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Role of the CBS enzymatic pathway in angiogenesis and apoptosis under normal conditions and pressure-induced stress through in vitro and ex vivo model

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Alle in dieser Dissertation vorgestellten Experimente und Datenanalysen wurden von Gabriela Pacheco Callirgos unter der Leitung von Dr. rer. nat. Thomas Clahsen durchgeführt, mit Ausnahme der qPCR-Analyse der Maus-Netzhäute (Abschnitte 7.2.6 und 7.2.7), die von Dr. rer. nat. Thomas Clahsen durchgeführt wurde.

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ABBREVIATION LIST

MSVI	Moderate and severe vision impairment
H2S	Hydrogen Sulfide
CBS	Cystathionine-beta-synthase
3MST	3-Mercaptopyruvate Sulfurtransferase
CSE	Cystathionin-gamma-Lyase
MMP	Matrix metalloproteinases
IOP	Intraocular pressure
RGC	Retinal ganglion cells
OAG	Open angle glaucoma
ACG	Angle closure glaucoma
POAG	Primary open glaucoma
CAT	Cysteine aminotransferase
SC	Schlemm Canal
CAP	Corneal Angiogenic Privilege
LSCD	Degeneration limbal stem cell deficiency
cNV	Corneal neovascularization
VEGF	Vascular Endothelial Growth Factor
AOAA	Aminooxyacetic acid
siRNA	Small interfering Ribonucleic acid
VEGF R2	Vascular endothelial growth factor receptor 2
VEGF R3	Vascular endothelial growth factor receptor 3
DAO	d-amino acid oxidase
qPCR	Quantitative polymerase chain reaction
bEND.3	Murine brain endothelioma cell line
NO	Nitric Oxide
HIF-1	Hypoxia inducible factor
RSP-29	Ribosomal Protein S29
FBS	Fetal Bovin Serum
DAPI	4',6-diamidino-2-phenylindole
PFA	Paraformaldehyde
7-AAD	7-amino-actinomycin D
cDNA	complementary DNA
HDLECs	Human Dermal Lymphatic Endothelial Cells
ROS	Reactive oxygen species

1. Summary

Various ocular diseases affecting the cornea, retina, or smaller structures such as the Schlemm's canal are of great interest in current research. These include conditions related to the pro-angiogenic and anti-angiogenic imbalance or the dysregulation of lymphatic endothelial structures. For example, the loss of corneal privilege and the onset of corneal neovascularization, or other diseases like glaucoma, where alterations in the Schlemm's canal, a structure considered to have both endothelial and lymphatic phenotypes, have been described.

For this reason, the modulation of lymphangiogenesis is of significant interest in identifying new therapeutic targets that could act at this level. Recent studies show that inhibition of the CBS enzyme plays a role in suppressing lymphangiogenesis in lymphatic cells by modulating growth factors and their respective receptors. The present study demonstrates the effect of the AOAA inhibitor as an anti-angiogenic agent in endothelial cells and outlines a potential mechanism of action through VEGF and its receptor (VEGFR).

Zusammenfassung

Verschiedene Augenerkrankungen, die die Hornhaut, die Netzhaut oder kleinere Strukturen wie den Schlemm-Kanal betreffen, stehen derzeit im Mittelpunkt der Forschung. Dazu gehören Erkrankungen, die mit einem Ungleichgewicht zwischen pro-angiogenen und anti-angiogenen Faktoren oder mit einer Dysregulation lymphatischer Endothelstrukturen verbunden sind. Ein Beispiel hierfür ist der Verlust des sogenannten “corneal immune privilege“ und die Entstehung einer Hornhautneovaskularisation. Auch bei anderen Erkrankungen, wie dem Glaukom, wurden Veränderungen des Schlemm-Kanals beschrieben – einer Struktur, die sowohl endotheliale als auch lymphatische Eigenschaften aufweist.

Aus diesem Grund ist die Modulation der Lymphangiogenese von großem Interesse, um neue therapeutische Zielstrukturen auf dieser Ebene zu identifizieren. Studien zeigen, dass die Hemmung des Enzyms CBS eine Rolle bei der Unterdrückung der Lymphangiogenese in lymphatischen Zellen spielt, indem sie Wachstumsfaktoren und deren jeweilige Rezeptoren moduliert. Die vorliegende Studie zeigt die Wirkung des Inhibitors AOAA als anti-angiogenen Wirkstoff in Endothelzellen und beschreibt einen möglichen Wirkmechanismus über den VEGF-Signalweg und dessen Rezeptor (VEGFR).

2. Introduction

With the increase in life expectancy, there is a greater tendency for the emergence of non-communicable diseases and disabilities.(1)
One of these is vision impairment and the most frequent causes of moderate and severe vision impairment (MSVI) are: undercorrected refractive error, cataract, age-related macular degeneration, glaucoma and diabetic retinopathy, being glaucoma the second leading cause of blindness. (1)

Additionally, blindness due to corneal disease is the third leading cause, after cataract and glaucoma. (2) Approximately 10 million people worldwide suffer from cornea diseases that can be treated with corneal transplantation(3), being it one of the most common transplanted tissues in the world. (4, 5) However, certain conditions can increase the risk of corneal transplant rejection, such as disruption of the corneal (lymph)angiogenic privilege, leading to pathological formation of corneal lymphatic and blood vessels. (6)

Currently, research is focused on the study of lymphangiogenesis and its role in pathological scenarios such as corneal neovascularization or diseases like glaucoma. It is currently known that Schlemm's canal has a dual phenotype, as a lymphangiogenic structure is a key element to discover novel therapeutic targets.(7)
Moreover, the mechanisms of the corneal (lymph)angiogenesis after disruption of the “corneal privilege” are of significant interest, for example; for the treatment of corneal transplant rejection. (8, 9)

Of particular interest are the molecule Hydrogen sulfide (H2S) and its enzymatic pathways involving Cystathionine-beta-synthase (CBS), 3-Mercaptopyruvate Sulfurtransferase (3MST), and Cystathionin-gamma-Lyase (CSE). Studies have described the involvement of the H2S in neuroprotection in animal models of glaucoma(7) and in the other hand, the CBS enzyme is as a novel regulator of lymphangiogenesis, (9) which is the focus of current research.

2.1. Glaucoma

2.1.1. Definition

Glaucoma is a chronic degenerative disease of the optic nerve, where there is a loss of retinal ganglion cells, thinning of the retinal nerve fiber layer and increasing excavation of the optic disc. (10-12) However it can be asymptomatic until it turns severe and for this reason leads to underdiagnosis. (13, 14)

The main goal in glaucoma treatment is to lower intraocular pressure (IOP), which can halt progression in 80% of the cases and IOP independent rescuing of RGCs (Retinal ganglion cells) (15), increasing evidence has suggested that vasoactive factors play a role in IOP regulation.

2.1.2. Epidemiology

Glaucoma is considered one of the leading causes of irreversible blindness worldwide. (1) Some factors influence the prevalence of Glaucoma, such as ethnicity. For instance Open angle glaucoma (OAG) is more prevalent in black populations, while angle closure glaucoma (ACG) is more prevalent in East Asian populations. (16) For this reason, it is difficult to describe the global trend of this neurodegenerative disease.

The studies vary in sample size, geographic region, ethnicity, examination methods, and definition of glaucoma. (17) However the prevalence of glaucoma worldwide, in Europe, and in Germany is constantly being updated.

Studies describe a global prevalence of Primary Open-Angle Glaucoma (POAG) in a population over the age of 40 as 2.4% (18), Whereas in Europe, the prevalence of POAG is 2.60% (19) and in Germany the one-year prevalence of POAG is 1.70% for the adult population (20). It is worth mentioning that some studies estimate the number of patients with glaucoma will increase to 112 million by 2040. (16)

2.1.3. Treatment

Glaucoma is treated according to its etiology, currently with pharmacological therapy, laser treatment, cyclodestructive procedures, or open surgeries. (16) But in recent years, new therapeutic targets have been described, opening the way for neuroprotection and regeneration of damage to the nerve fibers of the optic nerve (21). One of the molecules under study for neuroprotection is the H2S.

2.1.4. Glaucoma and New Therapeutic Perspectives: lymphatic-endothelial system and H2S

H2S and its producing enzymes CBS and Cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (CAT/3MST) might hit two birds with one stone and play an important role in the modulation of angiogenesis and neuroprotection in the eye. (7, 9, 22, 23)

Schlemm's canal (SC) exhibits both lymphatic and endothelial phenotypes (22, 24) and plays a crucial role in maintaining intraocular pressure homeostasis (24). This finding suggests that the lymphatic system is also involved in the pathogenesis of glaucoma (25-29). In terms of the endothelial vascular system, Wang et al. used a mouse model of glaucoma induced by occlusion of the three episcleral veins to demonstrate that elevated intraocular pressure leads to persistent endothelial dysfunction and impaired autoregulation in retinal arterioles (30). Liu et al. showed that the slow-releasing H2S donor GYY4137 protects RGCs against elevated pressure by increasing retinal vessel diameter, thereby illustrating the endothelial pathogenesis in a glaucoma model (7). Furthermore, studies showed that H2S is capable of lowering IOP (22) and on the other hand, an elevation of 3MST has been described in an in vivo glaucoma model in mouse retinas (7)

These findings confirm the involvement of the lymphatic endothelial system in the pathogenesis of glaucoma, and how it is influenced by the molecule H2S and its enzymatic pathways. Additionally, the neuroprotective role of H2S has been described in animal models, but here is no current information about the CBS enzyme and its implications in diseases such as glaucoma.

2.2. Disruption of the corneal privilege

2.2.1. Definition

The cornea, in healthy conditions, is transparent, avascular and immune-privileged. This corneal angiogenic privilege (CAP) refers to the avascular state of the cornea, which is maintained by the balance between anti-angiogenic and pro-angiogenic mechanisms. (24)

However, certain factors, such as infections or trauma, can disrupt this balance, leading to the growth and invasion of blood and lymphatic vessels into the cornea, which can result in vision impairment.(25) This phenomenon is known as neovascularization.

Additionally, there is another event known as corneal immune privilege, which is responsible for maintaining corneal transparency in response to inflammatory stimuli (26), making it one of the tissues with the lowest rates of rejection after transplantation and a high survival rate even under inflammatory conditions.(27)

2.2.2. Epidemiology

The etiology of corneal neovascularization varies depending on the population and location of the study and it varies across the world. Some causes of neovascularization include the use of contact lenses, corneal infections, trauma, corneal degeneration and limbal stem cell deficiency (LSCD). (28)

In a 15-year retrospective study conducted in Italy, the most common cause was non-infectious, with infectious causes being the second most frequent (29), other study indicate that contact lenses, particularly extended-wear soft contact lenses, are the most common non-infectious cause of corneal neovascularization (cNV) in the United States (30). In western countries, herpes simplex keratitis is the leading infectious cause of cNV. cNV is one of the leading causes of blindness worldwide and is a risk factor for corneal transplant rejection, that is why it has significant clinical relevance. (31) The most leading causes are in the Table N° 1 (32)

Table N° 1 Most leading causes of corneal neovascularization

Categories	Cause
Hypoxia	Contact lens wearing
Infectious Keratitis	Viral, Fungal, Bacterial, Parasitic
Inflamatory disorder	Mucous membrane pemphigoid, Stevens-Johnson syndrome, Atopic conjunctivitis, Rosacea, Lyell´s syndrome, corneal graft rekection
Loss of limbal barrier function	Limbal stem cell deficiency, Chemical burn, thermal burn
Other Disorders	Ocular surface neoplasia (papilloma, and conjunctival or corneal intraepithelial neoplasia) Pterygium

2.2.3. Treatment

The treatment can be through medications such as anti-inflammatory drugs. Recent studies mention the role of anti- Vascular Endothelial Growth Factor (VEGF) agents administered topically or subconjunctivally; however, it is still considered experimental therapy and off-label, and long-term studies are needed. MMP (matrix metalloproteinases) inhibitors, such as oral doxycycline in combination with topical corticosteroids, are used under inflammatory conditions. There are also surgical treatments such as diathermy and cautery; however, multicenter studies are still needed. (33)

2.2.4. Corneal neovacularization and New Therapeutic Perspectives: CBS

Hatami, et al. described that the inhibition of CBS in lymphatic endothelial cells by the specific blocker Aminooxyacetic acid (AOAA) and downregulation of CBS by specific Small interfering Ribonucleic acid (siRNA) results in reduced proliferation, migration, altered tube-formation capacity and decreased expression of Vascular endothelial growth factor receptor 2 (VEGF-R2) and Vascular endothelial growth factor receptor 3 (VEGF-R3).

In addition, C57BL/6 mice treated with AOAA showed significantly lower expression of VEGF-R2 and -R3. (9) Describing AOAA as a new modulating agent of lymphangiogenesis.

2.3. H2S and Enzymatic pathways: CBS, CSE and CAT/3MST

H2S is a gaseous signaling molecule involved in physiological processes of the nervous, cardiovascular, respiratory, and gastrointestinal systems (23, 34) However, their functions in the vasculature of the eye are still unclear.

H2S has been implicated in the regulation of homeostasis, vascular contractility, pro- and anti-inflammatory functions, as well as pro- and anti-apoptotic role. However, the effects of H2S are influenced by the concentration, reaction time, and cell/disease types.(35)

Enzymatic and non-enzymatic pathways are responsible for H2S production. The four enzymatic pathways that regulate endogenous H2S production are: CBS, CSE, CAT/3MST and d-amino acid oxidase (DAO/3MST). (Fig.1) Nevertheless, the pathway DAO/3MST has not been found in retinal tissues. (34)

The enzymatic pathway found in mammalian retina is 3MST/CAT, specifically in retinal neurons. (36) While the CBS enzymatic pathway has a strong expression and activity in the anterior segment (conjunctiva, iris, ciliary body and cornea-epithelial layer), however in the retina, this pathway changes with age, being its expression higher at older age. (37) Non-enzymatic production occurs through the oxidation of glucose in erythrocytes (38), which can be stimulated by increased oxidative stress and hyperglycemia. (39)

As mentioned earlier, H2S and its enzymatic pathways play an important role in the modulation of lymphangiogenesis and neuroprotection in the eye (7, 9), a current topic of research.

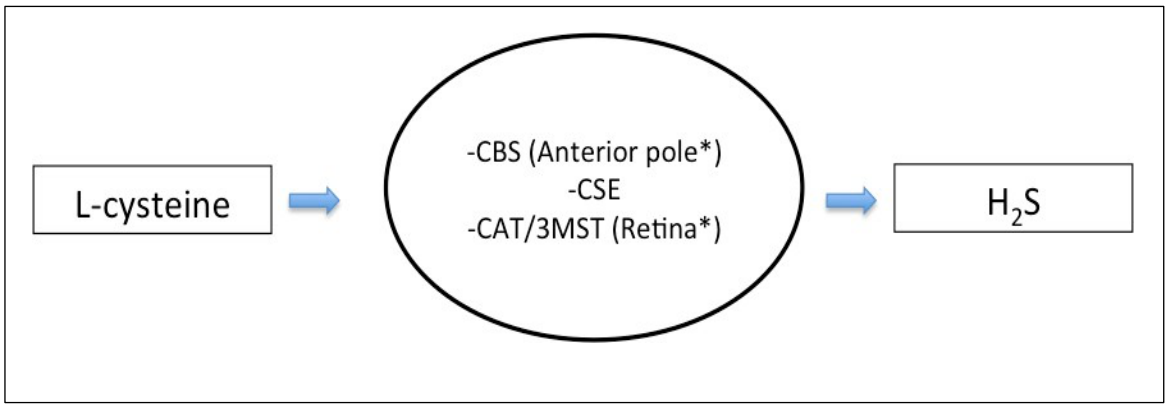

Figure 1 The three enzymatic pathways that regulate endogenous H2S production. Cystathionine-ß-synthase (CBS), cystathionine-y-lyase (CSE), cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (CAT/ 3MST).

Lymphatic vessels play an important role in the corneoscleral segment and alterations can lead to loss of corneal transparency and loss of vision, as well as an increased risk of corneal graft rejection.

In addition, Schlemm's canal, responsible for transporting aqueous humor to the episcleral veins, shows characteristics of an atypical lymphatic vessel, so that modulation of lymphangiogenesis could be related to the development of glaucoma and also alteration in vascular system in the retina was detected in glaucoma model in vivo.

Therefore, H2S and its enzymatic pathways, CBS, could be targets of study in the search for new therapeutic strategies for neuroprotection or modulation of angiogenesis.

