Erbayraktar, S., Erbayraktar, Z., Gokmen, N., Yilmaz, O., Cerami-Hand, C., Xie, Q. W., Coleman, T., Cerami, A. & Brines, M. (2004). Derivatives of Erythropoietin that are tissue protective but not erythropoietic. *Science*, *305*(5681), 239–242.

Leiva, M., Naranjo, C. & Peña, M. T. (2006). Comparison of the rebound tonometer (ICare) to the applanation tonometer (Tonopen XL) in normotensive dogs. *Veterinary Ophthalmology*, *9*(1), 17–21.

Leske, M. C., Connell, A. M., Wu, S. Y., Nemesure, B., Li, X., Schachat, A. & Hennis, A. (2001). Incidence of open-angle glaucoma: the Barbados Eye Studies. The Barbados Eye Studies Group. *Archives of Ophthalmology (Chicago, Ill. : 1960)*, *119*(1), 89–95.

Leske, M. C., Heijl, A., Hyman, L., Bengtsson, B., Dong, L., Yang, Z. & EMGT Group. (2007). Predictors of long-term progression in the early manifest glaucoma trial. *Ophthalmology*, *114*(11), 1965–1972.

Leske, M. C. (2009). Ocular perfusion pressure and glaucoma: clinical trial and epidemiologic findings. *Current Opinion in Ophthalmology*, *20*(2), 73–8.

Levin, L. A. (2016). Translational pharmacology in glaucoma neuroprotection. In *Handbook of experimental pharmacology*, 242, 209–230.

Levkovitch-Verbin, H., Quigley, H. A., Martin, K. R. G., Valenta, D., Baumrind, L. A. & Pease, M. E. (2002). Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Investigative Ophthalmology & Visual Science*, *43*(2), 402–10.

Lezoualc'h, F., Sagara, Y., Holsboer, F. & Behl, C. (1998). High constitutive NF-kB activity mediates resistance to oxidative stress in neuronal cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *18*(9), 3224–3232.

Li, G., Luna, C., Liton, P. B., Navarro, I., Epstein, D. L. & Gonzalez, P. (2007). Sustained stress response after oxidative stress in trabecular meshwork cells. *Molecular Vision*, *13*, 2282–8.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. & Wang, X. (1997). Cytochrome C and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, *91*(4), 479–89.

Li, S., Mealing, G. A., Morley, P. & Stys, P. K. (1999). Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na+-dependent glutamate transport. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(14), RC16.

Libby, R. T., Smith, R. S., Savinova, O. V, Zabaleta, A., Martin, J. E., Gonzalez, F. J. & John, S. W. M. (2003). Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science (New York)*, *299*(5612), 1578–81.

Liu, X., Xie, W., Liu, P., Duan, M., Jia, Z., Li, W. & Xu, J. (2006). Mechanism of the cardioprotection of rhEPO pretreatment on suppressing the inflammatory response in ischemia–reperfusion. *Life Sciences*, *78*(19), 2255–2264.

Lucas, D. R. & Newhouse, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. *Archives of Ophthalmology*, *58*(2), 193–201.

Lukasiewicz, P. (2005). Synaptic mechanisms that shape visual signaling at the inner retina. In *Progress in brain research*, 147, 205–218.

Luo, W., Hu, L. & Wang, F. (2015). The protective effect of erythropoietin on the retina. *Ophthalmic Research*, *200072*, 74–81.

Mabuchi, F., Sakurada, Y., Kashiwagi, K., Yamagata, Z., Iijima, H. & Tsukahara, S. (2011). Association between *SRBD1* and *ELOVL5* gene polymorphisms and primary open-angle glaucoma. *Investigative Opthalmology & Visual Science*, *52*(7), 4626.

Maes, C., Carmeliet, G. & Schipani, E. (2012). Hypoxia-driven pathways in bone development, regeneration and disease. *Nature Reviews. Rheumatology*, *8*(6), 358–66.

Maggio, F. (2015). Glaucomas. *Topics in Companion Animal Medicine*, *30*(3), 86–96.

Maggs, D., Miller, P. & Ofri, R. (2008). *Slatter's Fundamentals of Veterinary Ophthalmology* (4th ed.). St. Louis, Missouri: Saunders Elsevier.

Maiese, K., Li, F. & Chong, Z. Z. (2004). Erythropoietin in the brain: can the promise to protect be fulfilled? *Trends in Pharmacological Sciences*, *25*(11), 577–83.

Maiese, K., Chong, Z. Z., Shang, Y. C. & Wang, S. (2012). Erythropoietin: New directions for the nervous system. *International Journal of Molecular Sciences*, *13*(9), 11102–11129.

Mallet, R. T. & Ryou, M. G. (2017). Erythropoietin: endogenous protection of ischemic brain. In *Vitamins and hormones*, 105, 197–232.

Mark, H. H. (2010). Aqueous humor dynamics in historical perspective. *Survey of Ophthalmology*, *55*(1), 89–100.

Marti, H. H. (2004). Erythropoietin and the hypoxic brain. *Journal of Experimental Biology*, *207*(18), 3233–3242.

Martínez-Estrada, O. M., Rodríguez-Millán, E., González-De Vicente, E., Reina, M., Vilaró, S. & Fabre, M. (2003). Erythropoietin protects the *in vitro* blood-brain barrier against VEGF-induced permeability. *The European Journal of Neuroscience*, *18*(9), 2538–44.

McLellan, G. J. & Miller, P. E. (2011). Feline glaucoma-a comprehensive review. *Veterinary Ophthalmology*, *14*(s1), 15–29.

McVicar, C. M., Hamilton, R., Colhoun, L. M., Gardiner, T. A., Brines, M., Cerami, A. & Stitt, A. W. (2011). Intervention with an erythropoietin-derived peptide protects against neuroglial and vascular degeneration during diabetic retinopathy. *Diabetes*, *60*(11), 2995–3005.

Medeiros, F. A., Meira-Freitas, D., Lisboa, R., Kuang, T. M., Zangwill, L. M. & Weinreb, R. N. (2013). Corneal hysteresis as a risk factor for glaucoma progression: a prospective longitudinal study. *Ophthalmology*, *120*(8), 1533–40.

Meyer, C. H., Krohne, T. U., Charbel Issa, P., Liu, Z. & Holz, F. G. (2015). Routes for drug delivery to the eye and retina: Intravitreal injections. *Developments in Ophthalmology*, *55*, 63–70.

Miceli, M. V., Liles, M. R. & Newsome, D. A. (1994). Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. *Experimental Cell Research*, *214*(1), 242–249.

Miller, P. E., Pickett, J. P. & Majors, L. J. (1990). Evaluation of two applanation tonometers in horses. *American Journal of Veterinary Research*, *51*(6), 935–7.

Miyake, T., Kung, C. K. & Goldwasser, E. (1977). Purification of human erythropoietin. *The Journal of Biological Chemistry*, *252*(15), 5558–64.

Monemi, S., Spaeth, G., DaSilva, A., Popinchalk, S., Ilitchev, E., Liebmann, J., Ritch, R., Héon, E., Crick, R. P., Child, A. & Sarfarazi, M. (2005). Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1. *Human Molecular Genetics*, *14*(6), 725–33.

Morrison, J. C., Moore, C. G., Deppmeier, L. M. H., Gold, B. G., Meshul, C. K. & Johnson, E. C. (1997). A rat model of chronic pressure-induced optic nerve damage. *Experimental Eye Research*, *64*(1), 85–96.

Morrison, J. C., Cepurna Ying Guo, W. O. & Johnson, E. C. (2011). Pathophysiology of human glaucomatous optic nerve damage: Insights from rodent models of glaucoma. *Experimental Eye Research*, *93*(2), 156–164.

Morton, S., Hesson, L., Peggie, M. & Cohen, P. (2008). Enhanced binding of TBK1 by an optineurin mutant that causes a familial form of primary open angle glaucoma. *FEBS Letters*, *582*(6), 997–1002.