2.4. VEGF-R and lymph-angiogenesis

Vascular endothelial growth factor is responsible for lymphangiogenesis. Studies have shown that VEGF-C and VEGF-D, along with their respective receptors VEGF-R2 and VEGF-R3, play a key role in this process like promotors. (40) (25, 41)

Recent studies have shown that inhibition of the CBS enzymatic pathway in an In vitro lymphatic cell model results in downregulation of proliferation, migration, and tube formation, as well as a reduction in the expression of VEGF-R2 and VEGF-R3. A similar downregulation of these receptors was observed in an animal model upon inhibition of the same enzymatic pathway.(9)

However, the expression of VEGF and its ligands varies depending on the cell being studied, due to genetic heterogeneity, and their functions will depend on the pro-angiogenic or anti-angiogenic environment in which they are located (42)

Another described function of the VEGFC/VEGFR3 complex is its crucial role in the development and maintenance of the Schlemm's canal. The application of recombinant VEGF-C promotes the growth of the Schlemm's canal in mice and reduces intraocular pressure (43)

2.5. Endothelial System

Most tissues in living organisms depend on blood supply, and blood supply depends on endothelial cells that form blood vessels. Between the ectoderm and mesoderm, there is the embryonic mesoderm, which gives rise to the formation of connective tissue and endothelial cells. The endothelial system is characterized by its ability to extend and remodel the network of blood vessels. (44) For this reason, there is an interest in research, focusing on the capacity for repair, growth, and migration in diseases such as cancer or ocular conditions, such as proliferative diabetic retinopathy, retinal neovascular membranes, or after the loss of corneal privilege, leading to angiogenesis in the cornea.

Endothelial cells control the passage of materials and the transit of white blood cells. The pericytes, connective tissue, and smooth muscle are later formed under the influence of signals from endothelial cells, giving rise to veins and arteries. It is known that endothelial cells also have mechanoreceptors that allow them to sense shear stress caused by blood flow over their surface, leading to the adaptation of their diameter and thickness. This response is mediated by neural signals and the release of the gas nitric oxide (NO). (44)

The formation of new endothelial cells can occur through simple duplication or by endothelial precursor cells derived from the bone marrow, as they have the ability to divide and move, which is responsible for their repair and remodeling capacity. On the other hand, the formation of new capillaries through sprouting is well known. Irritating stimuli in the cornea induce blood vessel growth by emitting pseudopodia, which lead to the formation of tubes, and this process concludes when another capillary is formed.(44) **Figure 2.**

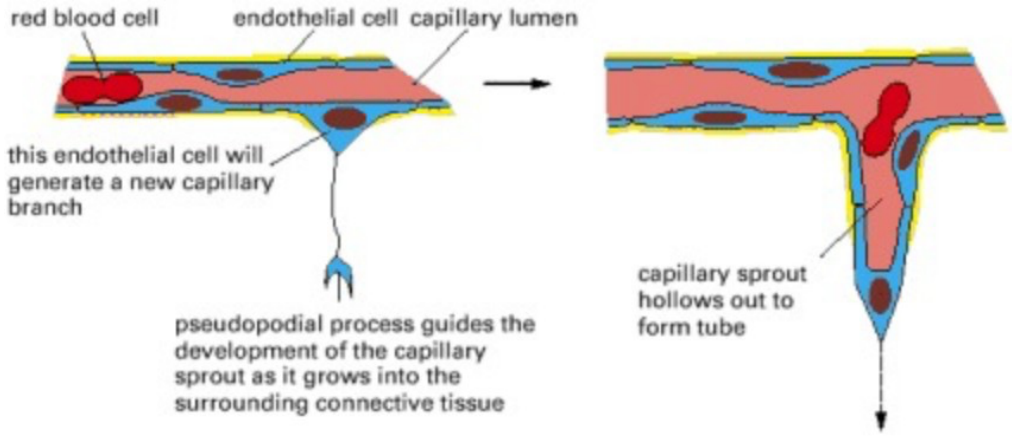

Figure 2. **Angiogenesis.** This image is based on the cells from the tail of a living tadpole. A new blood capillary forms from an endothelial cell. (Speidel, Am. J. Anat. 52:1–79, 1933.) (44)

External factors can influence the formation of blood vessels, such as local irritants and infections. VEGF plays a fundamental role in regulating blood vessel growth. A lack of oxygen causes an increase in the intracellular concentration of the active form of the gene-regulating protein called hypoxia-inducible factor 1 (HIF-1), which is responsible for stimulating the transcription of the VEGF gene. Four steps are important: the formation of protrusions to digest their way through the basal lamina, migration, proliferation, and the formation of tubes, ultimately leading to differentiation.(44) **Figure 3.**

Endothelial cells are classified into arterial, venous, and capillary types. There is a differentiated endothelial subpopulation called lymphatic endothelial cells. (45) (46, 47)
Due to this diversity and the microenvironments in which they are found, as well as the oxygen levels, nutrients, and mechanical forces, each cell has a distinct genetic expression pattern. As a result, angiogenesis is currently poorly defined (47)

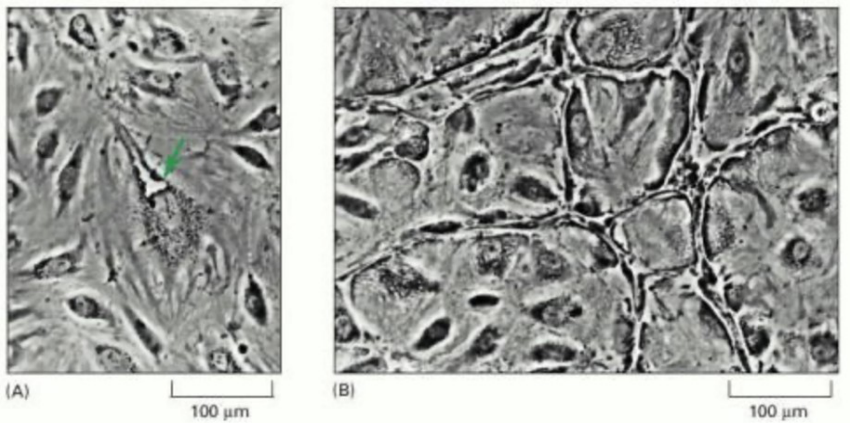

Figure 3. Angiogenesis in vitro. The arrow (A) points to a vacuole formed in a single endothelial cell. The colony spreads across the plate, and after 20 days, capillary tubes begin to form in the central regions. As seen in (B), an extensive network of tubes is formed. (44)

2.5.1. Tradicional angiogenesis model

In 1971, J Folkman proposed that "the growth of a tumor depends on angiogenesis and that substances which inhibit this process could be therapeutic."(48, 49) **Table 2**

The main stimulating factor of angiogenesis is hypoxia, which triggers the production of VEGF-A. Once new vasculature is formed, it increases the oxygen supply, alleviating local ischemia. Endothelial cells respond by growing and migrating towards the hypoxic area.(47)

There are two types of cells during angiogenesis: the cells that lead the sprout, called tip cells, and the supporting cells, called stalk cells, which together form a new capillary network. (47) **Figure 4.**

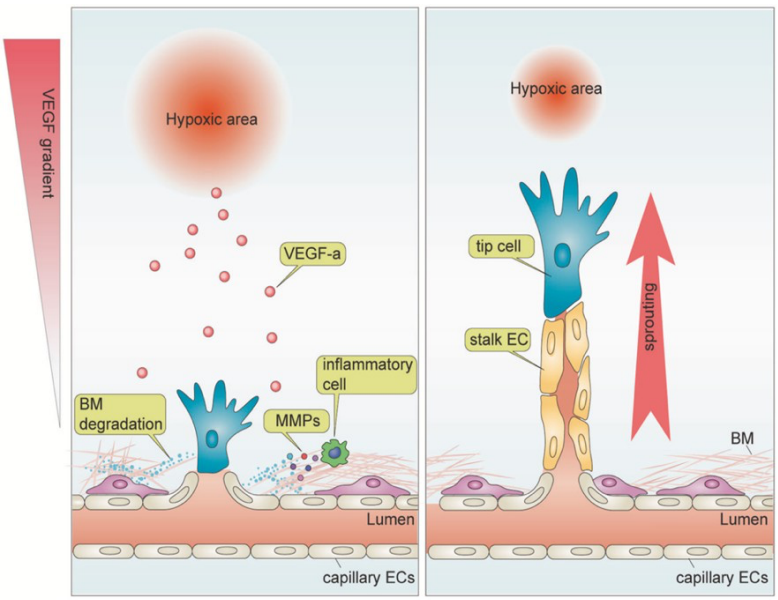

Figure 4. Tradicional model of angiogenesis. The expression of VEGF-A is increased under hypoxia, inducing the formation of new blood vessels and stimulating the recruitment of proinflammatory cells, which are responsible for the local production of metalloproteases (MMPs), facilitating the migration of endothelial cells. (47)

1980	Interferon alpha-betha
1982	Platelet factor 4/Protamine
1985	Angiostatic stereoids
1990	Fumagillin
1994	Angiostatin
1994	Thalidomide
1994	2-methoxyestradiol
1997	Endostatin
1999	Cleaved antithrombin III
2002	3-Aminotholidomide
2003	DBF-maf
2005	Caplostatin

Table 2. Angiogenesis inhibitors discovered in Folkman´s laboratory from 1980 to 2005 (50)

Therefore, studying lymph-angiogenesis at the molecular level is important to clarify which enzymes or proteins play an angiogenic, anti-angiogenic, or neuroprotective role, in order to treat current diseases such as glaucoma or corneal transplant rejection.

In the current study, experiments were conducted on murine endothelial cells to examine the effects on proliferation, migration, and apoptosis upon inhibition of the CBS enzymatic pathway. On the other hand, isolation of VEGF/R was performed using Quantitative polymerase chain reaction (qPCR) under both normal and stress conditions, with a pressure chamber. Finally, the same proteins were isolated from mouse retinal explants. This study aimed to evaluate, in vitro, the effect of inhibiting the CBS enzymatic pathway in murine endothelial cells, with the objective of identifying potential new antiproliferative or neuroprotective agents.

2.6. Questions and objectives of the thesis

The present study will examine the effect of inhibiting the CBS enzyme using its inhibitor, AOAA, on the proliferation and migration of murine endothelial cells. It will be analyzed through cell culture and imaging using the Incucyte system. Additionally, the apoptosis will be studied under both normal and stress conditions using a Pressure Chamber.

In the second part of the study, the expression of VEGF and its receptors will be analyzed to assess the impact of AOAA as a potent inhibitor of angiogenesis through these proteins. Finally, an animal model will be used where mouse retinas will be isolated and cultured to analyze the expression of these proteins under both normal and pressure conditions.

The primary objective of this study is to investigate the effect of CBS inhibition and its anti-angiogenic rol through VEGF and its respective receptors, as well as the potential anti-apoptotic effect under stress conditions.

3. MATERIAL AND METHODS

3.1. Material

Table 3: Primer and oligos

Mouse primers

Name	**Sequence (5´→ 3´)**
RPS29-for	GAG CAG ACG CGG CAA
RPS29-rv	CCT TTC TCC TCGTTG GG C
CBS_for	GATCGCCAGAAAGCTGAAGGAG
CBS_rv	CCACCTCATAGGCTGTTTGCTC
VEGF -A-for	CAT GGA TGT CTA CCA GCG AAG
VEGF-A-rv	CAT GGT GAT GTT GCT CTC TGAC
VEGF- C -for	AGA ACG TGT CCA AGA AAT CAG C
VEGF-C-rv	ATG TGG CCT TTT CCA ATA CG
VEGF- D -for	ATG GCG GCT AGG TGA TTC C
VEGF-D-rv	CCC TTC CTT TCT GAG TGC TG
VEGF-R1-for	TTG GTG GTG GCT GAC TCT CA

VEGF-R1-rv	TCT CCT TCG GCT GGC ATC TT
VEGF-R2-for	ATT CTG GAC TCT CCC TGC CTA C
VEGF-R2-rv	GCT CTT TCG CTT ACT GTT CTG G
VEGF-R3-for	GTC CCT CTA CTT CCA ACT GCT TC
VEGF-R3-rv	CACTCC TCC TCT GTG ACT TTG AG

Table 4: Cell culture

DMEM Dulbeco´s Modified Eagle Medium + Glutamax	Gibco
Fetal Bovine serum (FBS)	Gibco
Dulbecco's Phosphate-Buffered Saline (DPBS)	Gibco
T25 cell culture flask	Greiner Bio One
T75 cell culture flask	Greiner Bio One
96-Well Cell Culture Plates	Thermo Scientific™
Trypan blue solution 0.4%	Sigma-Aldrich
6-well Cell Culture Plates	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
[BEND3] endothelial cells	

Table 5: Antibodies

Mouse VEGFR1-Fit-1 Antibody	
DAPI	

Table 6: Kits, reagents and accesories

RNeasy Mini Kit	Qiagen, Hilden, Germany
RNeasy Micro Kit	Qiagen, Hilden, Germany
RNase-free DNase Set	Qiagen, Hilden, Germany
RevertAid First-Strand Synthesis cDNA Synthese Kit	ThermoScientific, Langenselbold, Germany

SsoFast EvaGreen Supermix kit	BioRad, Munich, Germany
PowerTrack SYBR Green Master Mix	ThermoScientific, Langenselbold, Germany
Aminooxy acetic acid(AOAA)	Sigma Aldrich, Taufkirchen, Germany
Chemicals Paraformaldehyde, 4 % in PBS (PFA)	Alfa Aesar, Kandel, Germany
Apotracker TM Green	
7-AAD Staining Solution	

Table 7: Devices

Name	Company	Function
IncuCyte TM Zoom	Essen Biosciences, Hertfordshire, UK	Live cell imaging
Zeiss Primo Vert inverted microscope fitted with an AxioCam ERc5s camera	Carl Zeiss Microscopy Gmb, Jena, Germany	Imaging
BioRad CFX96	BioRad, Munich, Germany	Detection and quantification of nucleic acid
Bioscience FACSCanto II Flow Cytometer	BD Biosciences, Heidelberg, Germany	Cell sorting analysis
Fluorescence microscope	Olympus BX63	Imaging
NanoDrop 2000c	Thermo Fisher Scientific	DNA quantification
Applied Bioscience Quantstudio 6	ThermoScientific, Langenselbold, Germany)	Detection and quantification of nucleic acid

Table 8: Software

Name	Company	Function
IncuCyteTM software Version 2016B and 2018A	Essen Biosciences, Hertfordshire, UK	Live cell image analysis
FACS DIVA 8.0.2	Beckton Dickenson, Ashland, USA	Cell sorting analysis
cell^F 3.4	Olympus Europe, Hamburg, Germany	Image analysis
NanoDrop 2000 (v.1.5)	Thermo Fisher Scientific	DNA quantification
GraphPad Prism software version 8	GraphPad Software, San Diego,CA	Statistical analysis
Image J (1.53K)	National group of Health, USA	Image analysis

Table 9: Animals

C57BL/6 NCrl Mice	Charles River Germany, Sulzfeld, Germany

3.2. Method

3.2.1. Cell Culture

For the in vitro experiments, endothelial cells (bEnd.3) isolated from the brain tissue of a mouse with endothelioma were used and cultivated under standardized conditions. The culture medium, DMEM Dulbecco's Modified Eagle Medium + GlutaMAX, containing 4.5 g/L glucose and L-glutamine but lacking sodium pyruvate, was supplemented with 10% FBS. The cells were maintained in an incubator at 37°C with 5% CO_2.

3.2.2. Proliferation Assay for Analyzing the Effect of CBS on Angiogenesis in Vitro

Naive 3000 Bend.3 cells were cultured in a 96-well plate with 100 µL of DMEM + GlutaMAX medium supplemented with 10% FBS. The plates were left in the hood for 30 minutes, and then incubated for 24 hours in an incubator at 37°C with 5% CO2. The following day, the Medium was removed and the cells were treated with different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM, in addition to a control group without AOAA exposure. Cell proliferation were determined by life-cell imaging every 4h for 24h using the IncuCyte Zoom Programm, monitoring the cell density.
Cell proliferation was assessed at 24, 48, and 72 hours messuring the fold change in density with the IncuCyte™ software (Version 2026B and 2018A, Essen Biosciences, Hertfordshire, UK).

3.2.3. Apoptosis Assay

350 000 bEnd.3 cells were seeded onto a 6-well plate in 5ml DMEM + Glutamax Medium supplemented with 10% FBS. The plates are incubated for 24 hours in an incubator at 37°C with 5% CO2. The day after the medium was removed and replaced with different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM, in addition 3 control groups/wells without AOAA exposure were needed and incubated for 24 hours.
The cells were harvested and stained using Apotracker solution to detect apoptosis and with 7-aminoactinomycin (7-ADD) to detect necrosis.
The appropriate volume of reconstituted Apotracker solution was taken to perform a 1:10 dilution with cell staining buffer. The cells were washed at least two times prior to running them on the cytometer, and this reagent was registered in the FITC channel of the flow cytometer. BD Biosciences FACSCanto II Flow Cytometer was used to analyze the cells samples.

3.2.4. Migration Assay

12 500 Bend.3 cells p were seeded in 100ul of DMEM+Glutamax Medium in 96-well plates. The plates were left under the hood for 30minutes to allow the cells to adhere and the incubated overnight. The WoundMaker® - Sartorius was used to create a precise wound in each well of an ImageLock 96-well plate, the medium was replaced to medium containing different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM.
Wound closure was monitored by cell imaging every 4 hours for 72 hours using the IncuCyte TM Zoom. ImageJ was used to calculate the wound surface area over a 72-hour period.

3.2.5. RNA Isolation and cDNA Synthesis

Cell Culture under normal Conditions

6-well plates were prepared with 125,000 bEnd.3 cells and incubated overnight at 37°C. The next day, the DMEM+GlutaMAX medium was replaced with a medium containing different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM. After 24 hours, the cells were washed twice with PBS. The Qiagen Mini Kit was used for RNA isolation, and the concentration was determined using the NanoDrop 1000. Finally, the RevertAid First-Strand cDNA Synthesis Kit was used for the synthesis of Complementary DNA (cDNA).

Cell Culture under Pressure

12 plates of 5 mL were prepared with 350,000 bEnd.3 cells and incubated overnight at 37°C. The following day, the DMEM+GlutaMAX medium was replaced with a medium containing different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM, and the plates were incubated in the pressure chamber at 120 mmHg. After 24 hours, the cells were washed twice with PBS. RNA isolation was performed using the Qiagen Mini Kit, and the concentration was determined using the NanoDrop 1000. Finally, complementary DNA (cDNA) was synthesized using the RevertAid First-Strand cDNA Synthesis Kit.

3.2.6. Analysis of mRNA Expression by Real Time Quantitative PCR

1000 ng of cDNA generated from each mRNA sample was used for real-time polymerase chain reaction (RT-PCR) in a BioRad CFX96 system using the SsoFast EvaGreen Supermix PCR Kit and the following primers: VEGF A, VEGF C, VEGF D, VEGF R1, VEGF R2, VEGF R3, and CBS. RSP29 was used as an endogenous control for normalization.

3.2.7. Inmunofluorescense Staining of Bend.3 for Examination of VEGF R1

Cell Culture:

30,000 bEnd.3 cells were cultured on coverslips in a 24-well plate with DMEM + GlutaMAX + FBS and incubated overnight at 37°C.

Fixation and Permeabilization:

The next day, cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS++).

Cells were fixed with 500 µL of 4% paraformaldehyde per well for 20 minutes.

Permeabilization was performed using 500 µL of PBS++ containing 0.1% Triton X-100 (PBS T++) for 5 minutes at room temperature.

Quenching and Blocking:

After permeabilization, cells were quenched with 1 mL of 50 mM NH_4Cl in PBS T++ for 5 minutes and then washed with PBS T++.

Cells were then blocked with 500 µL of PBS T++ containing 1% BSA for 1 hour at room temperature.

Primary Antibody Staining:

Humidified chambers were prepared using parafilm and moist tissue towels.

20 µL of the primary antibody solution containing VEGFR1 was placed on top of the parafilm. Coverslips were positioned over the antibody solution with the cell-facing side down and incubated for 60 minutes at room temperature.

Secondary Antibody Staining:

After incubation, coverslips were washed three times with 0.2% BSA/PBS T++.

20 µL of the secondary antibody (1:100) was added, and coverslips were incubated for another 45 minutes.

DAPI Staining:

To stain the total number of cells, DAPI (1:5000) was added for 2 minutes at room temperature.

Mounting and Storage:

Coverslips were mounted onto slides. The entire procedure was performed in darkness, and slides were stored at 4°C.

Experimental Conditions:

This protocol was conducted with both a control group and a group pre-treated with AOAA at 4 mM for 24 hours.

Imaging:

Images were captured using a fluorescent microscope (Olympus BX63).

3.2.8. Preparation of Retina Explants and Culture

Animals

C57BL/6N (animal provided by Center for Molecular Medicine Cologne, Germany). Age: 8-10 weeks. These animals will be used for the retina explants. The animals will be treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the Institutional Animal Care and Use Committee.

The mice C57BL/6N were sacrificed by cervical dislocation, after which their eyes were promptly enucleated and placed in Petri dishes containing ice-cold phosphate-buffered saline (PBS). The retinas were carefully extracted, ensuring their structural integrity, and the vitreous humor was removed. Each retina was then sectioned into four equal parts, with the ganglion cell layer oriented upward. These retinal explants were transferred into 6-well plates (Sarstedt, Nümbrecht, Germany) and maintained in a culture medium consisting of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco BRL, Eggenstein, Germany), supplemented with 10 µg/mL porcine insulin, 100 U/mL penicillin, and 100 µg/mL streptomycin.(51)

3.2.9. RNA Isolation from mouse Retinas and Reverse Transcription Polymerasa Chain Reaction (RT-PCR)

RNA was isolated after 24 hours of incubation using the RNeasy Micro Kit. To remove genomic DNA contamination, the RNase-Free DNase Set was applied, using 10 µL per sample. RNA concentrations were measured with the NanoDrop 2000 spectrophotometer, followed by quantification using Real-Time PCR. SYBR Green Master Mix was used for amplification. The primers utilized included RSP29, VEGF-A, VEGF-D, VEGF-R1, VEGF-R2, VEGF-R3, and CBS.

3.2.10. Elevated-Pressure Incubation Chamber

The pressure chamber is designed to culture cells or retinal explants under normal- or hypertensive conditions. (52) This Chamber involve different pressure conditions atmospheric + 0 mm Hg, 60 mm Hg and 120mmHg. In this study, the pressure chamber was used with a pressure of 60 mmHg and 120mmHg. (53)

A custom-engineered high-pressure incubation chamber was used. This metallic incubator features a screw-on lid and a one-way valve to regulate controlled air intake. The internal air pressure is precisely adjusted using a nanometer, displaying measurements in mmHg, ensuring stable conditions over extended periods.(53) The system allows for increasing air pressure up to 200 mmHg (266.64 Pascal), maintaining a steady level, or modifying pressure as required. A dedicated valve facilitates the introduction of a 5% CO_2-enriched atmosphere from the primary incubator (Heraeus, Germany). A pressure meter offers uninterrupted observation of internal pressure.(53)

3.2.11. Statistics and Reproducibility

All cell experiments were performed at least three times. For animal experiments, each eye (retina) was counted per individual. Statistical analysis was conducted using GraphPad Prism software Version 8 (GraphPad Software, San Diego, CA). Statistical significance was defined as follows:

$p < 0.05$ (*statistically significant*)
$p < 0.01$ (**highly significant**)
$p < 0.001$ (***very highly significant***)
$p < 0.0001$ (**extremely significant**)

4. RESULTS

4.1. Results under normal conditions without pressure chamber

4.1.1. Effect of CBS inhibition by AOAA on bEND.3 proliferation and migration

The effect of the AOAA inhibitor on proliferation in bEnd.3 cells was evaluated using various concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM. The results were analyzed after 24 hours, 48 hours, and 72 hours. A significant reduction in proliferation was observed at 2 mM and 4 mM after 24 hours and 72 hours, while at 48 hours, the reduction was significant at 1 mM, 2 mM, and 4 mM. No significant effects were observed at 0.25 mM and 0.5 mM.

Another essential step for angiogenesis is cell migration. After creating a scratch and exposing the cells to different concentrations of AOAA: 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM for 72 hours, a significant reduction of the migration was observed in bEnd.3 cells at concentrations of 1 mM, 2 mM, and 4 mM after 48hrs. No significant differences were observed at 0.25 mM and 0.5 mM **(Figure 5.)**

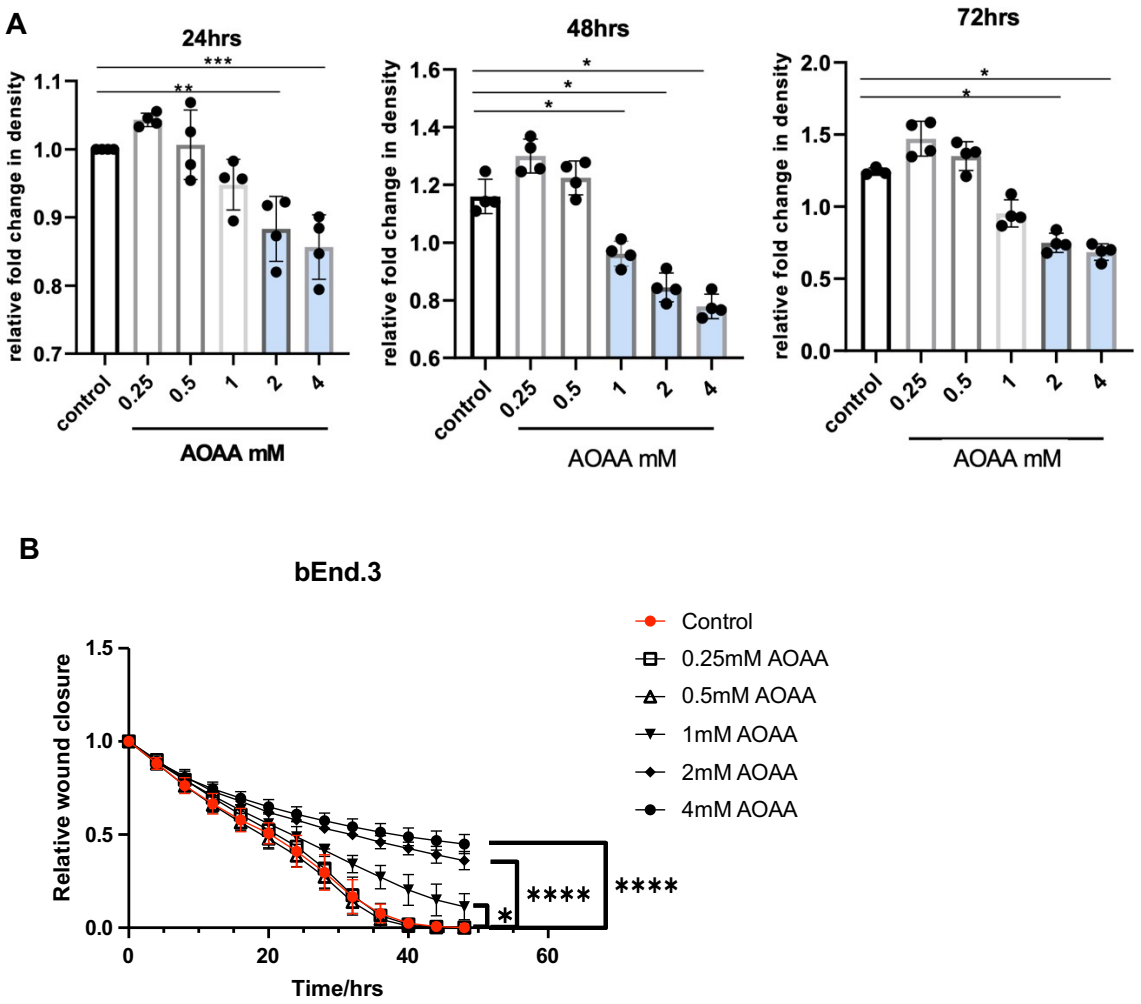

Figure 5. A Effect of AOAA on the proliferation of bEND.3 after 24 h-48h-72h. Proliferation was determined by using IncuCyte Zoom (n=4). **B** Migration Assay. AOAA treatment of bEND.3 decrease migration compared to control cells ($n = 3$). Statistical significance was analyzed with **(A, B)** one-way ANOVA. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

4.1.2. Effect of CBS inhibition by AOAA on bEND.3 without major effects on Apoptosis

The effect of AOAA on cell apoptosis was studied to rule it out as a possible cause of the reduction in proliferation and migration.

In the bEnd.3 control group, after 24 hours, less than 20% of Apotracker-positive cells were detected. An increase in apoptosis was observed at concentrations of 0.25 mM, 0.5 mM, and 1 mM. However, apoptosis was reduced compared to the control group at concentrations of 2 mM and 4 mM *without significant differences.* **(Figure 6.)**

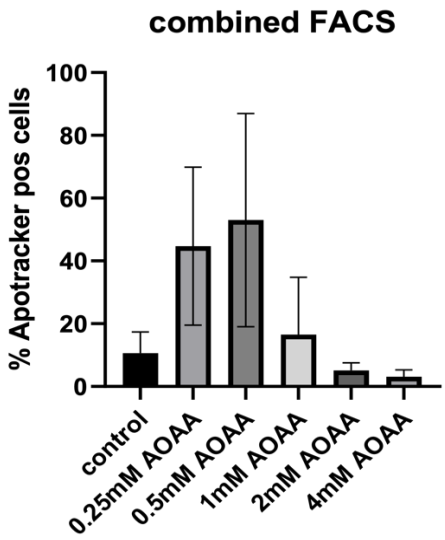

Figure 6. Effect of AOAA on apoptosis of bEND.3 after 24 h. (n=3). Apotracker was used to detect apoptosis and 7-Amino-Actinomycin D (7 ADD) to detect necrosis.

4.1.3. AOAA treatment increases expression of VEGF-A, VEGF-C and VEF-D in bEND.3

The expression of VEGF-A, VEGF-C, and VEGF-D and their respective receptors, VEGF R1, VEGF R2, and VEGF R3, which are involved in angiogenesis, was analyzed.

RT-PCR demonstrated a significant increase in VEGF-A expression at AOAA doses of 1 mM, 2 mM, and 4 mM. VEGF-C expression was significantly increased after exposure to AOAA at doses of 2 mM and 4 mM. Meanwhile, VEGF-D expression was significantly increased at a dose of 4 mM.

The inhibition of CBS using AOAA led to an upregulation of VEGF-A, VEGF-C, and VEGF-D on bEnd.3 cells, proteins described as promoters of angiogenesis. **(Figure 7.)** Based on these results, it would be important to further investigate the behavior of VEGF receptors in response to AOAA exposure.

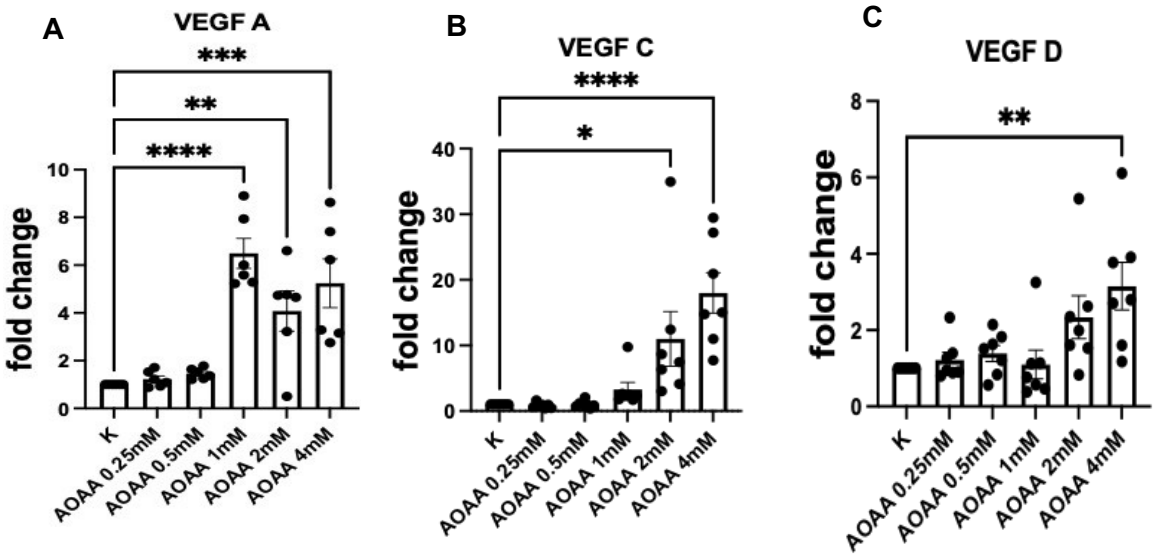

Figure 7. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-A*, and *VEGF-C* and *VEGF-D* was assessed using real-time PCR. (**A**)n = 6, (**B**) n=7, (**C**) n=7. Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test ($n = 5$). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

4.1.4. AOAA treatment reduces expression of VEGF-R1 in bEND.3s without major effects on VEGF-R2 and VEGF-R3

RT-PCR demonstrated a significant decrease in VEGF-R1 expression in bEnd.3 cells at AOAA doses of 0.5mM, 1 mM, 2 mM, and 4 mM. While no significant differences were found in the regulation of VEGF R2 and VEGF R3. Immunostaining of VEGF-R1 with Cy5 shows low signal intensity at an AOAA concentration of 4 mM (**Figure 8**.)