Nagarwal, R. C., Kant, S., Singh, P. N., Maiti, P. & Pandit, J. K. (2009). Polymeric nanoparticulate system: a potential approach for ocular drug delivery. *Journal of Controlled Release : Official Journal of the Controlled Release Society*, *136*(1), 2–13.

Nakazawa, T., Nakazawa, C., Matsubara, A., Noda, K., Hisatomi, T., She, H., Michaud ,N., Hafezi-Moghadam, A., Miller, J. W. & Benowitz, L. I. (2006). Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *26*(49), 12633–41.

Nita, M. & Grzybowski, A. (2016). The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of

the anterior and posterior eye segments in adults. *Oxidative Medicine and Cellular Longevity*, *2016*, 3164734.

Nomoto, H., Shiraga, F., Kuno, N., Kimura, E., Fujii, S., Shinomiya, K., Nugent, A. K., Hirooka, K. & Baba, T. (2009). Pharmacokinetics of bevacizumab after topical, subconjunctival, and intravitreal administration in rabbits. *Investigative Opthalmology & Visual Science*, *50*(10), 4807.

Nucci, C., Russo, R., Martucci, A., Giannini, C., Garaci, F., Floris, R., Bagetta, G. & Morrone, L. A. (2016). New strategies for neuroprotection in glaucoma, a disease that affects the central nervous system. *European Journal of Pharmacology*, *787*, 119–126.

Olney, J. W. & Ho, O. L. (1970). Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. *Nature*, *227*(5258), 609–611.

Olsen, T. W., Aaberg, S. Y., Geroski, D. H. & Edelhauser, H. F. (1998). Human sclera: thickness and surface area. *American Journal of Ophthalmology*, *125*(2), 237–241.

Palomba, L., Cerioni, L. & Cantoni, O. (2010). Arachidonic acid inhibits neuronal nitric oxide synthase elicited by proinflammatory stimuli and promotes astrocyte survival with both exogenous and endogenous peroxynitrite via different mechanisms. *Journal of Neuroscience Research*, *88*(11), 2459–68.

Particle Science (2009). *Development and validation of in vitro release testing methods for semisolid formulations*. *Technical Brief* (Vol. 10).

Pasutto, F., Matsumoto, T., Mardin, C. Y., Sticht, H., Brandstätter, J. H., Michels-Rautenstrauss, K., Weisschuh, N., Gramer, E., Ramdas, W. D., van Koolwijk, L. M., Klaver, C. C., Vingerling, J. R., Weber, B. H., Kruse, F. E., Rautenstrauss, B., Barde, Y. A. & Reis, A. (2009). Heterozygous NTF4 mutations impairing neurotrophin-4 signaling in patients with primary open-angle glaucoma. *American Journal of Human Genetics*, *85*(4), 447–56.

Patel, S., Rowe, M. J., Winters, S. a & Ohls, R. K. (2008). Elevated erythropoietin mRNA and protein concentrations in the developing human eye. *Pediatr Res*, *63*(4), 394–397.

Peng, Y. W., Blackstone, C. D., Huganir, R. L. & Yau, K. W. (1995). Distribution of glutamate receptor subtypes in the vertebrate retina. *Neuroscience*, *66*(2), 483–97.

Perez, M. T. & Caminos, E. (1995). Expression of brain-derived neurotrophic factor and of its functional receptor in neonatal and adult rat retina. *Neuroscience Letters*, *183*(1–2), 96–9.

Pescina, S., Ferrari, G., Govoni, P., Macaluso, C., Padula, C., Santi, P. & Nicoli, S. (2010). In-vitro permeation of bevacizumab through human sclera: effect of iontophoresis application. *Journal of Pharmacy and Pharmacology*, *62*(9), 1189–1194.

Pescina, S., Govoni, P., Antopolsky, M., Murtomaki, L., Padula, C., Santi, P. & Nicoli, S. (2015). Permeation of proteins, oligonucleotide and dextrans across ocular tissues: experimental studies and a literature update. *Journal of Pharmaceutical Sciences*, *104*(7), 2190–2202.

Ponce, L. L., Navarro, J. C., Ahmed, O. & Robertson, C. S. (2013). Erythropoietin neuroprotection with traumatic brain injury. *Pathophysiology*, *20*(1), 31–38.

Quaranta, L., Katsanos, A., Russo, A. & Riva, I. (2013). 24-hour intraocular pressure and ocular perfusion pressure in glaucoma. *Survey of Ophthalmology*, *58*(1), 26–41.

Quigley, H. A., Dunkelberger, G. R. & Green, W. R. (1989). Retinal ganglion cell atrophy correlated with automated perimetry in human eyes with glaucoma. *American Journal of Ophthalmology*, *107*(5), 453–464.

Quigley, H. A., Nickells, R. W., Kerrigan, L. A., Pease, M. E., Thibault, D. J. & Zack, D. J. (1995). Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Investigative Ophthalmology & Visual Science*, *36*(5), 774–86.

Quigley, H. A. (2005). Glaucoma: macrocosm to microcosm. *Investigative Ophthalmology and Visual Science*, *46*(8), 2663–2670.

Quigley, H. A. & Broman, A. T. (2006). Number of people with glaucoma worldwide. *British Journal of Ophthalmology*, *90*(5), 262–267.

Rabie, T. & Marti, H. H. (2008). Brain protection by erythropoietin: a manifold task. *Physiology (Bethesda, Md.)*, *23*(5), 263–74.

R Core Team (2016). A language and environment for statistical computing. Retrieved from https://www.r-project.org/.

Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. & Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science (New York)*, *262*(5134), 695–700.

Ranta, V. P. & Urtti, A. (2006). Transscleral drug delivery to the posterior eye: Prospects of pharmacokinetic modeling. *Advanced Drug Delivery Reviews*, *58*(11), 1164–1181.

Ranta, V. P., Mannermaa, E., Lummepuro, K., Subrizi, A., Laukkanen, A., Antopolsky, M., Murtomäki, L., Hornof, M. & Urtti, A. (2010). Barrier analysis of periocular drug delivery to the posterior segment. *Journal of Controlled Release*, *148*(1), 42–48.

Rathore, K. & Nema, R. (2009). An insight into ophthalmic drug delivery system. *International Journal of Pharmaceutical Sciences and Drug Research*, *1*, 1–5.

Raviola, G. (1977). The structural basis of the blood-ocular barriers. *Experimental Eye Research*, *25*(1), 27–63.

Remington, L. A. (2012). *Clinical anatomy and physiology of the visual system*. *The mouse nervous system* (3rd ed.). St. Louis, Missouri: Elsevier Inc.

Resende, A. P., São-Braz, B. & Delgado, E. (2012). Expression patterns of erythropoietin in the retina after subconjunctival administration. Abstracts: European Society of Veterinary Ophthalmologists, Prague, Czech Republic, October 14-16, 2011. *Veterinary Ophthalmology.*, *15*(1), 66–70.

Resende, A. P., São-Braz, B. & Delgado, E. (2013). Alternative route for erythropoietin ocular administration. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *251*(8), 2051–6.

Resende, A. P., São Braz, B. & Delgado, E. (2016). Ocular erythropoietin penetration after subconjunctival administration in glaucomatous rats. *Ophthalmic Research*, *56*(2), 104–110.

Resende, A. P., Silva, B., Braz, B. S., Nunes, T., Gonçalves, L. & Delgado, E. (2017). *Ex vivo* permeation of erythropoietin through porcine conjunctiva, cornea, and sclera. *Drug Delivery and Translational Research*, *7*(5), 625–631.

Resende, A. P., Rosolen, S. G., Nunes, T., São-Braz, B., & Delgado, E. (2018). Functional and structural effects of erythropoietin subconjunctival administration in glaucomatous animals. *Biomed Hub, 3*, 488970.