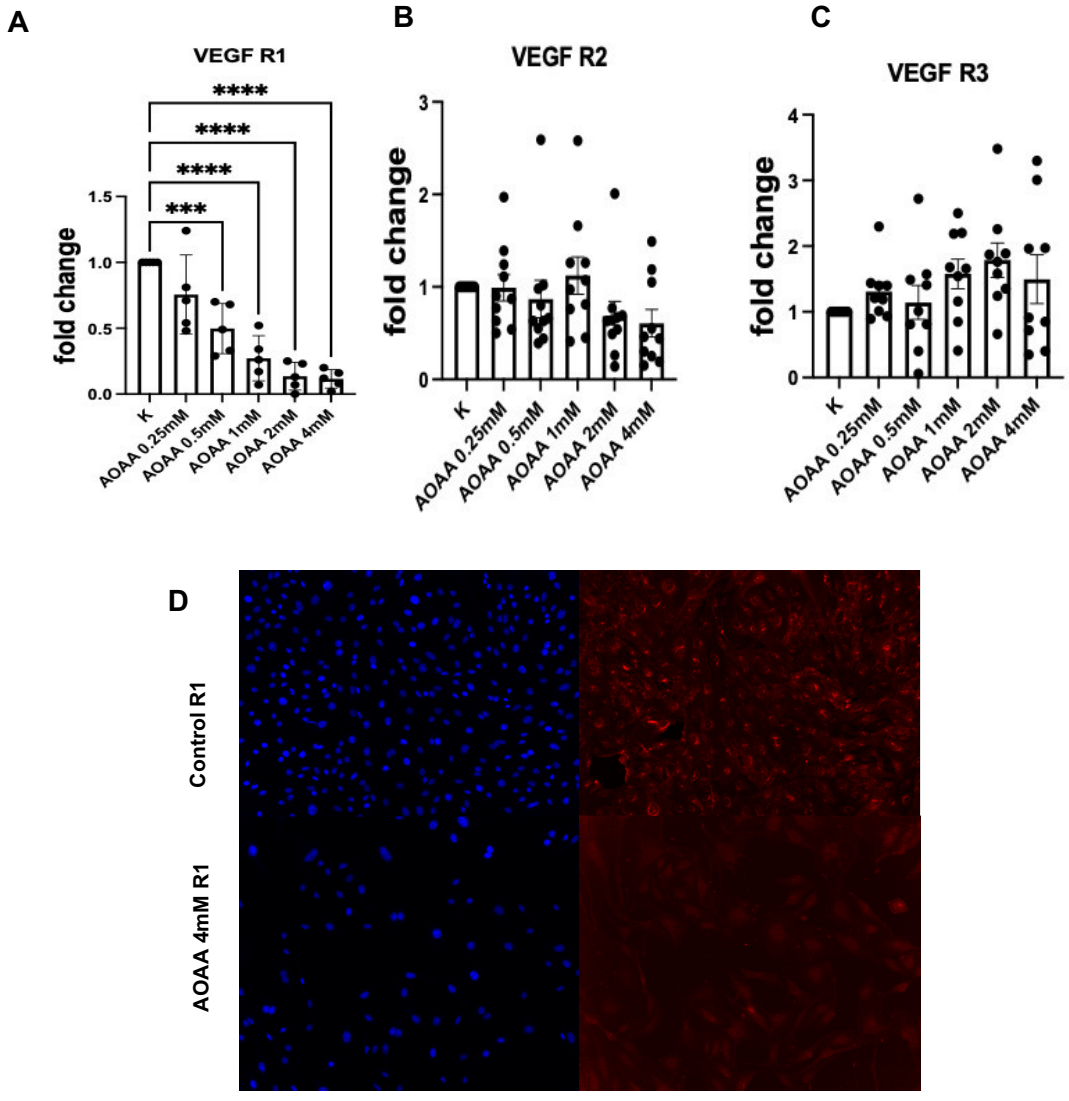

Figure 8. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-R1*, and *VEGF-R2* and *VEGF-R3* was assessed using real-time PCR. (**A**)n = 5, (**B**) n=10, (**C**) n=9. Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test ($n = 5$). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. (**D**) The immunostaining of VEGF-R1 shows a low signal in the group treated with AOAA at a concentration of 4 mM.

4.2. Results under stress conditions using a pressure chamber : murine endothelial cells

4.2.1. Effect of CBS inhibition by AOAA on cell morphology after incubation for 24 hours in a pressure chamber at 120 mmHg.

A change in cell morphology was observed after 24 hours of cell culture, with cells exposed to a pressure of 120 mmHg showing nuclear enlargement and a polyhedral shape in both the control group and the group treated with the AOAA inhibitor at a concentration of 0.5 mM, both under pressure.

In contrast, in the control group cultured under normal conditions, no nuclear enlargement or polyhedral shapes were observed, the cellular appearance remained unchanged. The cell morphology remained also unchanged in the group cultured under 120 mmHg pressure with the AOAA inhibitor at a concentration of 4 mM. These findings may suggest that the inhibition of CBS with AOAA in endothelial cells contributes to the maintenance of cellular morphology under high pressure, thereby indicating a potential protective role against stress.

This morphological pattern was consistently observed in experiments conducted using the pressure chamber, repeating across three independent experiments. (**Figure 9.)**

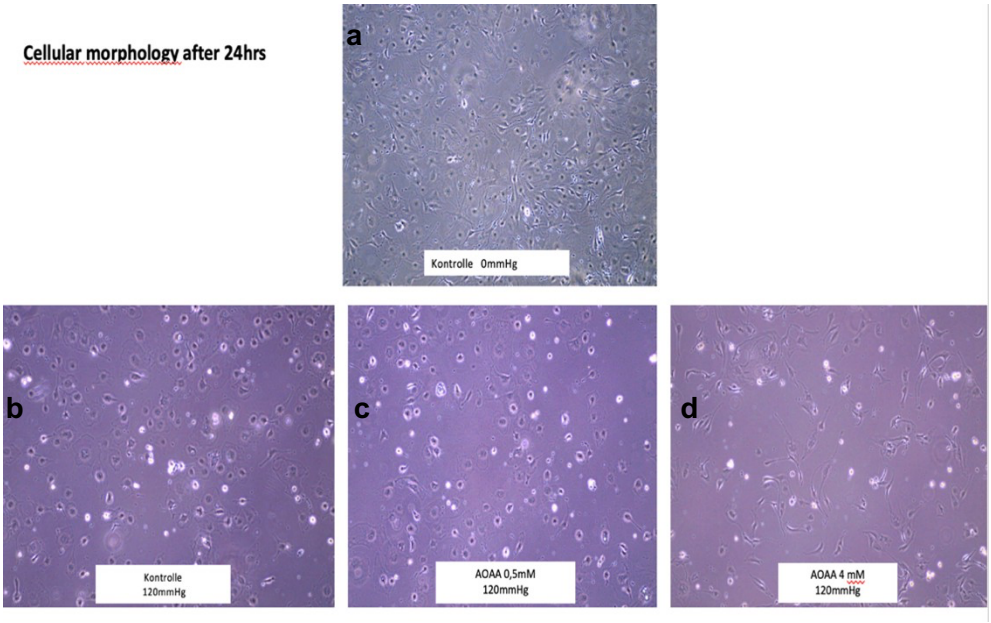

Figure 9. bEND3 were treated with indicated concentrations of AOAA for 24 h in the pressure chamber at 120mmHg**. (b)** In the Control group with 120mmHg pressure rounded and enlarged nuclei are observed, **(d)** in the group with AOAA at 4mM and 120mmHg pressure the nuclei maintain their shape and size compared to the control group without pressure **(a)**

4.2.2. Effect of CBS inhibition by AOAA on bEND.3 on apoptosis under pressure

An increase in apoptosis was observed in the control group cultured under 120 mmHg pressure compared to the control group cultured under normal conditions. Furthermore, a decrease in Apotracker detection was noted in the group cultured under 120 mmHg pressure with the AOAA inhibitor at a concentration of 4 mM (n = 3). However, the results were not statistically significant.

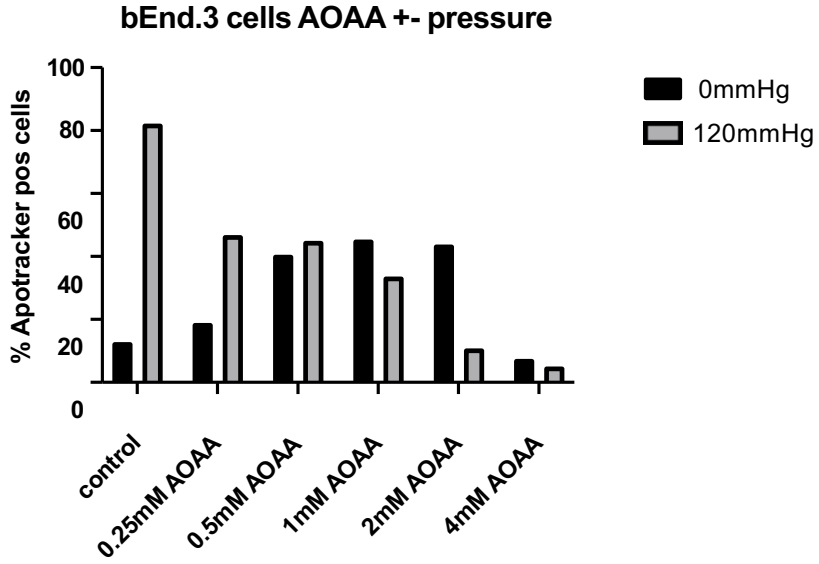

Figure 10. Effect of AOAA on apoptosis of bEND.3 after 24 h. (n=3). Apotracker with 1:10 Dilution was used to detect apoptosis and 7-Amino-Actinomycin D (7 ADD) to detect necrosis. The results were not statistically significant (n = 3).

4.2.3. AOAA treatment induces expression of VEGF A, VEGF C, VEGF D in bEND.3s in normal conditions and induces expression of VEGFA under 120mmHg pressure without major effects on VEGF C and VEGF D under 120mmHgh pressure

qPCR analysis detected a significant increase in the expression of VEGF-A, VEGF-C, and VEGF-D in normal conditions in murine endothelial cells. VEGF-A expression was significantly upregulated with AOAA concentrations of 1 mM and 4 mM, while VEGF-C and VEGF-D expression showed a significant increase at an AOAA concentration of 4mM.

In the group cultured under 120 mmHg pressure, a significant increase in VEGF-A expression was observed with an AOAA concentration of 2mM. Regarding the expression of VEGF-C and VEGF-D, the results were not significant. **(Figure 11.) (Table 10.)**

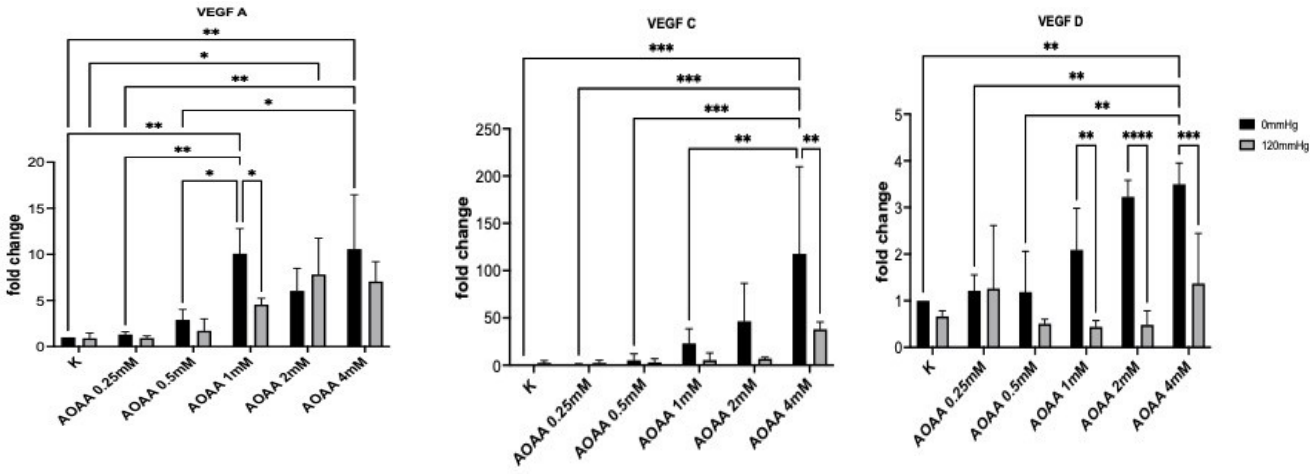

Figure 11. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-A*, and *VEGF-C* and *VEGF-D* was assessed using real-time PCR. **n = 3.** Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Table 10: VEGF Expression in Response to AOAA Treatment (qPCR Analysis)

Condition	AOAA	VEGF-A	VEGF-C	VEGF-D
Normal culture conditions (1)	1 mM	↑****	—	—
	2 mM	↑**	↑*	—
	4 mM	↑***	↑****	↑**
Normal culture conditions(2)	1 mM	↑**	—	—
	2 mM	—	—	—
	4 mM	↑**	↑***	↑**

(1) Figure 7. One group experiment

(2) Figure 11. In the two-group experiment, a significant increase in VEGF-A, VEGF-C, and VEGF-D expression was observed under normal conditions following treatment with 4 mM AOAA **Legend: ↑ = Significant increase, — = No significant change**

4.2.4. CBS Inhibition by AOAA reduces VEGF R1 Expression in bEND.3 cells in normal conditions without major effects on VEGF R2

The results of RT-PCR indicated no significant difference in the expression of VEGF-R2 in bEND.3 cells after treatment with AOAA compared to the untreated control (Figure 8A), in the group with and without pressure.

However, the expression of VEGF-R1 in bEND.3 cells treated with 4mM AOAA for 24 hours exhibited significant downregulation compared to untreated control cells without pressure. On the other hand, the expression of VEGF-R3 increased in the non-pressure group treated with AOAA at a concentration of 1 mM.

VEGF-R1, VEGF-R2, and VEGF-R3 in the group under 120 mmHg pressure did not show significant differences (**Figure 12**) (**Table 11)**

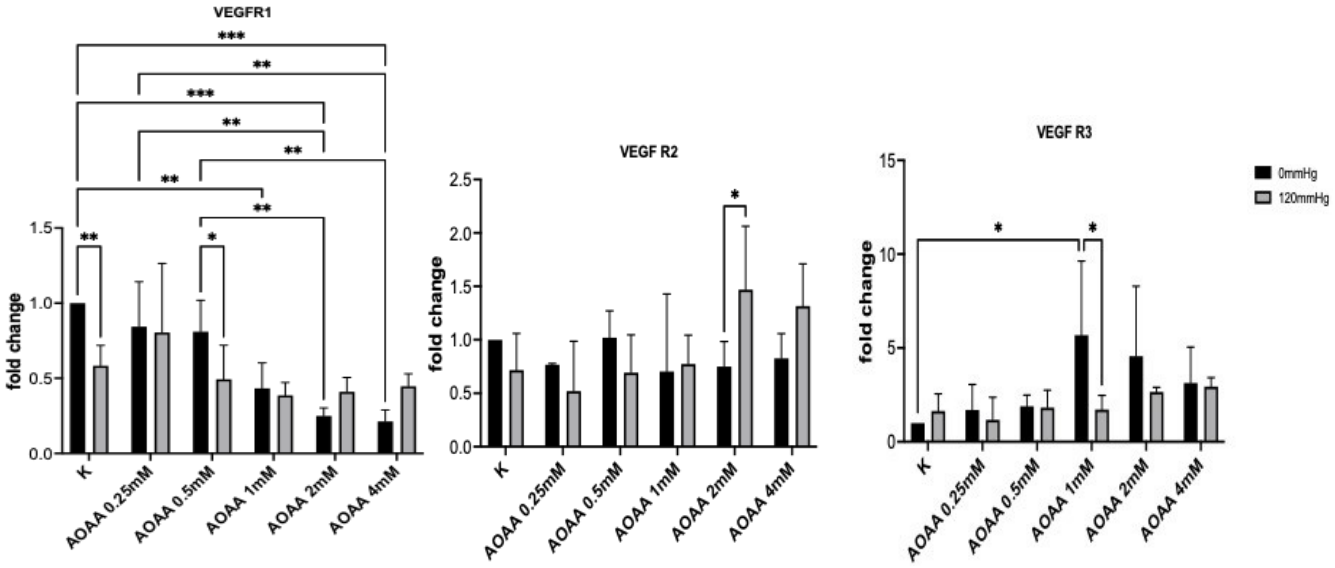

Figure 12. bEND3 were treated with indicated concentrations of AOAA for 24 h in normal conditions and under 120mmHg Pressure. Level of mRNA for *VEGF-R1*, and *VEGF-R2* and *VEGF-R3* was assessed using real-time PCR. (**a**)**n = 3.** Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test. **p* < 0.05, ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

Table 11: VEGF-R Expression in Response to AOAA Treatment (qPCR Analysis)

Condition	AOAA	VEGF-R1	VEGF-R2	VEGF-R3
Normal culture conditions (1)	0,5mM	↓***	—	—
	1 mM	↓****	—	—
	2 mM	↓****	—	—
	4 mM	↓****	—	—
120 mmHg pressure (2)	0,5mM – 4mM	—	—	—

(1) Figure 8. One group experiment

(2) Figure 12. In the two-group experiment, a significant decrease in VEGF-R1 expression was observed under normal conditions following treatment with 1 mM, 2 mM, and 4 mM AOAA. In contrast, no significant changes were observed in the group exposed to 120 mmHg pressure

Legend: ↓ = Significant decrease, — = No significant change

4.2.5. AOAA reduces CBS expression in bEnd.3 cells

RT-PCR demonstrated a significant downregulation of CBS expression in bEnd.3 cells at AOAA doses of 0.5mM, 1 mM, 2 mM, and 4 mM after 24 hours. This suggests that AOAA effectively inhibits CBS expression in a dose-dependent manner.