Rex, T. S., Allocca, M., Domenici, L., Surace, E. M., Maguire, A. M., Lyubarsky, A., Cellerino, A., Bennett, J. & Auricchio, A. (2004). Systemic but not intraocular Epo gene transfer protects the retina from light-and genetic-induced degeneration. *Molecular Therapy*, *10*(5), 855–861.

Rex, T. S., Wong, Y., Kodali, K. & Merry, S. (2009). Neuroprotection of photoreceptors by direct delivery of erythropoietin to the retina of the retinal degeneration slow mouse. *Experimental Eye Research*, *89*(5), 735–40.

Rezaie, T., Child, A., Hitchings, R., Brice, G., Miller, L., Coca-Prados, M., Héon, E., Krupin, T., Ritch, R., Kreutzer, D., Crick, R. P. & Sarfarazi, M. (2002). Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science (New York)*, *295*(5557), 1077–9.

Ritch, R., Liebmann, J. & Tello, C. (1995). A construct for understanding angle-closure glaucoma. *Ophthalmology Clinics of North America*, *8*(2), 281–293.

Ritch, R., Shields, M. B. & Krupin, T. (1996). *The glaucomas: clinical science* (2nd ed). Mosby.

Rosolen, S. G., Rigaudiere, F., Le Gargasson, J. F. & Brigell, M. G. (2005). Recommendations for a toxicological screening ERG procedure in laboratory animals. *Documenta Ophthalmologica*, *110*(1), 57–66.

Rusanen, E., Florin, M., Hässig, M. & Spiess, B. M. (2010). Evaluation of a rebound tonometer (Tonovet) in clinically normal cat eyes. *Veterinary Ophthalmology*, *13*(1), 31–6.

Saelens, X., Festjens, N., Walle, L. Vande, Gurp, M. van, Loo, G. van & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene*, *23*(16), 2861–2874.

Sahoo, S. K., Dilnawaz, F. & Krishnakumar, S. (2008). Nanotechnology in ocular drug delivery. *Drug Discovery Today*, *13*(3–4), 144–51.

Salinas-Navarro, M., Alarcón-Martínez, L., Valiente-Soriano, F. J., Ortín-Martínez, A., Jiménez-López, M., Avilés-Trigueros, M., Villegas-Pérez, M. P., Villa, P. & Vidal-Sanz, M. (2009). Functional and morphological effects of laser-induced ocular hypertension in retinas of adult albino Swiss mice. *Molecular Vision*, *15*, 2578–98.

Samuelson, D., Smith, P. & Brooks, D. (1989). Morphologic features of the aqueous humor drainage pathways in horses. *American Journal of Veterinary Research*, *50*(5), 720–7.

Sättler, M. B., Merkler, D., Maier, K., Stadelmann, C., Ehrenreich, H., Bähr, M. & Diem, R. (2004). Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis. *Cell Death and Differentiation*, *11 Suppl 2*, S181-92.

Segal, R. A. & Greenberg, M. E. (1996). Intracellular signaling pathways activated by neuropathic factors. *Annual Review of Neuroscience*, *19*(1), 463–489.

Seki, M. & Lipton, S. A. (2008). Targeting excitotoxic/free radical signaling pathways for therapeutic intervention in glaucoma. *Progress in Brain Research*, *173*(8), 495–510.

Semenza, G. L. & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology*, *12*(12), 5447–5454.

Semenza, G. L. (2000). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *88*(4), 1474–80.

Shah, S. S., Tsang, S. H. & Mahajan, V. B. (2009). Erythropoetin receptor expression in the human diabetic retina. *BMC Research Notes*, *2*(1), 234.

Shahidullah, M., Wilson, W. S., Yap, M. & To, C. (2003). Effects of ion transport and channel-blocking drugs on aqueous humor formation in isolated bovine eye. *Investigative Opthalmology & Visual Science*, *44*(3), 1185.

Shareef, S. R., Garcia-Valenzuela, E., Salierno, A., Walsh, J. & Sharma, S. C. (1995). Chronic ocular hypertension following episcleral venous occlusion in rats. *Experimental Eye Research*, *61*(3), 379–82.

Shingo, T., Sorokan, S. T., Shimazaki, T. & Weiss, S. (2001). Erythropoietin regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(24), 9733–43.

Shirley Ding, S. L., Leow, S. N., Munisvaradass, R., Koh, E. H., Bastion, M. L. C., Then, K. Y., Kumar, S. & Mok, P. L. (2016). Revisiting the role of erythropoietin for treatment of ocular disorders. *Eye*, (June), 1–17.

Sirén, A. L., Fratelli, M., Brines, M., Goemans, C., Casagrande, S., Lewczuk, P., Keenan, S., Gleiter, C., Pasquali, C., Capobianco, A., Mennini, T., Heumann, R., Cerami, A., Ehrenreich, H. & Ghezzi, P. (2001). Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(7), 4044–9.

Smith, P. J., Samuelson, D. A., Brooks, D. E. & Whitley, R. D. (1986). Unconventional aqueous humor outflow of microspheres perfused into the equine eye. *American Journal of Veterinary Research*, *47*(11), 2445–53.

Sola, A., Rogido, M., Lee, B. H., Genetta, T. & Wen, T. C. (2005). Erythropoietin after focal cerebral ischemia activates the Janus kinase–signal transducer and activator of transcription signaling pathway and improves brain injury in postnatal day 7 rats. *Pediatric Research*, *57*(4), 481–487.

Song, B. J., Cai, H., Tsai, J. C., Chang, S., Forbes, M. & Del Priore, L. V. (2008). Intravitreal recombinant human erythropoietin: a safety study in rabbits. *Current Eye Research*, *33*(9), 750–60.

Squadrito, F., Altavilla, D., Squadrito, G., Campo, G. M., Arlotta, M., Quartarone, C., Saitta, A. & Caputi, A. P. (1999). Recombinant human erythropoietin inhibits iNOS activity and reverts vascular dysfunction in splanchnic artery occlusion shock. *British Journal of Pharmacology*, *127*(2), 482–488.

Srirangam, R. & Majumdar, S. (2012). Transscleral drug delivery to the posterior segment of the eye : Particulate and colloidal formulations and biopharmaceutical considerations. *Advances in Ocular Drug Delivery, 37*(661), 33-63.

Stone, E. M., Fingert, J. H., Alward, W. L., Nguyen, T. D., Polansky, J. R., Sunden, S. L., Nishimura, D., Clark, A. F., Nystuen, A., Nichols, B. E., Mackey, D. A., Ritch, R., Kalenak, J. W., Craven, E. R. & Sheffield, V. C. (1997). Identification of a gene that causes primary open angle glaucoma. *Science (New York)*, *275*(5300), 668–70.

Strom, A. R., Hässig, M., Iburg, T. M. & Spiess, B. M. (2011a). Epidemiology of canine glaucoma presented to University of Zurich from 1995 to 2009. Part 1: Congenital and primary glaucoma (4 and 123 cases). *Veterinary Ophthalmology*, *14*(2), 121–126.

Strom, A. R., Hässig, M., Iburg, T. M. & Spiess, B. M. (2011b). Epidemiology of canine glaucoma presented to University of Zurich from 1995 to 2009. Part 2: secondary glaucoma (217 cases). *Veterinary Ophthalmology*, *14*(2), 127–132.

Suárez, F., Jockovich, M. E., Hernandez, E., Feuer, W., Parel, J. M. & Murray, T. G. (2007). Paclitaxel in the treatment of retinal tumors of LH beta-Tag murine transgenic model of retinoblastoma. *Investigative Ophthalmology and Visual Science*, *48*(8), 3437–3440.

Sugano, K., Kansy, M., Artursson, P., Avdeef, A., Bendels, S., Di, L., Di, L., Ecker, G. F., Faller, B., Fischer, H., Gerebtzoff, G., Lennernaes, H. & Senner, F. (2010). Coexistence of passive and carrier-mediated processes in drug transport. *Nature Reviews. Drug Discovery*, *9*(8), 597–614.