Additionally, a reduction in CBS expression was observed in all groups treated with AOAA subjected to a pressure of 120mmHg. The significant decrease in CBS expression in the control group exposed to 120 mmHg pressure compared to normal conditions could indicates that elevated pressure itself may contribute to the reduction of CBS expression in endothelial cells or that AOAA, in combination with high pressure, may have a synergistic effect in downregulating CBS expression.

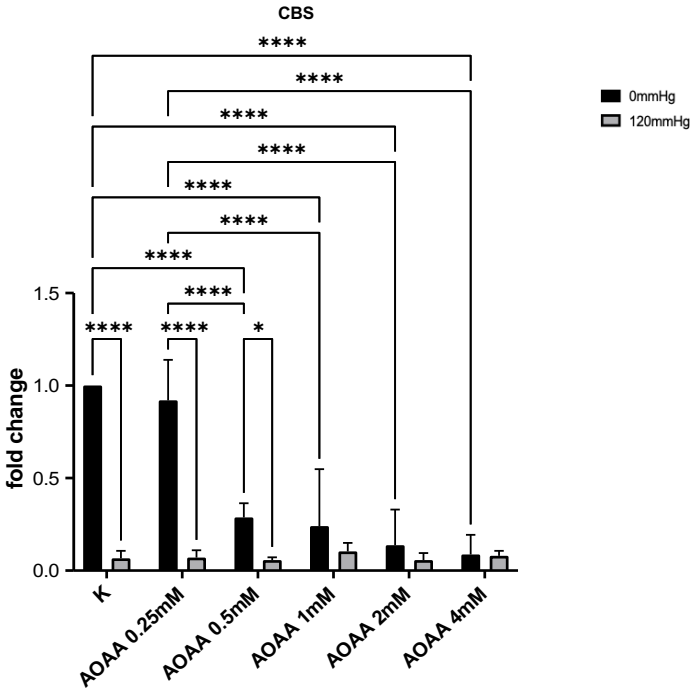

Figure 13. bEND3 were treated with indicated concentrations of AOAA for 24 h in normal conditions and under 120mmHg Pressure. Level of mRNA for *CBS* was assessed using real-time PCR. (**a**) n = 3. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

4.3. Results under stress conditions using a pressure chamber: Mouse retinas

4.3.1. High pressure and CBS, VEGF-R1, VEGFR-2, and VEGFR-3 expression in mouse retinas

High pressure may induce CBS expression in mouse retinas, accompanied by an increase in VEGFR-1, VEGFR-2, and VEGFR-3. CBS is involved in cellular stress responses and may contribute to protecting cells under pressure-induced stress conditions, possibly through the regulation of sulfur-containing molecules such as hydrogen sulfide (H_2S), which has been implicated in vascular health and cellular protection. VEGF receptors are crucial for endothelial cell signaling, driving both angiogenesis and lymphangiogenesis in response to various stressors, including pressure. However, the results were not statistically significant, underscoring the need for further studies to elucidate the underlying mechanisms and interactions in greater detail.

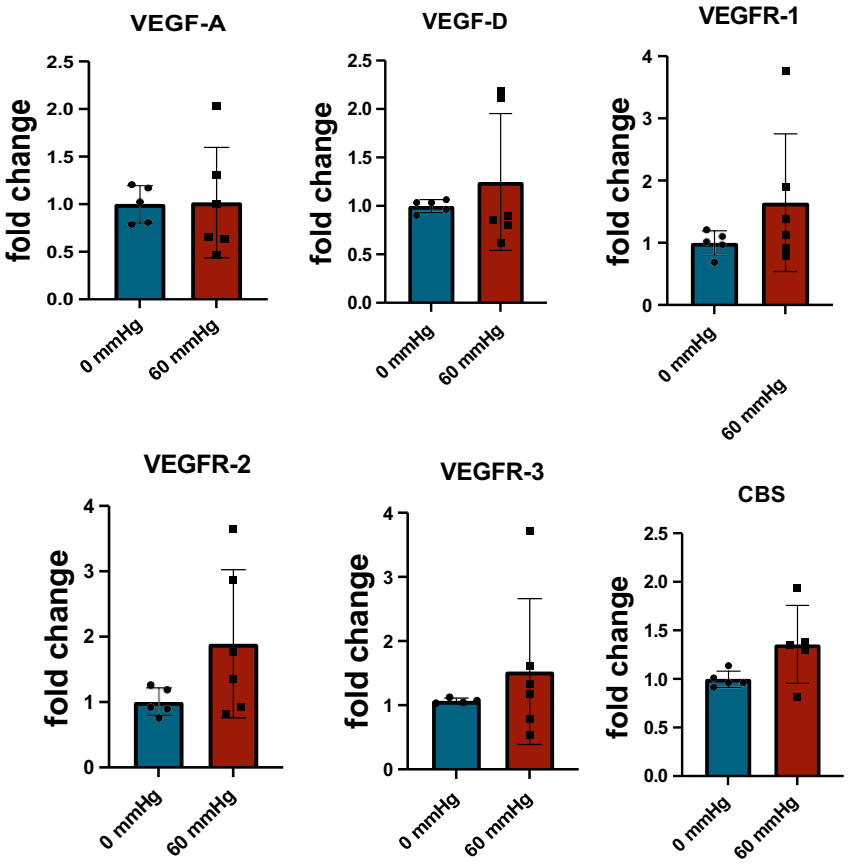

Figure 14. qPCR of mouse retina explants cultured under normal conditions and under 60 mmHg pressure. (n = 5) Results were not significant.

4.3.2. AOAA and CBS, VEGF-R1 and VEGFR-2 expression in mouse retinas under pressure

AOAA may induce CBS expression in mouse retinas in normal conditions and under pressure, along with and decrese in VEGF-R1 and VEGFR-2 under pressure. This suggests that AOAA may trigger a compensatory response in the mouse retina, where CBS expression increases in an attempt to counteract stress or maintain cellular homeostasis.

However, under pressure conditions and with AOAA, there is a decrease in the expression of VEGF-R1 and VEGFR-2.

Nevertheless, the results were not statistically significant, emphasizing the need for further experiments to thoroughly investigate the potential compensatory mechanisms and interactions. (**Figure 15**.)

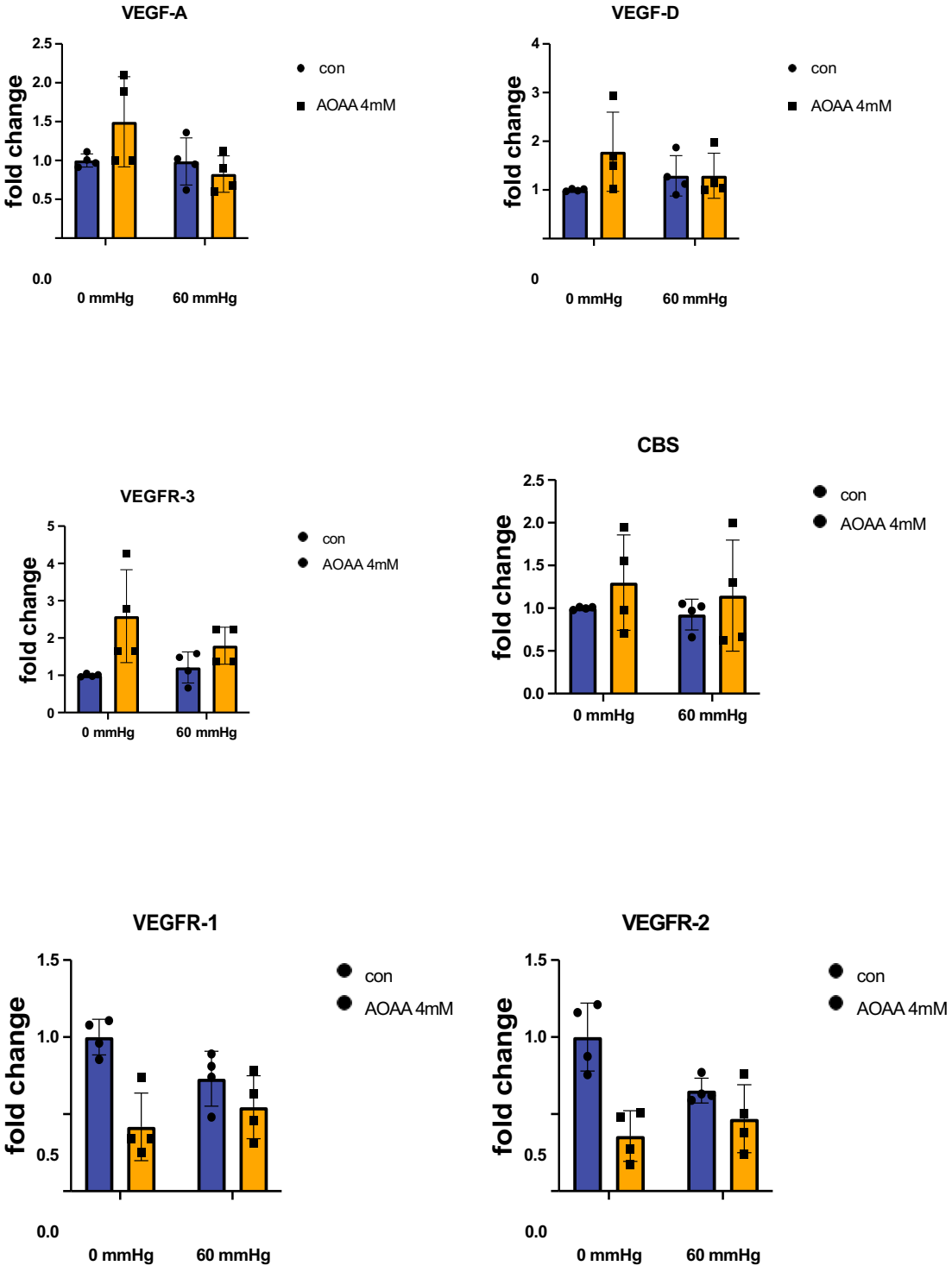

Figure 15. qPCR of mouse retina explants cultured under normal conditions and under 60 mmHg pressure, with a control group and a group treated with AOAA 4mM (n = 5) Results were not significant.

5. Discussion

In 2022, it was demonstrated that treating Human Dermal Lymphatic Endothelial Cells (HDLECs) with either AOAA or transfection with specific siRNA targeting CBS reduces the transcriptional expression of VEGF-R2 and VEGF-R3, thereby decreasing proliferation, migration, and tube formation. This describes an anti-lymphangiogenic role of CBS inhibition at the cellular level. (9)

Human cystathionine beta-synthase (CBS) is an enzyme that functions as a tetramer composed of four subunits of approximately 64 kDa each.(54) The gene encoding CBS is located on human chromosome 21. (55) CBS plays a central role in the transsulfuration pathway, catalyzing the conversion of methionine to cysteine. Deficiency of CBS results in homocystinuria, a hereditary metabolic disorder marked by cognitive impairment, seizures, neuropsychiatric symptoms, skeletal deformities, and vascular complications.(56)

At the ocular level, patients affected by homocystinuria frequently exhibit lens dislocation (ectopia lentis) and high myopia, whereas other ocular complications—such as degeneration or detachment of the retina, optic nerve atrophy, glaucoma, corneal irregularities, and cataract formation—are observed less commonly.(37) CBS enzymatic activity shows strong expression in anterior segment including the conjunctiva, iris, ciliary body and cornea-epithelial layer. In Contrast, retinal expression of CBS appears to vary with age, showing increased levels in older individuals. (37) However, the functions of CBS in the human eye are still not fully understood.

AOAA is currently one of the most potent and commonly used inhibitors of CBS, despite its lack of specificity, as it also inhibits enzymes such as CSE and various transaminases.(54) Although it also has its limitations, Petrosino et al. highlight the constraints of AOAA as a selective CBS inhibitor and suggest that its efficacy in the cellular context—under both physiological and pathological conditions—may be significantly influenced by the surrounding biochemical environment, particularly the availability of serine. (57)

The significance of CBS inhibition using AOAA at the cellular level has gained attention following the results published by Hatami et al., who reported an anti-lymphangiogenic role of CBS inhibition in vitro using lymphatic endothelial cells, as well as ex-vivo through the analysis of murine corneas.(9)

Corneal neovascularization is a clear example of the imbalance between pro- and anti-angiogenic factors, characterized by the formation of new blood vessels in the normally avascular cornea.(26) Additionally, other ocular structures, such as the Schlemm's canal, have been shown to exhibit a lymphatic-endothelial phenotype.(7) This highlights the

importance of investigating enzymatic pathways like CBS at the endothelial level and their potential roles in angiogenesis and in response to induced stress.

In the present study, a reduction in proliferation was observed in murine endothelial cells after 24 and 48 hours of exposure to AOAA at concentrations of 1 mM, 2 mM, and 4 mM. Similarly, a decrease in cell migration was detected after 48 hours at the same concentrations. Hatami et al. (9) reported comparable results, observing reduced proliferation, migration, and tube formation in human lymphatic endothelial cells. Other studies have also shown that AOAA suppresses the proliferation of colon cancer cells in vitro and reduces tumor growth in vivo (11, 58). Moreover, silencing of CBS has been associated with a significant reduction in proliferation across all ovarian cancer cell lines examined. (59) These findings indicate that CBS inhibition through AOAA impacts key steps in the angiogenic process, exerting an anti-angiogenic effect by reducing both cell proliferation and migration. According to Hatamie et al., AOAA treatment did not increase apoptosis or necrosis in HDLECs, as determined by Annexin V staining, nor did it induce cellular senescence, as evidenced by the absence of significant changes in $p16^{INK4A}$ expression. (9) In contrast, the results of the present study with bEnd.3 cells showed no significant changes in apoptosis under normal conditions. However, the cells cultured under 120 mmHg pressure exhibited an increase in Apotracker concentration compared to cells maintained under normal conditions. Following treatment with 4 mM AOAA, a reduction in Apotracker signal was observed, suggesting a potential anti-apoptotic effect under stress conditions. Nonetheless, these findings did not reach statistical significance and there was no clear evidence of increased apoptosis that could account for the observed reduction in proliferation and migration.

Following the findings of Hatamie et al.(9), the effect of the inhibitor AOAA on VEGF and VEGF receptors was studied in the present study under normal conditions and under 120 mmHg pressure, in order to investigate a possible correlation between the decrease in proliferation and migration via VEGF/VEGF-R signaling and its modulation under stress conditions.

In both the single-group control experiments and the two-group design (with and without pressure), the results remained consistent and statistically significant, demonstrating an increase in the expression of VEGF-A, VEGF-C, and VEGF-D under treatment with 4 mM AOAA in normal conditions. However, in the two-group experiments, a significant increase of VEGF-A was observed at the 2 mM concentration in the group exposed to 120 mmHg pressure. No statistically significant changes were observed in the pressure-exposed control

group for VEGF-C and VEGF-D receptors. These findings suggest that inhibition of the CBS enzyme through AOAA leads to increased expression of VEGF-A, VEGF-C, and VEGF-D under normal culture conditions.

VEGF-A, VEGF-C, and VEGF-D are well-known pro-angiogenic factors(60, 61); therefore, the observed effect of AOAA appears paradoxical. While AOAA treatment decreases proliferation and migration—both essential steps in angiogenesis(9)—it simultaneously stimulates the expression of these pro-angiogenic factors. This paradox may reflect a compensatory response to metabolic stress or redox imbalance induced by CBS inhibition.(54) Moreover, the elevation of VEGF-A, -C, and -D would not be able to exert their angiogenic effect in the absence of expression or activation of their receptors VEGFR-1, VEGFR-2, and VEGFR-3.

The binding of these ligands to their specific receptors is crucial for initiating the signaling pathways that lead to angiogenic responses.(62-64)

Furthermore, CBS inhibition could lead to elevated homocysteine levels, thereby reducing antioxidant capacity and contributing to oxidative stress.(65) Excessive reactive oxygen species (ROS) at high concentrations can damage proteins, DNA, and cellular membranes, ultimately impairing critical cellular functions such as migration and proliferation.(65) Thus, it may lead to increased levels of VEGF-A, VEGF-C, and VEGF-D without resulting in a functional pro-angiogenic effect.

Additionally, the effect of AOAA on VEGF receptors 1, 2, and 3 was studied. The results were consistent and significant in both single-group and two-group experiments (with and without pressure), showing that AOAA treatment is associated with a decrease in VEGF-R1 expression at concentrations of 1, 2, and 4 mM in the group in normal Conditions. No significant differences were found in VEGF-R2 and VEGF-R3 expression and the bEnd.3 cells exposed to 120 mmHg pressure did not show significant changes.