Sugiyama, T., Moriya, S., Oku, H. & Azuma, I. (1995). Association of endothelin-1 with normal tension glaucoma: Clinical and fundamental studies. *Survey of Ophthalmology*, *39*, S49–S56.

Sühs, K. W., Hein, K., Sättler, M. B., Görlitz, A., Ciupka, C., Scholz, K., Käsmann-Kellner, B., Papanagiotou, P., Schäffler, N., Restemeyer, C., Bittersohl, D., Hassenstein, A., Seitz, B., Reith, W., Fassbender, K., Hilgers, R., Heesen, C., Bähr, M. & Diem, R. (2012). A randomized, double-blind, phase 2 study of erythropoietin in optic neuritis. *Annals of Neurology*, *72*(2), 199–210.

Sullivan, T. a, Geisert, E. E., Templeton, J. P. & Rex, T. S. (2012). Dose-dependent treatment of optic nerve crush by exogenous systemic mutant erythropoietin. *Experimental Eye Research*, *96*(1), 36–41.

Szabo, A., Vegvari, D., Deak, G., Lukats, A., Berta, I. A. & Szel, A. (2008). The expression of erythropoietin and its receptor in the developing rat retina. *Invesiigative Ophthalmology & Visual Science*, *49*(13), 5896.

Tachikawa, M., Takeda, Y., Tomi, M. & Hosoya, K. (2010). Involvement of OCTN2 in the transport of acetyl-l-carnitine across the inner blood-retinal barrier. *Investigative Opthalmology & Visual Science*, *51*(1), 430.

Tamm, E. R. (2002). Myocilin and glaucoma: facts and ideas. *Progress in Retinal and Eye Research*, *21*(4), 395–428.

Tang, Z., Sun, X., Huo, G., Xie, Y., Shi, Q., Chen, S., wang, X. & Liao, Z. (2013). Protective effects of erythropoietin on astrocytic swelling after oxygen–glucose deprivation and reoxygenation: Mediation through AQP4 expression and MAPK pathway. *Neuropharmacology*, *67*, 8–15.

Teng, K. K. & Hempstead, B. L. (2004). Neurotrophins and their receptors: signaling trios in complex biological systems. *Cellular and Molecular Life Sciences (CMLS)*, *61*(1), 35–48.

Tezel, G. & Wax, M. B. (2000). Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(23), 8693–700.

Tezel, G., Li, L. Y., Patil, R. V & Wax, M. B. (2001). TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Investigative Ophthalmology & Visual Science*, *42*(8), 1787–94.

Tezel, G., Yang, X., Yang, J. & Wax, M. B. (2004). Role of tumor necrosis factor receptor-1 in the death of retinal ganglion cells following optic nerve crush injury in mice. *Brain Research*, *996*(2), 202–12.

Tezel, G. (2006). Oxidative stress in glaucomatous neurodegeneration: Mechanisms and consequences. *Progress in Retinal and Eye Research*, *25*(5), 490–513.

Tian, K., Shibata-Germanos, S., Pahlitzsch, M. & Cordeiro, M. F. (2015). Current perspective of neuroprotection and glaucoma. *Clinical Ophthalmology*, *9*, 2109–2118.

Topper, J. E. & Brubaker, R. F. (1985). Effects of timolol, epinephrine, and acetozolomide on aqueous flow during sleep. *Investigative Ophthalmology & Visual Science*, *26*(10), 1315–1319.

Tsai, J. C., Wu, L., Worgul, B., Forbes, M. & Cao, J. (2005). Intravitreal administration of erythropoietin and preservation of retinal ganglion cells in an experimental rat model of glaucoma. *Current Eye Research*, *30*(11), 1025–31.

Tsai, J. C., Song, B. J., Wu, L. & Forbes, M. (2007). Erythropoietin: a candidate neuroprotective agent in the treatment of glaucoma. *Journal of Glaucoma*, *16*(6), 567–71.

Tsai, J. C. (2008). Safety of intravitreally administered recombinant erythropoietin (an AOS thesis). *Transactions of the American Ophthalmological Society*, *106*, 459–72.

Tsuji, A., Tamai, I. & Sasaki, K. (1988). Intraocular penetration kinetics of prednisolone after subconjunctival injection in rabbits. *Ophthalmic Research*, *20*, 31–43.

Uzüm, G., Sarper Diler, A., Bahçekapili, N. & Ziya Ziylan, Y. (2006). Erythropoietin prevents the increase in blood-brain barrier permeability during pentylentetrazol induced seizures. *Life Sciences*, *78*(22), 2571–6.

Vasudevan, S. K., Gupta, V. & Crowston, J. G. (2011). Neuroprotection in glaucoma. *Indian Journal of Ophthalmology*, *59 Suppl*(7), S102-13.

Vecino, E., Caminos, E., Ugarte, M., Martín-Zanca, D. & Osborne, N. N. (1998). Immunohistochemical distribution of neurotrophins and their receptors in the rat retina and the effects of ischemia and reperfusion. *General Pharmacology*, *30*(3), 305–14.

Villa, P., Bigini, P., Mennini, T., Agnello, D., Laragione, T., Cagnotto, A., Viviani, B., Marinovich, M., Cerami, A., Coleman, T. R., Brines, M. & Ghezzi, P. (2003). Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *The Journal of Experimental Medicine*, *198*(6), 971–5.

Vohra, R., Tsai, J. C. & Kolko, M. (2013). The role of inflammation in the pathogenesis of glaucoma. *Survey of Ophthalmology*, *58*(4), 311–320.

Wang, W. H., McNatt, L. G., Pang, I. H., Hellberg, P. E., Fingert, J. H., McCartney, M. D. & Clark, A. F. (2008). Increased expression of serum amyloid A in glaucoma and its effect on intraocular pressure. *Investigative Ophthalmology & Visual Science*, *49*(5), 1916–23.

Wang, Z., Shen, L., Tu, L., Hu, D., Liu, G. Y., Zhou, Z. L., Lin, Y., Chen, L. H. & Qu, J. (2009). Erythropoietin protects retinal pigment epithelial cells from oxidative damage. *Free Radical Biology & Medicine*, *46*(8), 1032–41.

Wei, L., Han, B. H., Li, Y., Keogh, C. L., Holtzman, D. M. & Yu, S. P. (2006). Cell death mechanism and protective effect of erythropoietin after focal ischemia in the whisker-barrel cortex of neonatal rats. *The Journal of Pharmacology and Experimental Therapeutics*, *317*(1), 109–16.

Weijtens, O., Schoemaker, R. C., Lentjes, E. G., Romijn, F. P., Cohen, A. F. & van Meurs, J. C. (2000). Dexamethasone concentration in the subretinal fluid after a subconjunctival injection, a peribulbar injection, or an oral dose. *Ophthalmology*, *107*(10), 1932–8.

Weiner, A. L. & Gilger, B. C. (2010). Advancements in ocular drug delivery. *Veterinary Ophthalmology*, *13*(6), 395–406.

Weinreb, R. N., Aung, T. & Medeiros, F. A. (2014). The pathophysiology and treatment of glaucoma. *Journal of American Medical Association*, *311*(18), 1901.

Weishaupt, J. H. (2004). Effect of erythropoietin axotomy-induced apoptosis in rat retinal ganglion cells. *Investigative Ophthalmology & Visual Science*, *45*(5), 1514–1522.

Wen, H., Hao, J. & Li, S. K. (2013). Characterization of human sclera barrier properties for transscleral delivery of bevacizumab and ranibizumab. *Journal of Pharmaceutical Sciences*, *102*(3), 892–903.

Wolfs, R. C., Klaver, C. C., Ramrattan, R. S., van Duijn, C. M., Hofman, A. & de Jong, P. T. (1998). Genetic risk of primary open-angle glaucoma. Population-based familial

aggregation study. *Archives of Ophthalmology (Chicago, Ill. : 1960)*, *116*(12), 1640–5.

Wu, L., Chang, S., Chen, Y., Xia, Q., Forbes, M. & Tsai, J. C. (2008). Erythropoietin receptor plays a role in the cell differentiation of retina and lens during eye development. *Investigative Ophthalmology & Visual Science*, *49*(13), 3078–3078.

Wu, Q., Zhang, M., Song, B., Lu, B. & Hu, P. (2007). Expression of ciliary neurotrophic factor after induction of ocular hypertension in the retina of rats. *Chinese Medical Journal*, *120*(20), 1825–9.

Wu, Y., Shang, Y., Sun, S. & Liu, R. (2007). Antioxidant effect of erythropoietin on 1-methyl-4-phenylpyridinium-induced neurotoxicity in PC12 cells. *European Journal of Pharmacology*, *564*(1–3), 47–56.

Xie, Z., Chen, F., Wu, X., Zhuang, C., Zhu, J., Wang, J., … Hua, X. (2012). Effects of supplemental erythropoietin on its receptor expression and signal transduction pathways in rat model of retinal detachment. *Current Eye Research*, *37*(2), 138–144.

Xie, Z., Chen, F., Wu, X., Zhuang, C., Zhu, J., Wang, J., … Hua, X. (2012). Safety and efficacy of intravitreal injection of recombinant erythropoietin for protection of photoreceptor cells in a rat model of retinal detachment. *Eye*, *26*(1), 144–152.

Yan, Q., Wang, J., Matheson, C. R. & Urich, J. L. (1999). Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of axotomized retinal ganglion cells in adult rats: comparison to and combination with brain-derived neurotrophic factor (BDNF). *Journal of Neurobiology*, *38*(3), 382–90.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I. Jones, D. P. & Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science (New York)*, *275*(5303), 1129–32.

Zeevalk, G. D. & Nicklas, W. J. (1992). Evidence that the loss of the voltage-dependent Mg2+ block at the N-methyl-D-aspartate receptor underlies receptor activation during inhibition of neuronal metabolism. *Journal of Neurochemistry*, *59*(4), 1211–20.

Zhang, C., Wang, H., Nie, J. & Wang, F. (2014). Protective factors in diabetic retinopathy: focus on blood-retinal barrier. *Discovery Medicine*, *18*(98), 105–12.

Zhang, F., Signore, A. P., Zhou, Z., Wang, S., Cao, G. & Chen, J. (2006). Erythropoietin protects CA1 neurons against global cerebral ischemia in rat: potential signaling mechanisms. *Journal of Neuroscience Research*, *83*(7), 1241–51.

Zhang, F., Wang, S., Cao, G., Gao, Y. & Chen, J. (2007). Signal transducers and activators of transcription 5 contributes to erythropoietin-mediated neuroprotection against hippocampal neuronal death after transient global cerebral ischemia. *Neurobiology of Disease*, *25*(1), 45–53.

Zhang, J., Wu, Y., Jin, Y., Ji, F., Sinclair, S. H., Luo, Y., Xu, G., Lu, L., Dai, W., Yanoff, M., Li, W. & Xu, G. T. (2008). Intravitreal injection of erythropoietin protects both retinal vascular and neuronal cells in early diabetes. *Investigative Ophthalmology and Visual Science*, *49*(2), 732–742.

Zhang, J., Hu, L. M., Xu, G., Wu, Y., Shen, J., Luo, Y., Zhong, Y., Sinclair, S. H., Yanoff, M., Li, W. & Xu, G. T. (2010). Anti-VEGF effects of intravitreal erythropoietin in early diabetic retinopathy. *Frontiers in Bioscience (Elite Edition)*, *2*, 912–27.

Zhang, S. X., Sima, J., Wang, J. J., Shao, C., Fant, J. & Ma, J. X. (2005). Systemic and periocular deliveries of plasminogen kringle 5 reduce vascular leakage in rat models of oxygen-induced retinopathy and diabetes. *Current Eye Research*, *30*(8), 681–9.

Zhong, L., Bradley, J., Schubert, W., Ahmed, E., Adamis, A. P., Shima, D. T., Robinson, G. S. & Ng, Y. S. (2007). Erythropoietin promotes survival of retinal ganglion cells in DBA/2J glaucoma mice. *Investigative Ophthalmology & Visual Science*, *48*(3), 1212–8.

Zhong, Y. S., Yao, H., Deng, L., Cheng, Y. & Zhou, X. (2007). Promotion of neurite outgrowth and protective effect of erythropoietin on the retinal neurons of rats. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *245*(12), 1859–67.

Zhong, Y. S., Liu, X. H., Cheng, Y. & Min, Y. J. (2008). Erythropoietin with retrobulbar administration protects retinal ganglion cells from acute elevated intraocular pressure in rats. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *24*(5), 453–9.

Zhu, A., Martosella, J. & Duong, P. T. (2013). Peptide mapping of glycoprotein erythropoietin by HILIC LC-MS and RPLC-MS. *LC-GC North America*, 26(7), 8–10.

Zimmerman, T. J. (1993). Topical ophthalmic beta blockers: a comparative review. *Journal of Ocular Pharmacology*, *9*(4), 373–84.

# ANNEXES a

# Annexe I

## ERYTHROPOIETIN REACHES THE RETINA AFTER SUBCONJUNCTIVAL ADMINISTRATION

**Resende AP (1), São Braz B (1), Delgado E (1)**

(1) CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. Da Universidade Técnica, 1300-477 Lisboa

### Purpose

Erythropoietin (EPO), a naturally occurring cytokine known by its erythropoietic properties, has been recently shown to have neuroprotective and neuroregenerative effects. EPO is actually considered a promising therapeutic agent for ischemic retinal diseases, such as glaucoma, where the retinal ganglion cells (RGCs) death results in the progressive loss of visual function. All the previous studies used the systemic, intravitreal or retrobulbar administration routes to achieve therapeutic concentrations on the retina, all of these difficult to use in clinical practice. We aimed to investigate the subconjunctival injection as an alternative ocular delivery route for EPO administration.

### Material & Methods

Wistar Hannover female albino rats (n=9) were used. Complete ophthalmic examinations were carried out before and immediatly after the injections (Fig. 1). 1000 IU of recombinant erythropoietin (NeoRecormon®, Roche Faramcêutica Química, Amadora, Portugal) were administered subconjunctivally, under general anaesthesia, in the right eye of each animal while the left eye served as control (Fig. 2) . Rats were humanely euthanized and eyes were enucleated at 12, 24 and 36 hours after EPO administration. Immunohistochemistry was used to detect EPO expression in the different retinal cell layers (sc-1310 , Santa Cruz Biotechnology; sc-2094, Santa Cruz Biotechnology).

### Results

The evaluation of EPO expression after subconjunctival administration yielded an immunostaining signal in the rat's retinas. Twelve hours after the subconjunctival administration, EPO was detected in all retinal cell layers, showing a higher concentration in the photoreceptor layer (Fig. 3). Twenty four hours after EPO administration the signal was concentrated in the RGCs layer (Fig. 4, 5) and 36 hours after EPO administration the presence of the protein, detected by immunohistochemistry, was residual (Fig. 6). EPO was not detected in any of the control eyes.

### Conclusion & Discussion

After subconjunctival injection EPO reached all the neuroretinal cell layers in an animal rat model. Our findings prove that the subconjunctival administration is a possible alternative ocular EPO delivery route. Further studies are necessary to assess the kinetics of subconjunctival administration of EPO, both in physiological conditions and ischemic ocular disease conditions.

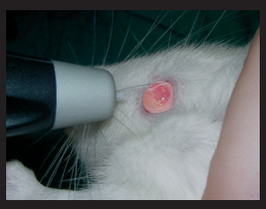

Fig. 1 – Rebound tonometry (Tonolab®, Icare) during the ophthalmic examination.

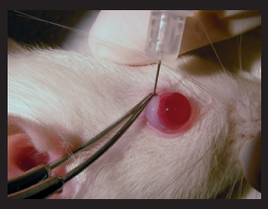

Fig. 2 – Subconjunctival administration of EPO under general anesthesia.