VEGF-R1 acts as a modulator of angiogenesis by binding to VEGF-A. By sequestering VEGF-A, it decreases its binding to VEGF-R2, which has a strong angiogenic role.(66)

A decrease in VEGF-R1 expression following AOAA exposure in bEnd.3 endothelial cells could explain the observed increase in VEGF-A concentration, as the ligand may remain unbound due to reduced receptor availability. However, despite this elevated VEGF-A level, cell proliferation and migration are reduced after AOAA treatment, suggesting that AOAA may exert its effects through alternative signaling pathways in endothelial cells.(67-69)

This contrasts with the findings of Hatamie et al., who reported a reduction in VEGFR-2 and VEGFR-3 expression in human lymphatic endothelial cells following a similar treatment, while observing no significant changes in the expression levels of VEGF-C and VEGF-D in HDLECs after treatment with 4 mM AOAA compared to the control group. It is well established that VEGF-R3 plays a central role in lymphangiogenesis.
In other studies, AOAA did not exert any significant effect on the migratory capacity of EO771 cells, a murine epithelial-like carcinoma cell line. (70)

The discrepancy in VEGF and VEGF receptor expression patterns may be attributed to cell type–specific differences, as endothelial and lymphatic endothelial cells exhibit distinct However, there are currently no other studies describing the effect of CBS inhibition on angiogenesis and/or the expression of VEGF and VEGF-R in endothelial cells.

On the other hand, a change in cell morphology was observed in Bend.3 cells cultured under 120 mmHg pressure for 24 hours, showing nuclear enlargement and a polyhedral shape in both the control group and the group treated with the AOAA inhibitor at a concentration of 0.5 mM. In contrast, in the control group cultured under normal conditions, no nuclear enlargement or polyhedral shapes were observed, and the cellular appearance remained unchanged. Cell morphology also remained unchanged in the group cultured under 120 mmHg pressure with the AOAA inhibitor at a concentration of 4 mM (Fig. 9), suggesting a potential protective effect of the inhibitor against stress. However, further studies are needed to evaluate cellular morphology in more detail, such as analyzing cytoskeletal organizatio, and cell viability assays to better understand the underlying mechanisms.(71, 72)

Finally, the effect of the CBS inhibitor was studied in mouse retinal explants cultured under normal conditions and at a pressure of 60 mmHg. Previous studies have shown that viability for retinal explant culture at 60 mmHg is still possible (73, 74). However elevated pressure can impact gene expression, which may affect the reliability of results in protein isolation and qPCR analyses. (74) Studies have demonstrated that increased pressure alters gene expression patterns in retinal progenitor cells, as shown by RT-qPCR.(74)

In the present study, an initial experiment with the retinal explants without the expose of AOAA was conducted with a control group in normal conditions and another group cultured at 60 mmHg. (Fig. 14) An increasing trend was observed in VEGFR1, VEGFR2, and VEGFR3, as well as in CBS expression in cells cultured under pressure. No changes were detected in VEGF A and VEGF-D expression. However, these results were not statistically significant.

In a subsequent experiment, two groups were analysed (Fig 15): Retinal explants exposed to 4 mM AOAA, cultured under normal conditions and in the other Hand at 60 mmHg pressure, each with their respective control groups. The results were contradictory compared to the first experiment without AOAA exposure, as in the control groups there was a trend toward reduced expression of VEGFR1 and VEGFR2, and in the other hand no variation in the VEGFR3 expression under 60 mmHg pressure was founded. Additionally, CBS, VEGFA, and VEGFD expression remained unchanged between control and pressure groups and no significant differences were observed.

It is worth mentioning that, for the analysis of protein expression in retinal explants—specifically VEGF and VEGF receptors—additional techniques such as Western blot can be employed to confirm their presence in specific cell types. Moreover, complementary methods like immunofluorescence can be used to detect the spatial localization of VEGF and VEGFRs in mouse retinas. Furthermore, FACS (Fluorescence-Activated Cell Sorting) can be applied to isolate specific cell populations, allowing for subsequent analysis of their individual expression profiles.(75)

The present study demonstrated that the inhibitor AOAA exerts anti-proliferative and anti-migratory effects on bEnd.3 cells—both of which are key steps in angiogenesis—suggesting that AOAA may act as an anti-angiogenic agent. Furthermore, AOAA appears to play a protective role under stress conditions, such as exposure to 120 mmHg pressure, as cell structure remained unaltered following treatment with 4 mM AOAA. The involvement of the VEGF and VEGF-R pathways remains unclear, as no significant decrease in VEGF or VEGF-R with a role in angiogenesis, such us VEGF-R2 and VEG-R3 expression was observed, suggesting that AOAA may act through alternative molecular pathways. The experiments conducted on mouse retinas did not yield significant results; therefore, it is recommended to repeat these experiments and to include additional techniques such as Western blot or immunofluorescence in mouse retinas to specifically investigate protein expression.

In summary, the results of this thesis demonstrate that the CBS enzyme inhibitor, AOAA, exhibits anti-angiogenic effects by inhibiting the migration and proliferation of endothelial cells (bEnd.3), which are key steps in angiogenesis. The expression of VEGF and VEGF receptors (VEGF-R) may vary depending on the cell type studied. In this thesis, experiments carried out on bEnd.3 cells showed an increase in VEGF-A, VEGF-C, and VEGF-D, as well as a decrease in VEGFR-1 following CBS inhibition. These findings open the possibility that AOAA might act on additional enzymatic targets to inhibit angiogenesis and that VEGF and VEGF-R expression may differ according to the cellular context.

Cell morphology remained unchanged in the group exposed to stress conditions (incubation under 120 mmHg pressure) with AOAA treatment, suggesting a potential protective role of CBS inhibition under stress conditions. These findings warrant further investigation into the CBS enzymatic pathway, either through the analysis of cellular morphology under different stress conditions or by exploring the involvement of oxidative stress at the molecular level. Future studies could include detailed assessments of mitochondrial function, reactive oxygen species (ROS) production, and antioxidant enzyme expression (such as superoxide dismutase, catalase, and glutathione peroxidase) to better understand the redox balance influenced by CBS inhibition. Additionally, high-resolution imaging techniques and molecular markers of cytoskeletal integrity could be employed to evaluate morphological changes in response to AOAA treatment.

Future research should focus on the third step of angiogenesis: the formation of tubes in response to AOAA exposure in vitro. In addition, immunofluorescence studies—both on cells and retinal explants—should be performed to localize VEGF and VEGF-R expression. Western blot experiments could be used to confirm protein expression levels obtained from qPCR results.

To determine whether AOAA exerts a protective effect under stress, further experiments investigating cellular apoptosis and necrosis are recommended. In diseases such as glaucoma, changes in retinal vascularization have been observed in experimental mouse models. Therefore, immunofluorescence analysis of retinal explants subjected to pressure and AOAA exposure could provide valuable insights into VEGF and VEGF-R protein expression under such conditions.

Finally, it is important to emphasize the potential anti-angiogenic role that the AOAA inhibitor may play, as well as its possible implications in conditions such as corneal neovascularization. Therefore, preclinical studies in animal models, followed by clinical studies, will be necessary.

In the case of glaucoma, if AOAA proves to have a neuroprotective effect, this would represent another promising avenue for future research. The study of retinal vasculature as well as the expression of VEGF and its receptors under stress conditions using cell culture with a pressure chamber in vitro or in glaucoma animal models would be necessary

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7. Anhang

7.1. Abbildungsverzeichnis

Figure 1: Page 18

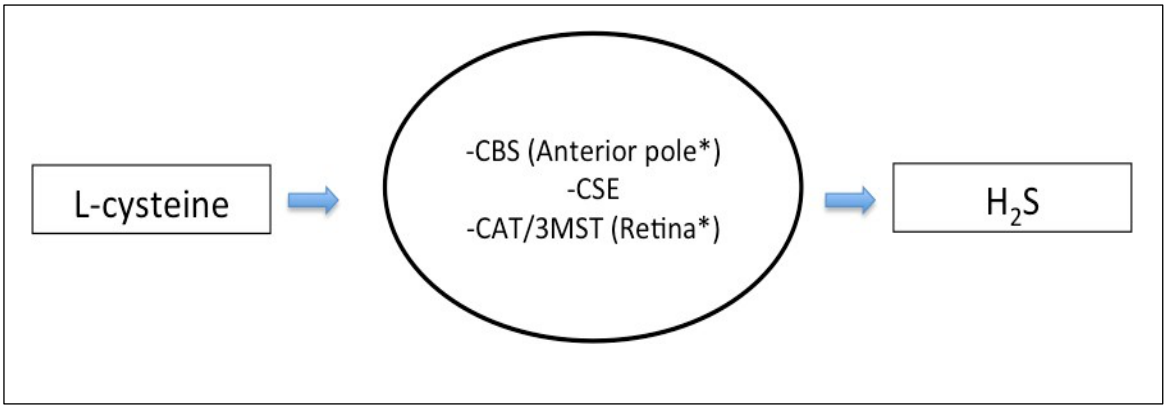

Figure 1 The three enzymatic pathways that regulate endogenous H2S production. Cystathionine-ß-synthase (CBS), cystathionine-y-lyase (CSE), cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (CAT/ 3MST).
**Strong expression*

Figure 2: Page 20

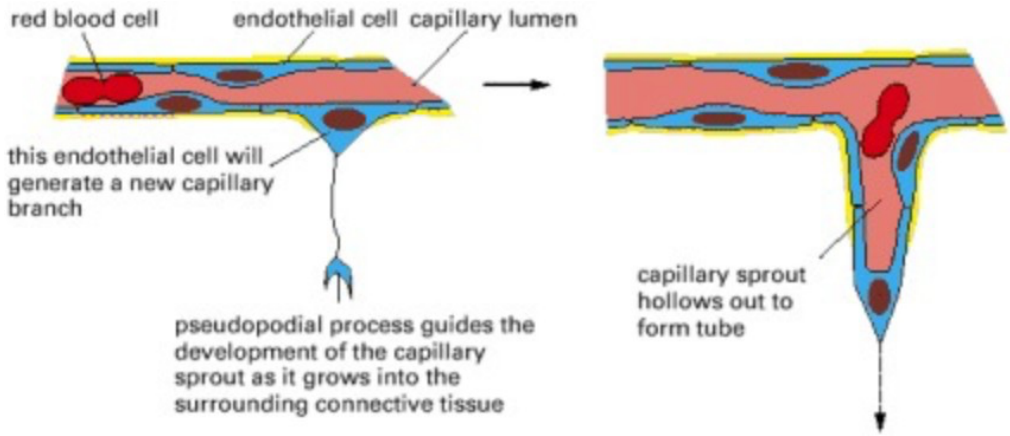

Figure 2. **Angiogenesis.** This image is based on the cells from the tail of a living tadpole. A new blood capillary forms from an endothelial cell. (Speidel, Am. J. Anat. 52:1–79, 1933.) (44)

Figure 3: Page 21

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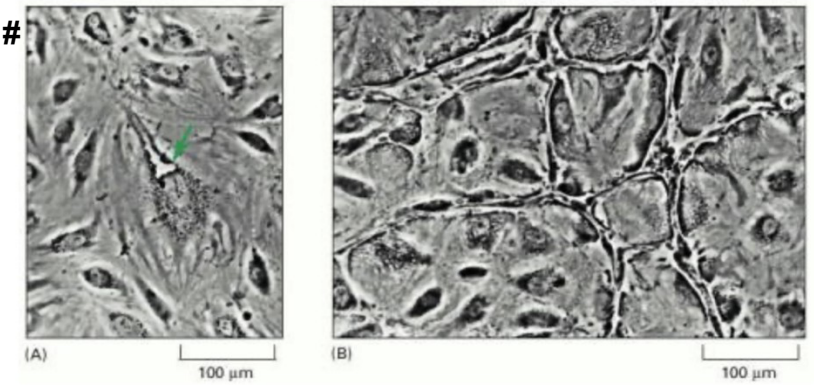

Figure 3. Angiogenesis in vitro. The arrow (A) points to a vacuole formed in a single endothelial cell. The colony spreads across the plate, and after 20 days, capillary tubes begin to form in the central regions. As seen in (B), an extensive network of tubes is formed. (44)

Figure 4: Page 21

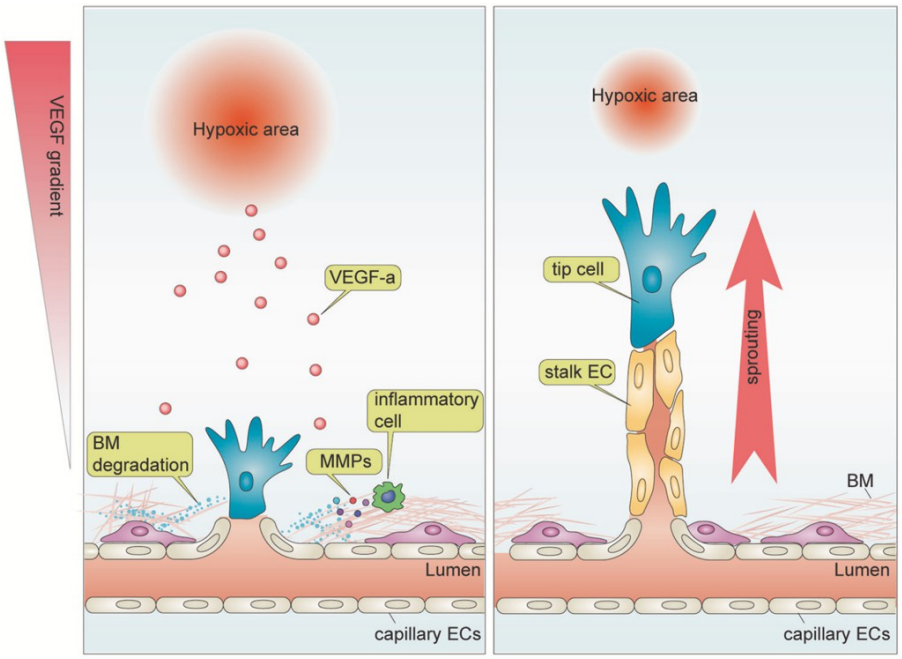

Figure 4. Tradicional model of angiogenesis. The expression of VEGF-A is increased under hypoxia, inducing the formation of new blood vessels and stimulating the recruitment of proinflammatory cells, which are responsible for the local production of metalloproteases (MMPs), facilitating the migration of endothelial cells. (47)

Figure 5: Page 33

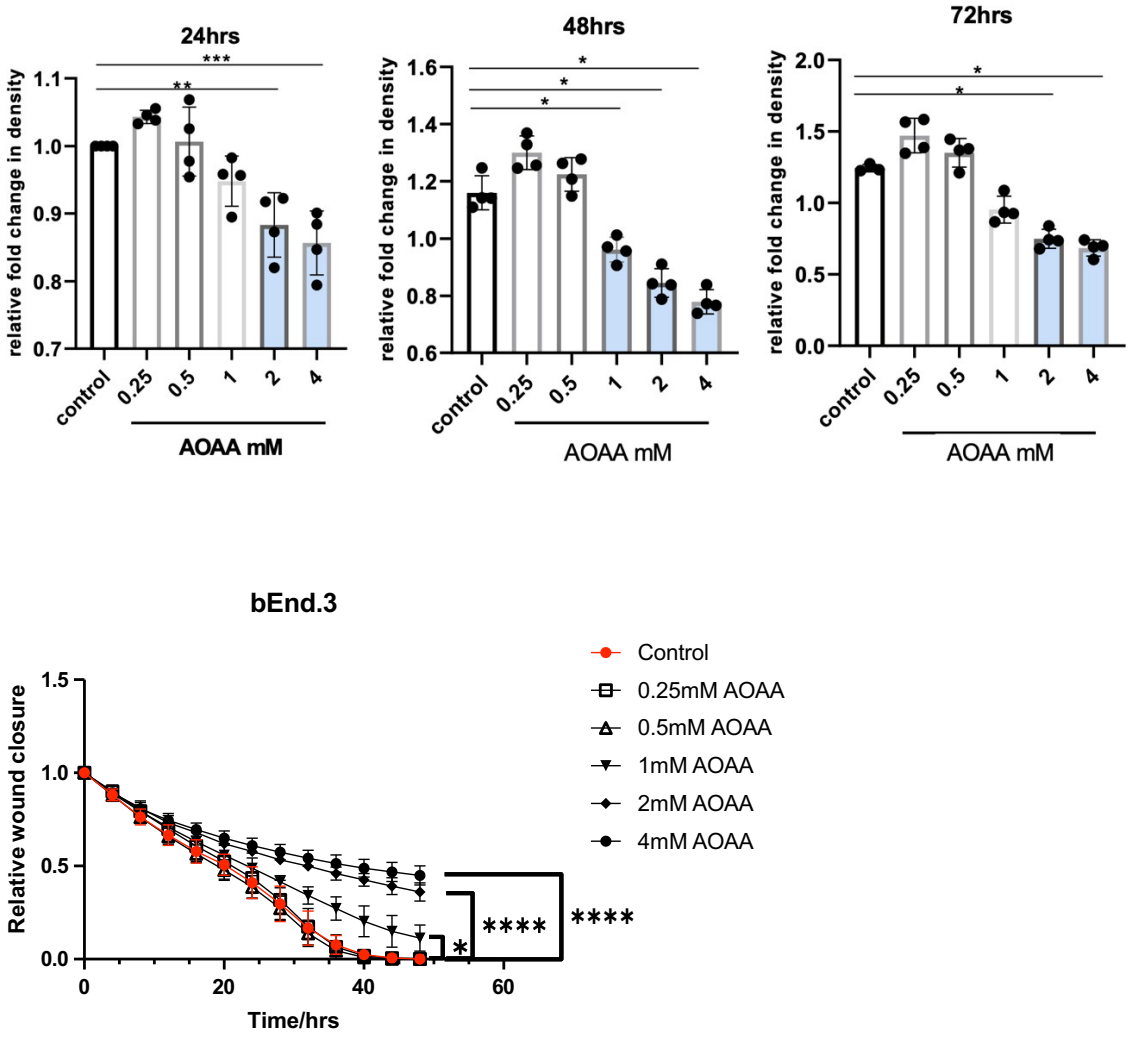

Figure 5. A Effect of AOAA on the proliferation of bEND.3 after 24 h-48h-72h. Proliferation was determined by using IncuCyte Zoom (n=4). **B** Migration Assay. AOAA treatment of bEND.3 decrease migration compared to control cells ($n = 3$). Statistical significance was analyzed with **(A, B)** one-way ANOVA. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 6: Page 34

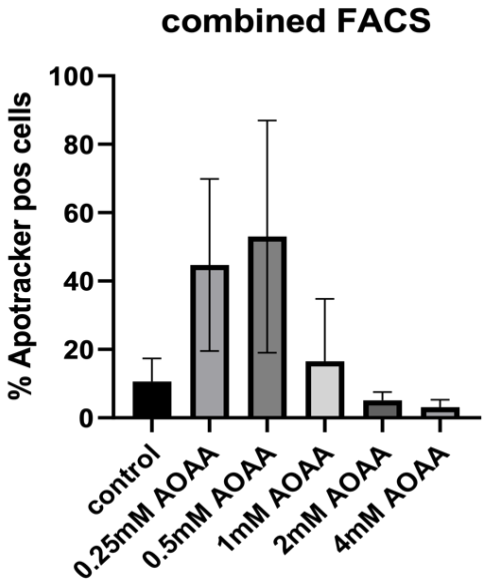

Figure 6. Effect of AOAA on apoptosis of bEND.3 after 24 h. (n=3). Apotracker was used to detect apoptosis and 7-Amino-Actinomycin D (7 ADD) to detect necrosis.