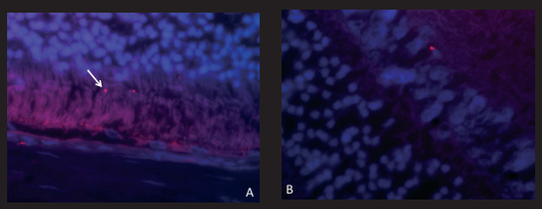


Fig. 3 –The immunohistochemical staining images of EPO 12 hours after subconjunctival administration. An EPO immunoreactivity staining (arrow) was observed in the photoreceptors layer (A) and in some cases in the inner nuclear layer (B) (Immunohistochemistry staining with rhodamine conjugated secondary antibody, 1000 x).

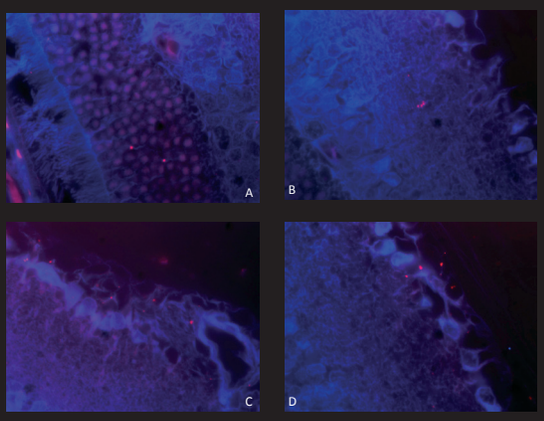


Fig. 4 –The immunohistochemical staining images of EPO 24 hours after subconjunctival administration. An EPO immunoreactivity staining was observed in several retina layers, including the outer nuclear layer (A) and the inner plexiform layer (B). A strong immunoreactivity staining was observed in the RGC (C, D) (Immunohistochemistry staining with rhodamine conjugated secondary antibody, 1000 x).

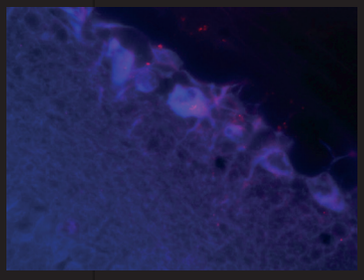

Fig. 5 – The immunohistochemical staining images of EPO 24 hours after subconjunctival administration. Immunofluorescence staining of RGC showing cytoplasmic staining (Immunohistochemistry staining with rhodamine conjugated secondary antibody, 1000 x).

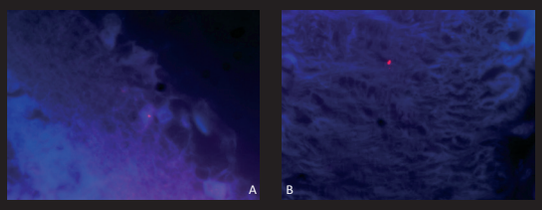


Fig. 6 –The immunohistochemical staining images of EPO 36 hours after subconjunctival administration. The EPO immunoreactivity staining was residual but it was still present in the RGC layer (A) and in the optic nerve (B) (Immunohistochemistry staining with rhodamine conjugated secondary antibody, 1000 x).

Acknowledgements: CIISA (Centro de Investigação Interdisciplinar em Sanidade Animal).

Champalimaud Neuroscience Symposium 2011

# Annexe II

## EXPRESSION PATTERNS OF ERYTHROPOIETIN IN THE RETINA AFTER SUBCONJUNCTIVAL ADMINISTRATION

A.P. Resende[1], B. São-Brás[1], E. Delgado[1]
1CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa

**Purpose:** Recently Erythropoietin (EPO) has been shown to have neuroprotective and neuroregenerative effects on retinal ganglion cells (RGC), apart from its erythropoietic properties. For this reason EPO is actually considered a promising therapeutic agent for ischemic retinal diseases. The death of RGC, which results in the progressive loss of visual function, occurs in glaucoma and other ocular diseases caused by hypoxia and ischemia. All the previous studies used the systemic, intravitreal or retrobulbar administration to acquire therapeutic concentrations on the retina, both difficult to use in clinical practice. With the present study we evaluated the efficacy of the subconjunctival injection as an alternative ocular delivery route for EPO.

**Material/Methods:** Wistar Hannover female albino rats were used (n=6). Using a 27 Gauge needle and a surgical microscope, EPO (NeoRecormon®, Roche Faramcêutica Química, Amadora, Portugal) in a dosage of 1000 IU was administered into each of the rat's eyes, under general anesthesia. The subconjunctival route was used in the right eye of each animal and the intravitreal route was used in the left eye of each animal. A control group was also included in the study (n=2). All rats were humanely euthanized and the eyes were enucleated at 12, 24 and 36 hours after EPO administration. The location of the EPO protein was immunohistochemically analyzed in the retinas.

**Results:** The evaluation of EPO expression in the animal's retinas after subconjunctival an intravitreal administration yielded a strong immunostaining signal. Among the retina's several layers, EPO expression was more evident in the RGC layer with both administration routes

used. With the intravitreal EPO administration route, the immunostaining signal was stronger in the RGC layer and its expression was lower in the vitreous. With the subconjunctival administration route we observed a strong signal in the RGC layer despite the presence of a low signal expression in all neuroretinal layers, and almost an absence of EPO signal in the vitreous.

**Conclusion/Discussion:** The subconjunctival injection of EPO allowed the delivery of EPO to RGC and all neuroretinal layers. Our findings prove that the subconjunctival route is a possible alternative route of administration for EPO on ischemic retinal diseases. Further studies are necessary to assess the kinetics of subconjunctival administration of EPO, both in physiological conditions and ischemic ocular disease conditions.

# Annexe III

## IMMUNOHISTOCHEMISTRY OF THE RAT'S RETINA AFTER SUBCONJUNCTIVAL ERYTHROPOIETIN ADMINISTRATION

Ana Paula Resende[1], Berta São Braz[1] & Esmeralda Delgado[1]

[1] CIISA, Faculty of Veterinary Medicine, Technical University of Lisbon, Alameda da Universidade Técnica, 1300-477 Lisbon, Portugal

**Purpose:** Due to its neuroprotective and neuroregenerative properties, Erythropoietin (EPO) is a promising drug to preserve vision in glaucoma patients. EPO ocular penetration and potential side effects on haematocrit after its subconjunctival (SCO) injection were assessed in a normotensive rat model.

**Methods:** Wistar Hannover female albino rats (n=42) divided into 7 groups, were used. One group (n=6) served as control. Six groups of 6 animals (n=36) received 1000 IU of EPO (NeoRecormon 5000®, Roche Diagnostics GmbH, Manheim, Germany) through the SCO route in one eye. Rats' human euthanasia and eyes enucleations were performed at 12, 24, 36, 48 and 60 hours after EPO administration. Retinal EPO distribution was analyzed by immunohistochemistry. Another group (n=6) was used to blood samples collection and haematocrit analysis at 0, 7, 14, 21 and 28 days after EPO administration.

**Results:** EPO retinal expression after subconjunctival administration yielded a strong immunostaining signal in several neuronal cells in all retinal layers. EPO expression was more evident in Retinal Ganglion Cell layer 24 hours after the administration but was present till the end of the study (60 hours). Subconjunctival EPO administration did not cause any significant changes in the haematocrit values.

**Conclusion:** When administered subconjunctivally EPO reached retinal ganglion cell layers 24 hours after administration and was still present at least until 60 hours. Considering that this administration didn’t cause any haematopoietic significant side effects the SCO route showed to be a promising alternative for EPO ocular delivery. Further studies will be performed in a glaucoma animal model (rat).

# Annexe IV

(in portuguese)

## ERITROPOIETINA POR VIA SUBCONJUNTIVAL – ALTERNATIVA PROMISSORA NO GLAUCOMA CANINO?

A. P. Resende[1], B. São-Braz[1] e E. Delgado[1]

[1] CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. Universidade Técnica, 1300-477 Lisboa

O glaucoma é uma causa frequente de cegueira irreversível no cão devido à morte por apoptose das células ganglionares da retina (RGC). Estudos recentes demonstram que a Eritropoietina (EPO), para além do seu conhecido efeito eritropoiético, tem acção neuroprotectora e neuroregenerativa sobre as RGC, sendo considerada um promissor agente terapêutico no glaucoma.

O presente estudo teve como objetivo avaliar a penetração ocular da EPO através da via subconjuntival e os potenciais efeitos secundários ao nível do hematócrito.

Foram utilizados ratos Wistar Hanover (n=42) divididos em 7 grupos (n=6). Seis grupos (n=36) receberam 1000 UI de EPO através da via subconjuntival num dos olhos e, de acordo com o grupo, foram eutanasiados às 12, 24, 36, 48 e 60 horas, num total de 30 animais. Foi realizada enucleação bilateral e a distribuição da proteína EPO nas retinas foi analisada por imuno-histoquímica. Num grupo de animais (n=6) foi realizado hematócritos aos 0, 7, 14, 21 e 28 dias após a administração subconjuntival de EPO. Um grupo (n=6) serviu como controle.

A EPO foi identificada por imuno-marcação em diferentes camadas da retina, sendo mais evidente na camada de RGC 24 horas após a administração e mantendo-se presente até ao

final do estudo (60 horas). Não se verificaram efeitos adversos da administração subconjuntival de EPO a nível ocular nem alterações significativas nos valores de hematócrito durante um período de 28 dias nos animais em estudo.

Estudos anteriores utilizaram as vias sistémica, intravítrea ou retrobulbar para atingirem concentrações terapêuticas de EPO ao nível da retina, sendo que todas elas apresentam efeitos secundários ou riscos para o paciente. Neste estudo foi demonstrado que a EPO, quando administrada pela via subconjuntival, atinge as camadas de células ganglionares da retina, sem efeitos secundários ao nível do hematócrito. Esta nova via mostrou ser uma alternativa segura para administração de EPO a nível ocular, o que poderá representar uma terapêutica promissora nas doenças isquémicas retinianas no cão.

# Annexe V

## ELECTRORRETINOGRAPHIC CHANGES AFTER ERYTHROPOIETIN SUBCONJUNCTIVAL ADMINISTRATION IN GLAUCOMATOUS ANIMALS

A.P. Resende[1], S. Rosolen[2,3], T. Nunes[1], B. São-Brás[1], E. Delgado[1]

[1] CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa

[2] Sorbonne Universités, UPMC Univ Paris 06, UMR_S 968, Institut de la Vision, Paris, France

[3] Clinique Veterinaire Voltaire, Asnières sur Seine, France

**Purpose:** to assess potential benefits of erythropoietin (EPO) in the retina of glaucomatous rats by electroretinography (ERG).

**Methods:** Glaucoma was induced by coagulation of three episcleral veins in one eye of Wistar Hannover female albino rats (n=14). One group (n=7) received a unique subconjunctival injection of 1000 IU of EPO in the glaucomatous eye while the other group (n=7) received only saline, which served as control. In all animals, binocular full-field ERGs photopic and scotopic ERGs were recorded under general anesthesia at 21 days after glaucoma induction. Rods response was elicited in scotopic conditions after a 12 hours dark adaptation period with the use of dim flashes (intensity:-3.02 log cds/m2). Cones response was elicited in photopic conditions with the use of flashes (0.98 log cds/m2) and 6.2 Hz flicker. ERG parameters were measured for each eye.

**Results:** In scotopic conditions, b-wave amplitude mean (SD) values were 152.4±49.6 µV in treated group and 133.3±38.8 µV in control group, respectively. In photopic conditions, wave amplitude mean values (SD) for flashes and flicker, in treated and control group, were 73.8±19.1µ and 55.5±11.7µVand, 59.7±18.9 µV and 45.2±17.1 µV, respectively. These differences were statistically significant ($p<0.05$) in photopic conditions.

**Discussion:** Subconjunctival EPO administration in glaucomatous animals seems to have beneficial effects on both cones and rods and their outputs after glaucoma induction Studies are in progress in order to correlate structure and function and to assess EPO neuroprotective and neuroregenerative effects in these conditions.

# Annexe VI

(in portuguese)

## ALTERAÇÕES NA ELETRORRETINOGRAFIA EM RATOS GLAUCOMATOSOS APÓS ADMINISTRAÇÃO DE ERITROPOIETINA POR VIA SUBCONJUNTIVAL

A.P. Resende[1], S. Rosolen[2,3], T. Nunes[1], B. São-Brás[1], E. Delgado[1]

[1] CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa

[2] Sorbonne Universités, UPMC Univ Paris 06, UMR_S 968, Institut de la Vision, Paris, France

[3] Clinique Veterinaire Voltaire, Asnières sur Seine, France

O glaucoma é a principal causa de cegueira irreversível no mundo. A morte das células ganglionares da retina (RGC) resulta numa perda progressiva de visão e ocorre tanto no glaucoma como noutras doenças oculares, causadas por hipóxia e isquémia. Estudos recentes demonstraram que a eritropoietina (EPO), para além do seu conhecido efeito eritropoiético, tem ação neuroprotectora e neuroregenerativa sobre as RGC, sendo actualmente considerada um agente terapêutico promissor nas doenças isquémicas retinianas. Os estudos já efectuados utilizam as vias sistémica, intravítrea ou retrobulbar para atingirem concentrações terapêuticas ao nível da retina, sendo pouco exequíveis na prática clínica. Com este projecto pretende-se estudar a via de administração subconjuntival em condições de glaucoma, num modelo experimental.

Após indução experimental de glaucoma em ratos, estudamos os potenciais benefícios da administração de eritropoietina (EPO) ao nível da retina utilizando a electrorretinografia (ERG). O glaucoma foi induzido experimentalmente por coagulação de três veias episclerais num dos olhos de ratos albinos Wistar Hannover (n=14). Um grupo (n=7) recebeu uma injecção subconjuntival única de 1000 UI de EPO no olho glaucomatoso enquanto que o outro grupo (n=7) recebeu apenas soro fisiológico, e serviu como controlo. Em todos os animais foram

efectuados ERGs binoculares de campo total em condições fotópicas e escotópicas, aos 0 dias e 21 dias após a indução de glaucoma. A resposta dos bastonetes foi avaliada em condições escotópicas, depois de um período de adaptação ao escuro de 12 horas, com o uso de flashes de intensidade -3,02 log cd / m2. A resposta dos cones foi avaliada em condições fotópicas com o uso de flashes de intensidade 0,98 log cd / m2 e de um flicker de 6,2 Hz. Foram avaliados os parâmetros de ERG para os 2 olhos de todos os animais. O estudo estatístico foi efectuado recorrendo à análise de variância para medidas repetidas e os resultados são apresentados como média±desvio padrão.

Em condições escotópicas, a amplitude média da onda b foi de 152,4 ± 49,6 μV no grupo tratado e 133,3 ± 38,8 μV no grupo controlo. Em condições fotópicas, a amplitude média da onda b (flash) foi de 73,8 ± 19.1μV nos ratos tratados e 55,5 ± 11.7μV no controlo e a amplitude média da onda b (flicker) foi de 59,7 ± 18,9 μV nos animais tratados e e 45,2 ± 17,1 μV nos animais controlo. Estas diferenças foram estatisticamente significativas ($p < 0,05$).

A administração subconjuntival de EPO em animais glaucomatosos teve efeitos benéficos na retina, tanto ao nível da resposta dos cones como dos bastonetes. Estes resultados permitem correlacionar a estrutura e a função da retina. Com este estudo esperamos dar um contributo valioso para a futura utilização da EPO no glaucoma tanto em animais como humanos.