Figure 7: Page 35

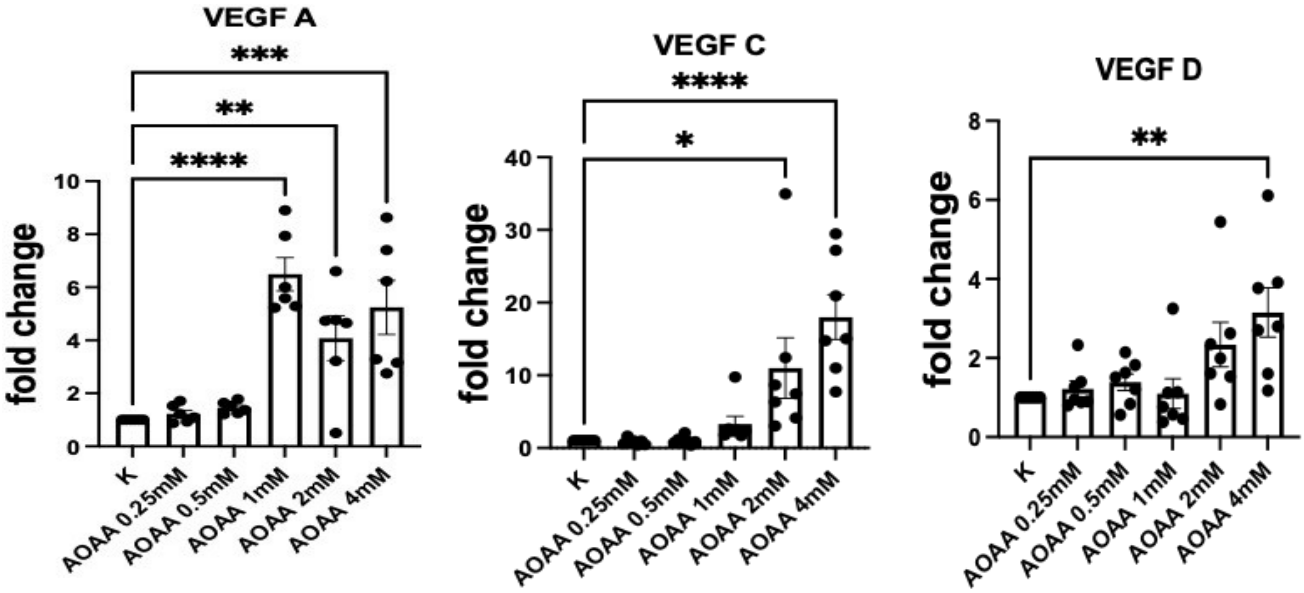

Figure 7. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-A*, and *VEGF-C* and *VEGF-D* was assessed using real-time PCR. (**A**)n = 6, (**B**) n=7, (**C**) n=7. Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$;

Figure 8: Page 36

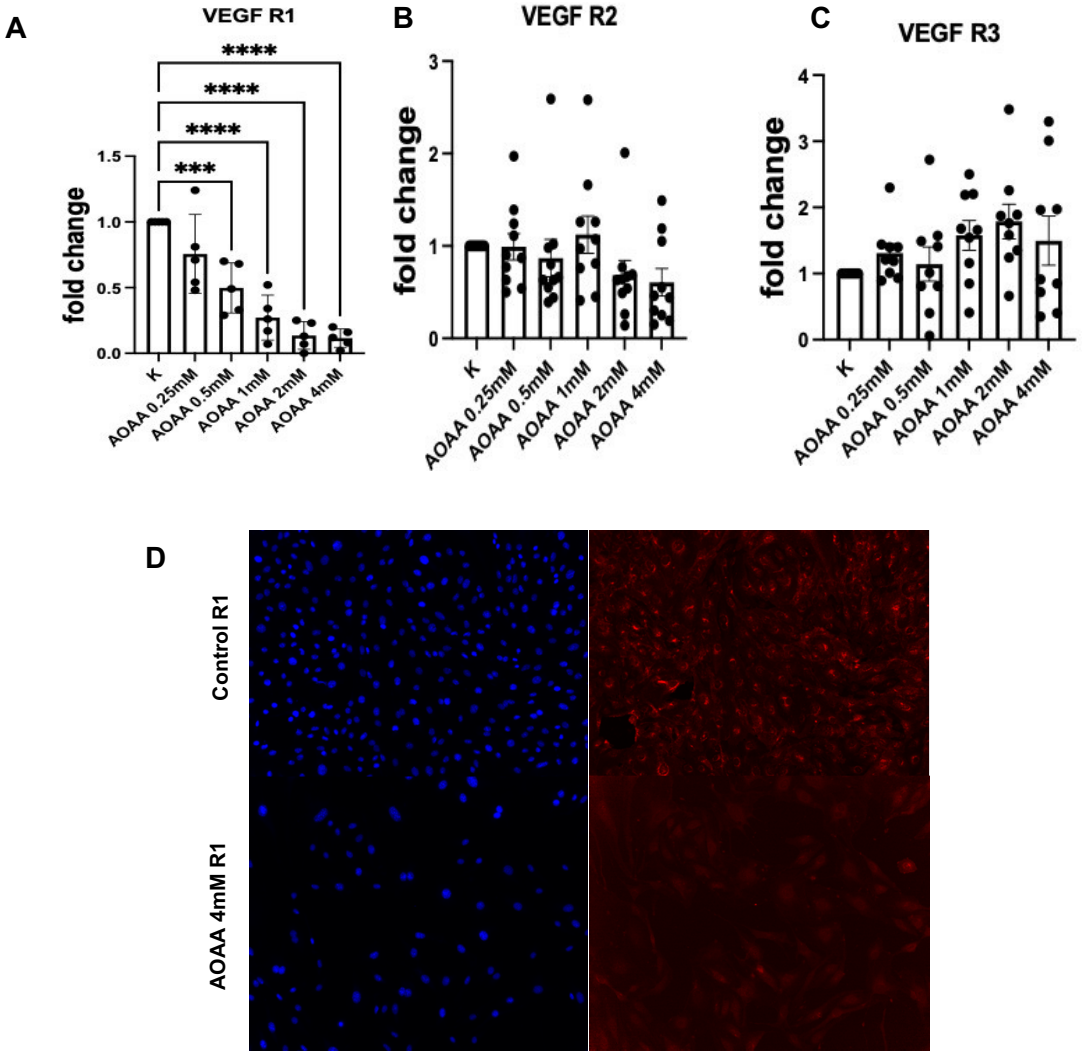

Figure 8. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-R1*, and *VEGF-R2* and *VEGF-R3* was assessed using real-time PCR. (**A**)n = 5, (**B**) n=10, (**C**) n=9. Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test ($n = 5$). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. (**D**) The immunostaining of VEGF-R1 shows a low signal in the group treated with AOAA at a concentration of 4 mM.

Figure 9: Page 37

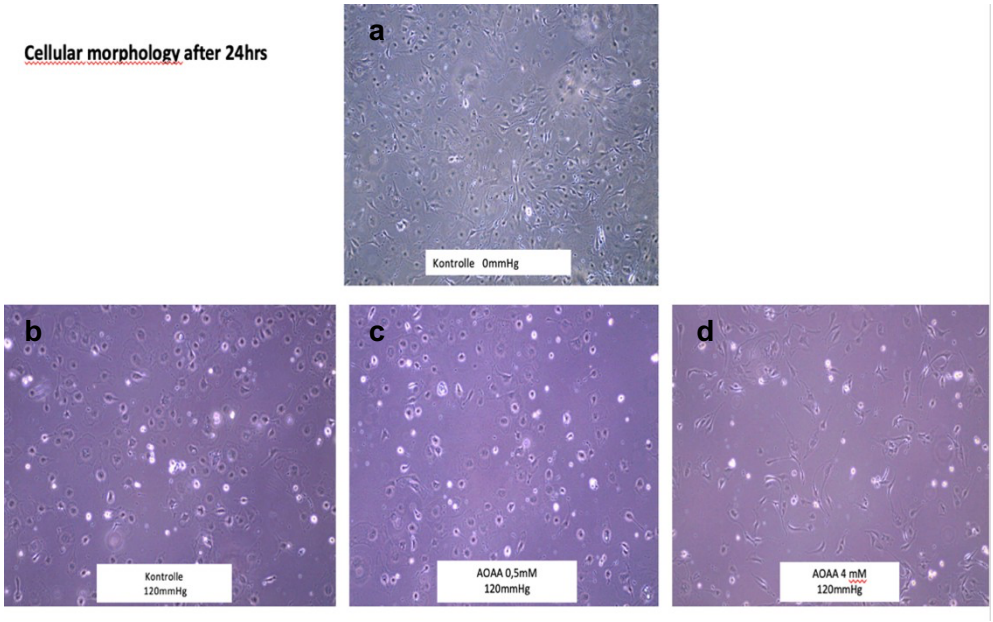

Figure 9. bEND3 were treated with indicated concentrations of AOAA for 24 h in the pressure chamber at 120mmHg**. (b)** In the Control group with 120mmHg pressure rounded and enlarged nuclei are observed, **(d)** in the group with AOAA at 4mM and 120mmHg pressure the nuclei maintain their shape and size compared to the control group without pressure **(a)**

Figure 10: Page 38

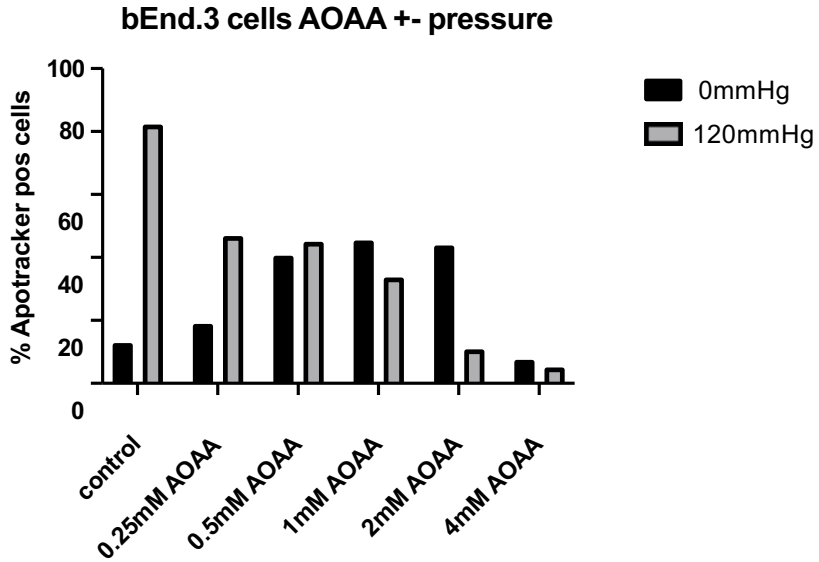

Figure 10. Effect of AOAA on apoptosis of bEND.3 after 24 h. (n=3). Apotracker with 1:10 Dilution was used to detect apoptosis and 7-Amino-Actinomycin D (7 ADD) to detect necrosis. The results were not statistically significant (n = 3).

Figure 11: Page 39

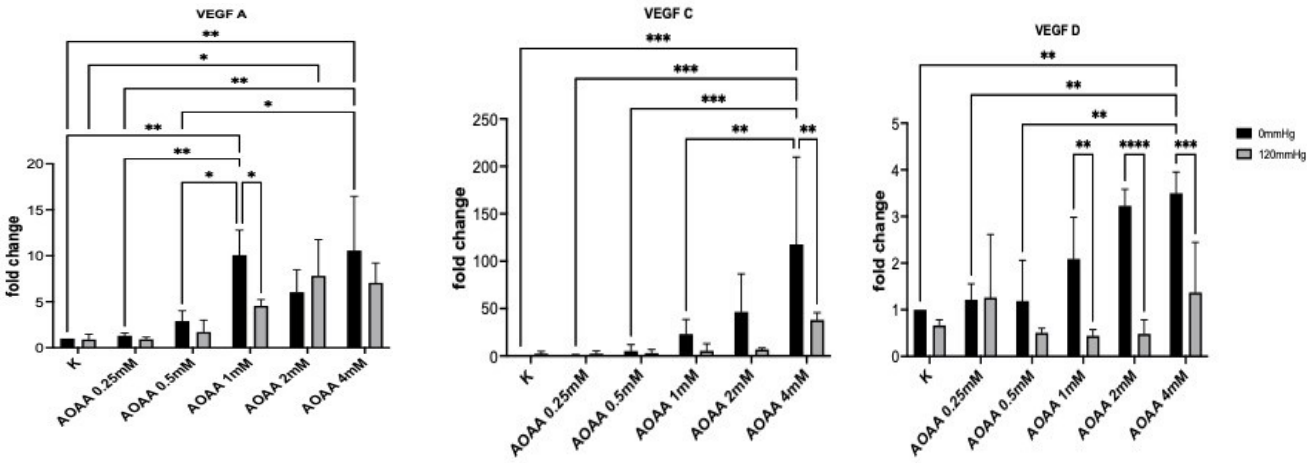

Figure 11. bEND3 were treated with indicated concentrations of AOAA for 24 h. Level of mRNA for *VEGF-A*, and *VEGF-C* and *VEGF-D* was assessed using real-time PCR. **n = 3.** Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 12: Page 41

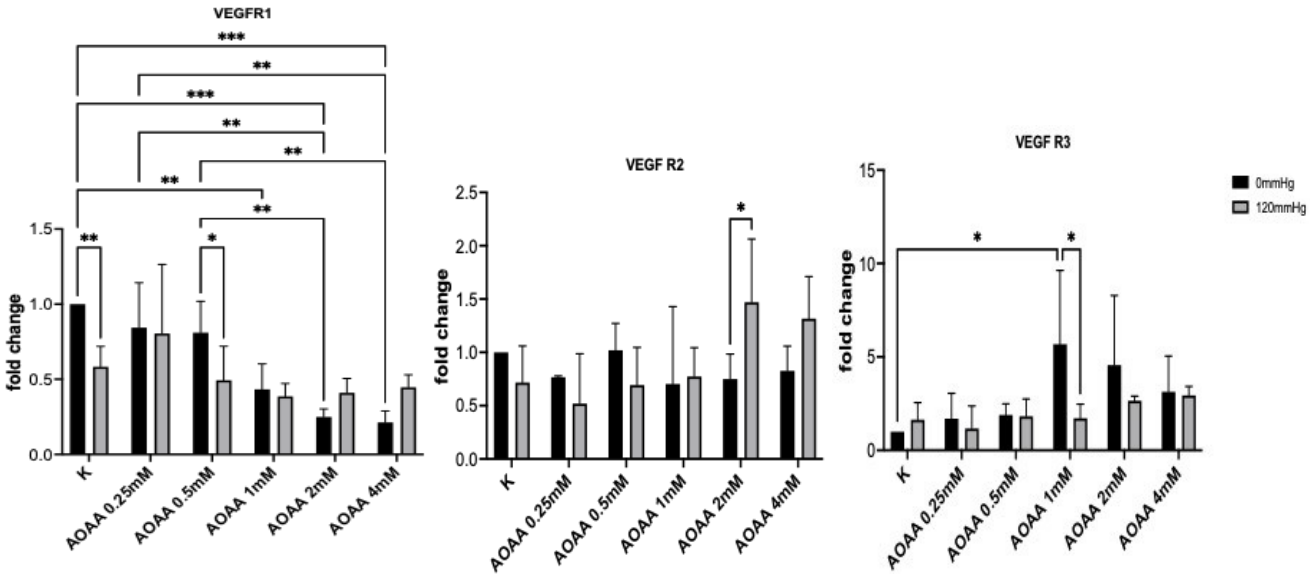

Figure 12. bEND3 were treated with indicated concentrations of AOAA for 24 h in normal conditions and under 120mmHg Pressure. Level of mRNA for *VEGF-R1*, and *VEGF-R2* and *VEGF-R3* was assessed using real-time PCR. (**a**)**n = 3.** Data are presented as means ± SEM. Statistical significance was analyzed with two-tailed *t*-test. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 13: Page 42

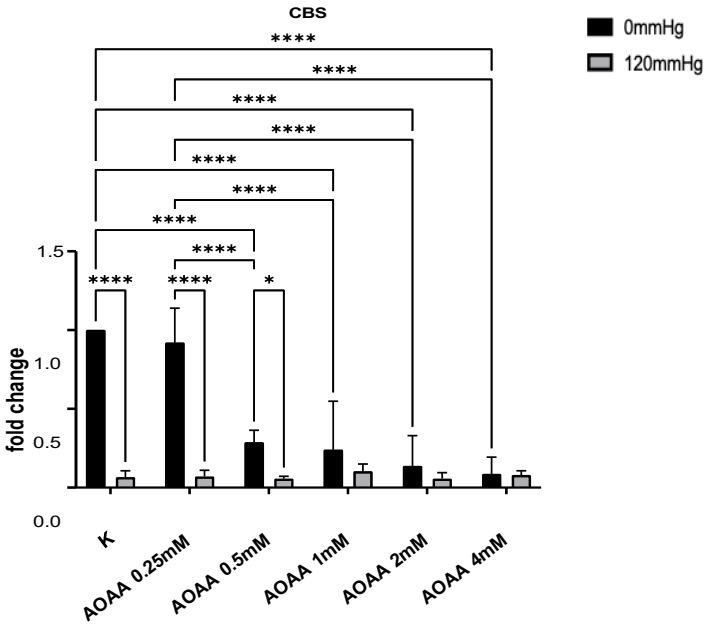

Figure 13. bEND3 were treated with indicated concentrations of AOAA for 24 h in normal conditions and under 120mmHg Pressure. Level of mRNA for *CBS* was assessed using real-time PCR. (**a**) n = 3. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 14: Page 43

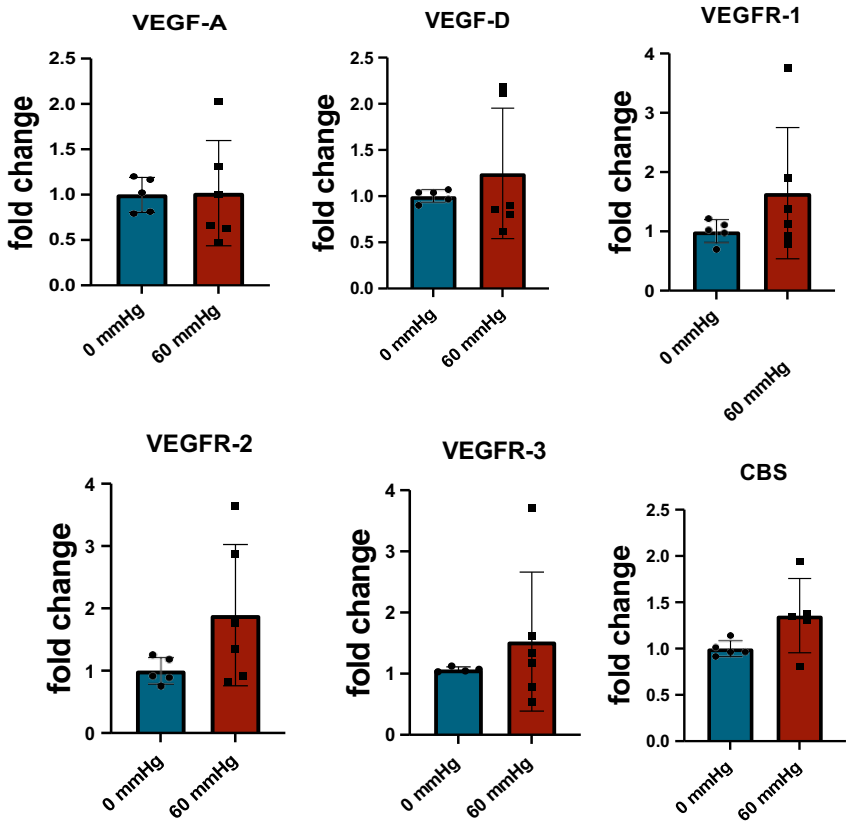

Figure 14. qPCR of mouse retina explants cultured under normal conditions and under 60 mmHg pressure. (n = 5) Results were not significant.

Figure 15: Page 45

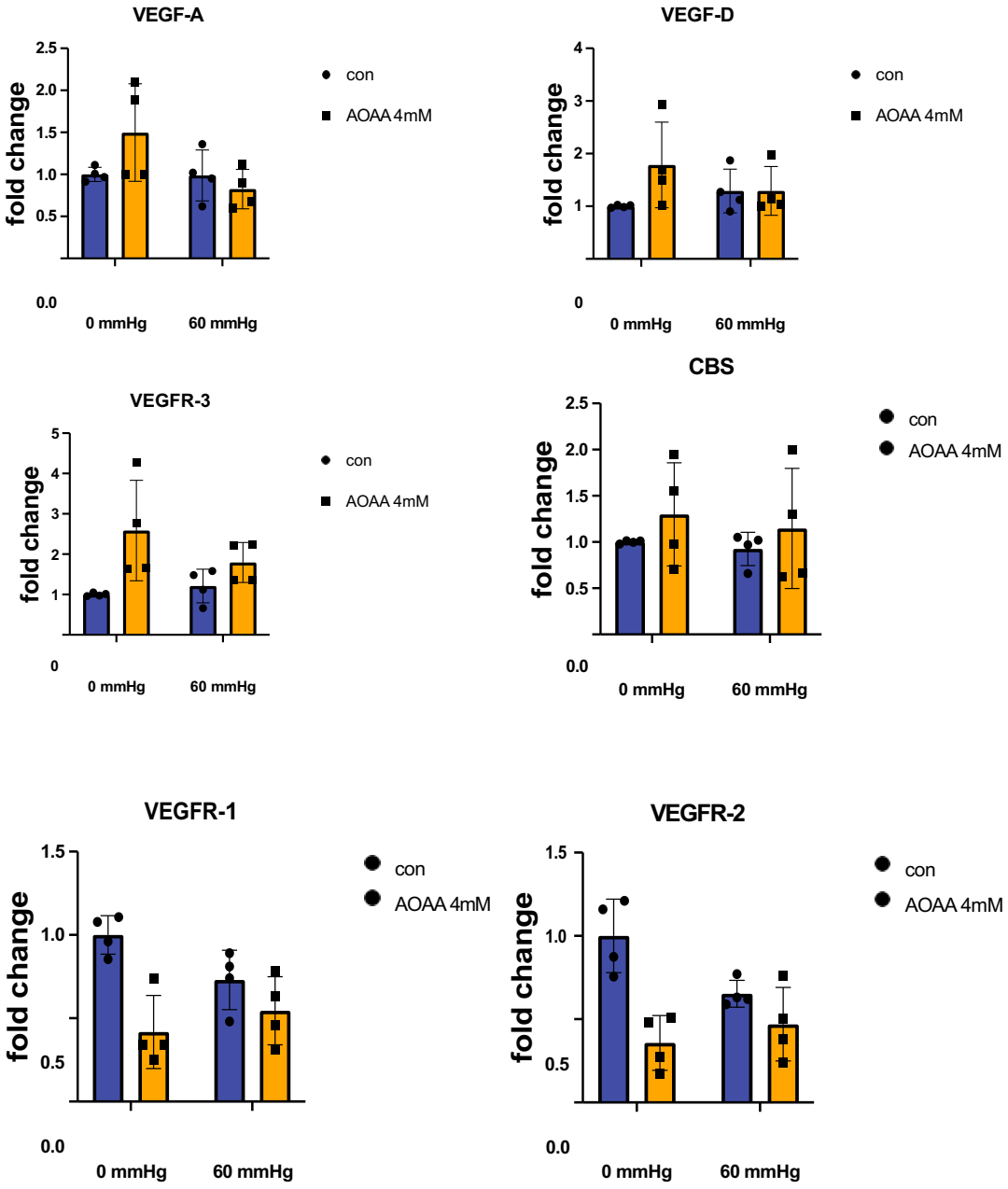

Figure 15. qPCR of mouse retina explants cultured under normal conditions and under 60 mmHg pressure, with a control group and a group treated with AOAA 4mM (n = 5) Results were not significant.

7.2. Tabellenverzeichnis

Table N° 1: Page 16

Table N° 1: Most leading causes of corneal neovascularization

Categories	Cause
Hypoxia	Contact lens wearing
Infectious Keratitis	Viral, Fungal, Bacterial, Parasitic
Inflamatory disorder	Mucous membrane pemphigoid, Stevens-Johnson syndrome, Atopic conjunctivitis, Rosacea, Lyell´s syndrome, corneal graft rekection
Loss of limbal barrier function	Limbal stem cell deficiency, Chemical burn, thermal burn
Other Disorders	Ocular surface neoplasia (papilloma, and conjunctival or corneal intraepithelial neoplasia) Pterygium

Table 2: Page 22

Table 2. Angiogenesis inhibitors discovered in Folkman´s laboratory from 1980 to 2005 (50)

1980	Interferon alpha-betha
1982	Platelet factor 4/Protamine
1985	Angiostatic stereoids
1990	Fumagillin
1994	Angiostatin
1994	Thalidomide
1994	2-methoxyestradiol
1997	Endostatin
1999	Cleaved antithrombin III
2002	3-Aminotholidomide
2003	DBF-maf
2005	Caplostatin

Table 3: Page 23

Table 3: Primer and oligos - Mouse primers

Name	Sequence (5´→ 3´)
RPS29-for	GAG CAG ACG CGG CAA
RPS29-rv	CCT TTC TCC TCGTTG GG C
CBS_for	GATCGCCAGAAAGCTGAAGGAG
CBS_rv	CCACCTCATAGGCTGTTTGCTC
VEGF -A-for	CAT GGA TGT CTA CCA GCG AAG
VEGF-A-rv	CAT GGT GAT GTT GCT CTC TGAC
VEGF- C -for	AGA ACG TGT CCA AGA AAT CAG C
VEGF-C-rv	ATG TGG CCT TTT CCA ATA CG
VEGF- D -for	ATG GCG GCT AGG TGA TTC C
VEGF-D-rv	CCC TTC CTT TCT GAG TGC TG
VEGF-R1-for	TTG GTG GTG GCT GAC TCT CA
VEGF-R1-rv	TCT CCT TCG GCT GGC ATC TT
VEGF-R2-for	ATT CTG GAC TCT CCC TGC CTA C
VEGF-R2-rv	GCT CTT TCG CTT ACT GTT CTG G
VEGF-R3-for	GTC CCT CTA CTT CCA ACT GCT TC
VEGF-R3-rv	CACTCC TCC TCT GTG ACT TTG AG

Table 4: Page 24

Table 4: Cell culture

DMEM Dulbeco´s Modified Eagle Medium + Glutamax	Gibco
Fetal Bovine serum (FBS)	Gibco
Dulbecco's Phosphate-Buffered Saline (DPBS)	Gibco
T25 cell culture flask	Greiner Bio One
T75 cell culture flask	Greiner Bio One
96-Well Cell Culture Plates	Thermo Scientific™
Trypan blue solution 0.4%	Sigma-Aldrich
6-well Cell Culture Plates	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
[BEND3] endothelial cells	

Table 5: Page 24

Table 5: Antibodies

Mouse VEGFR1-Fit-1 Antibody
DAPI

Table 6: Page 24

Table 6: Kits, reagents and accesories

RNeasy Mini Kit	Qiagen, Hilden, Germany
RNeasy Micro Kit	Qiagen, Hilden, Germany
RNase-free DNase Set	Qiagen, Hilden, Germany
RevertAid First-Strand Synthesis cDNA Synthese Kit	ThermoScientific, Langenselbold, Germany
SsoFast EvaGreen Supermix kit	BioRad, Munich, Germany
PowerTrack SYBR Green Master Mix	ThermoScientific, Langenselbold, Germany
Aminooxy acetic acid(AOAA)	Sigma Aldrich, Taufkirchen, Germany
Chemicals Paraformaldehyde, 4 % in PBS (PFA)	Alfa Aesar, Kandel, Germany

Apotracker TM Green	
7-AAD Staining Solution	

Table 7: Page 25

Table 7: Devices

Name	Company	Function
IncuCyte TM Zoom	Essen Biosciences, Hertfordshire, UK	Live cell imaging
Zeiss Primo Vert inverted microscope fitted with an AxioCam ERc5s camera	Carl Zeiss Microscopy Gmb, Jena, Germany	Imaging
BioRad CFX96	BioRad, Munich, Germany	Detection and quantification of nucleic acid
Bioscience FACSCanto II Flow Cytometer	BD Biosciences, Heidelberg, Germany	Cell sorting analysis
Fluorescence microscope	Olympus BX63	Imaging
NanoDrop 2000c	Thermo Fisher Scientific	DNA quantification
Applied Bioscience Quantstudio 6	ThermoScientific, Langenselbold, Germany)	Detection and quantification of nucleic acid

Table 8: Page 26

Table 8: Software

Name	Company	Function
IncuCyteTM software Version 2016B and 2018A	Essen Biosciences, Hertfordshire, UK	Live cell image analysis
FACS DIVA 8.0.2	Beckton Dickenson, Ashland, USA	Cell sorting analysis
cell^F 3.4	Olympus Europe, Hamburg, Germany	Image analysis
NanoDrop 2000 (v.1.5)	Thermo Fisher Scientific	DNA quantification
GraphPad Prism software version 8	GraphPad Software, San Diego,CA	Statistical analysis
Image J (1.53K)	National group of Health, USA	Image analysis

Table 9: Page 26

Table 9: Animals

C57BL/6 NCrl Mice	Charles River Germany, Sulzfeld, Germany

Table 10: Page 40

Table 10: VEGF Expression in Response to AOAA Treatment (qPCR Analysis)

Condition	AOAA	VEGF-A	VEGF-C	VEGF-D
Normal culture conditions (1)	1 mM	↑****	—	—
	2 mM	↑**	↑*	—
	4 mM	↑***	↑****	↑**
Normal culture conditions(2)	1 mM	↑**	—	—
	2 mM	—	—	—
	4 mM	↑**	↑***	↑**

(1) Figure 7. One group experiment

(2) Figure 11. In the two-group experiment, a significant increase in VEGF-A, VEGF-C, and VEGF-D expression was observed under normal conditions following treatment with 4 mM AOAA **Legend: ↑ = Significant increase, — = No significant change**

Table 11: Page 41

Table 11: VEGF-R Expression in Response to AOAA Treatment (qPCR Analysis)

Condition	AOAA	VEGF-R1	VEGF-R2	VEGF-R3
			—	—
	0,5mM	↓***		
Normal culture conditions (1)	1 mM	↓****	—	—
	2 mM	↓****	—	—
	4 mM	↓****	—	—
120 mmHg pressure (2)	0,5mM – 4mM	—	—	—

(1) Figure 8. One group experiment

(2) Figure 12. In the two-group experiment, a significant decrease in VEGF-R1 expression was observed under normal conditions following treatment with 1 mM, 2 mM, and 4 mM AOAA. In contrast, no significant changes were observed in the group exposed to 120 mmHg pressure

Legend: ↓ = Significant decrease, — = No significant change

7.3. Acknowledgments

I would like to express my sincere and profound gratitude to my two thesis supervisors. I am deeply thankful to Professor Dr. Prokosch for granting me the opportunity to participate in an experimental research project and for introducing me to the fascinating world of laboratory work. Her guidance allowed me to experience firsthand the generation of new knowledge and the rigor required in scientific research.

My heartfelt appreciation also goes to Dr. Clahsen, who not only served as my mentor but also as a constant source of support and scientific inspiration in the laboratory. His patience, dedication, and commitment to research have left a lasting impression on me and have played a key role in shaping my academic path. The passion for science shared by both of my supervisors has been a decisive factor in my decision to continue pursuing doctoral research.

I would also like to express my deepest gratitude to my parents, whose unwavering trust and support have accompanied me throughout the journey. To my husband, I extend my warmest thanks for his continued encouragement, for motivating me to always follow my passion, and for standing by me through every challenge and accomplishment.

Furthermore, I wish to thank all the members of the laboratory—biologists, technicians, and physicians—for their collaboration, professionalism, and the supportive environment they provided.