# Annexe VII

(in portuguese)

## PERMEABILIDADE DE TRÊS MEMBRANAS OCULARES À ERITROPOIETINA NUM MODELO *IN VITRO* DE OLHO DE PORCO

B. Silva[1], A.P. Resende[1], L. Gonçalves[2], T. Nunes[1], B. São-Brás[1], E. Delgado[1]

[1] CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa

[2] iMed, Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal.

**Introdução:** Recentemente, a eritropoietina (EPO) revelou propriedades neuro-protetoras e neuro-regenerativas na retina devido a um efeito anti-apoptótico [1,2]. Estudos prévios demonstraram que a eritropoietina recombinante humana (rHuEPO) se distribui nas várias camadas da retina após administração subconjuntival [1,3,4], não induzindo efeitos sistémicos significativos [3]. Apesar da administração subconjuntival ser, atualmente, a melhor forma de evitar os efeitos sistémicos da EPO, a aplicação tópica de gotas oftálmicas seria o método de eleição. Por conseguinte, é de extrema importância compreender a permeação da EPO através das principais barreiras oculares, nomeadamente conjuntiva, esclera e córnea.
Objetivos: O objetivo deste estudo foi testar a permeabilidade da conjuntiva, esclera e córnea à rHuEPO num modelo de olho de porco *in vitro*.

**Metodologia:** Olhos frescos de porco (n=60) foram colhidos no matadouro, e mantidos hidratados e refrigerados até a sua utilização. Selecionaram-se 30 olhos e procedeu-se à dissecção da conjuntiva (n=10), córnea (n=10) e esclera (n=10) dos tecidos adjacentes. As membranas oculares foram colocadas em células de Franz (volume recetor = 3 ml; área de permeação = 1 $cm^2$) e hidratadas com tampão fosfato-salino (PBS) 10 mM pH 7,4. A

rHuEPO (100 UI) foi adicionada à fase dadora de cada célula de Franz, exceto nos controlos, procedendo-se de seguida à incubação a 37ºC e 300 rpm. Da fase recetora foram colhidas amostras desde os 30 minutos até às 6 horas após o início da incubação. A quantificação da EPO foi efetuada por ELISA nas amostras da fase recetora e procedeu-se à análise imunohistoquímica das membranas oculares. A análise estatística foi realizada com o programa "R for Windows", usando os testes estatísticos "Kruskal-Wallis rank sum test" e "Tukey Contrasts" para comparação das médias. Os dados são apresentados como média ± desvio-padrão.

**Resultados:** Após 6 horas de permeação, a conjuntiva foi a membrana mais permeável à EPO (509,3±89,84 mUI/cm$^2$, correspondendo a 2,6%), seguida da esclera (359,1±123,68 mUI/cm$^2$, correspondendo a 1,8%) e da córnea (71,0±31,78 mUI/cm$^2$, correspondendo a 0,4%). As diferenças entre a permeabilidade das membranas oculares foram estatisticamente significativas ($p<0.001$). A marcação por imunohistoquímico para a EPO foi positiva para as três membranas oculares.

**Conclusão:** Os resultados obtidos permitem concluir que, no modelo utilizado, a conjuntiva, a esclera e a córnea são permeáveis à rHuEPO tópica. Estes resultados são promissores e poderão conduzir à formulação de EPO tópica para uso oftálmico. Todavia, deverão ser realizados mais estudos para a avaliar a farmacodinâmica e farmacocinética da EPO a nível ocular, de forma a assegurar que doses terapêuticas são atingidas na retina.

# Annexe VIII

## PERMEABILITY OF OCULAR MEMBRANES TO TOPIC HUMAN RECOMBINANT ERYTHROPOIETIN USING A PIG EYE EX-VIVO MODEL.

Beatriz Silva[1], Ana Paula Resende[1], Lídia Gonçalves[2], T. Nunes[1], Berta São Braz[1], Esmeralda Delgado[1]

[1] Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon (FMV-UL). Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal;
[2] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, Lisbon, 1649-003, Portugal.

**Purpose:** To determine the permeability of the ocular membranes conjunctiva, sclera and cornea in a pig eye *ex vivo* model, to human recombinant erythropoietin (rHuEPO), using topical administration.

Methods: 60 fresh pig eyes were collected from a slaughterhouse. Conjunctivas (n=10), corneas (n=10) and scleras (n=10) were surgically dissected from surrounding tissues. Ocular membranes were placed into Franz diffusion cells (receptor volume 3 ml, permeation area 1 $cm^2$) and immediately hydrated with 10mM phosphate-buffered saline (PBS) pH 7.4. rHuEPO (100IU) was administered in the donor phase of each Franz cell, except for control samples. Cells were incubated at 37ºC and 300 rpm. Samples were collected from the receptor phase at 9 time points, from 15 min until 6 hours of incubation. Human Erythropoietin ELISA kit (Abcam®, Cambridge, England) was used for rHuEPO quantification in the receptor phase. Statistical analysis was performed with R for Windows using Kruskal-Wallis rank sum test and Tukey Contrasts for comparisons of means. Data are presented as mean± standard deviation.

**Results:** rHuEPO was detected in all study samples at some timepoint after topical administration. After 6 hours of permeation, conjunctiva was the ocular membrane most permeable to EPO (509.3±89.84 mU/cm$^2$, corresponding to 2.6%), followed by sclera (359.1±123.68 mU/cm$^2$, corresponding to 1.8%) and finally cornea (71.0±31.78 mU/cm$^2$, corresponding to 0.4%). Differences between ocular membranes' permeation were statistically significant ($p<0.001$).

**Conclusion:** We have demonstrated that in a pig eye *ex vivo* model conjunctiva, sclera and cornea are permeable to topical rHuEPO. These are promising results concerning ocular topical route of administration for EPO protein. More studies should be performed on EPO pharmacodynamics and pharmacokinetics to assure that therapeutical dosages are achieved in the retina.

# Annexe IX

## DID SUBCONJUNCTIVAL ADMINISTRATION OF ERYTHROPOIETIN INDUCE A NEUROPROTECTIVE EFFECT IN GLAUCOMATOUS RATS?

A.P. Resende[1], S. Rosolen[2,3], T. Nunes[1], B. São-Braz[1], E. Delgado[1]

[1] CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa

[2] Sorbonne Universités, UPMC Univ Paris 06, UMR_S 968, Institut de la Vision, Paris, France

3Clinique Veterinaire Voltaire, Asnières sur Seine, France

.

**Purpose:** It has been previous demonstrated that erythropoietin (EPO) can reach the retina, in glaucomatous rats, after subconjunctival administration, with functional benefits. We aimed to assess structural effects of EPO, in the same conditions.

**Methods:** Twelve albino Wistar Hannover rats were used in the study. Treated group (n=6) received a unilateral single subconjunctival injection of 1000 IU of EPO while the control group (n=6) received only saline. Forty-eight hours after subconjunctival injections, glaucoma was experimentally induced by coagulation of three episcleral veins in one eye (n=12). Twenty-one days after glaucoma induction, rats were euthanized and the eyes were processed for histological observation. After paraffin embedding, longitudinal sections (3 µm) were cut through the globe along the anterior-posterior axis and haematoxylin and eosin staining technique was applied. Dorsal and ventral retinal thickness was measured at 500 µm and at 1500 µm from the optic nerve (ON) head in all retinal sections.

**Results:** Retinal thickness at 500 µm from the ON corresponded to 206,3±14,8 µm on treated group and 176,9±20,1 µm on control group and at 1500 µm from the ON corresponded to 175,9±21,2 µm on treated group and 154,0±15,4 µm on control group. The treated group showed thicker retinas when compared to the control group and these differences were statistically significant ($p<0.05$).

**Conclusions:** Subconjunctival EPO administration in glaucomatous rats showed beneficial effects on retinal structure. Accordingly, with previous beneficial functional results, the role of EPO suggests a neuroprotective effect on the retina in glaucoma induced in albino rats.