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THE ANTI-VEGF THERAPY IN OCULAR NEOVASCULARIZATION

ABSTRACT

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ABSTRACT

Introduction

Sight is the most important of our senses, as it is the vital element for connecting and integrating with the world around us. In this context, vision loss is one of the events that profoundly and irremediably affects the quality of life.

In the first report dedicated to vision by the World Health Organization, published in 2019, it is shown that at least 2.2 billion people suffer from visual impairments, of which tens of millions of patients have severe conditions that threaten the loss of vision [1].

The world-renowned scientific prize, the António Champalimaud Vision Award, often called the 'Nobel Prize of Vision,' recognized the revolutionary development of anti-angiogenic therapy for retinal diseases. It was awarded in 2014 to researchers for their contributions to the identification of vascular endothelial growth factor (VEGF) as a major trigger of ocular angiogenesis—a phenomenon underlying retinal pathology associated with vision loss, including age-related macular degeneration and diabetic retinopathy. These contributions have led to the development of new anti-VEGF-type treatments for these diseases [2].

The general part

Ocular neovascularization, also known as pathological angiogenesis, is the leading cause of blindness in developed countries. While the process of neovascularization can affect various parts of the eye (such as the cornea, iris, retina, and choroid), proliferative diseases of the retina play a central role in conditions associated with vision loss.

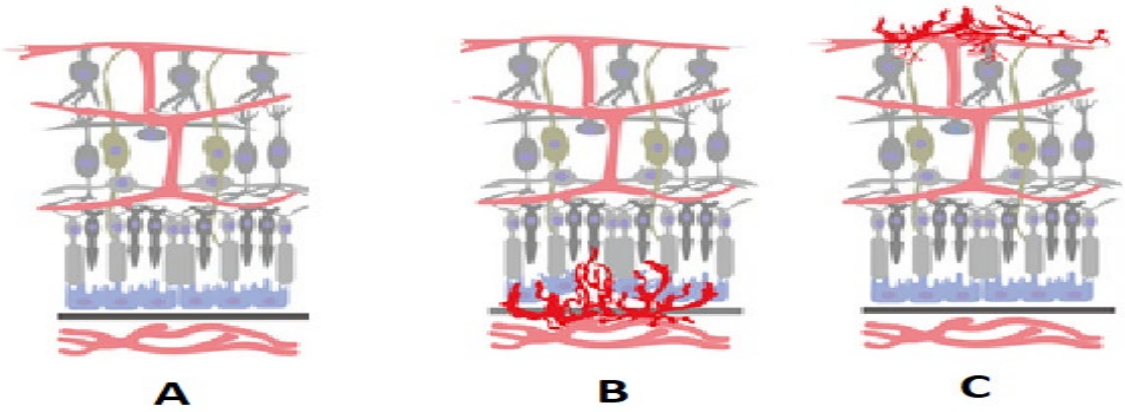
Proliferative diseases of the retina are generally categorized based on the location of the neovascularization process: retinal or choroidal. Retinal neovascularization refers to a condition in which new pathological blood vessels emerge from existing retinal veins and extend along the inner surface of the retina. On the other hand, choroidal neovascularization (CNV) originates from the choroid and grows by breaking through Bruch's membrane beneath the retinal pigmented epithelium (RPE) or retina. Virtually any pathological process involving the RPE and causing damage to Bruch's membrane can potentially lead to complications involving CNV.

In the process of vision, light must reach the photoreceptors. For this reason, the outer retina is largely avascular; having blood vessels located immediately in front of the

photoreceptors would affect the image formation. Instead, the retina is highly vascularized through a dual network: blood vessels in the inner layers of the retina and the choroidal network.

The vascularization of the inner retina is composed of deep and superficial capillaries, responsible for nourishing the inner two-thirds of the retina. Meanwhile, the choroidal vasculature supplies the outer third of the retina. These two vascular networks are separated by the retinal pigmented epithelium. In this context, neovascularization can arise from either the internal vasculature of the retina (retinal neovascularization) or the choroidal vasculature (choroidal or subretinal neovascularization). In both cases, new blood vessels arise, invading previously avascular regions.

Neovascularization is a protective mechanism found in many tissues throughout the body in response to injury or ischemia. For instance, wound repair in the skin involves the creation of new blood vessels to compensate the damaged ones. At the retinal level, processes leading to injuries that affect its normal vascular system result in retinal ischemia and non-perfusion, which in turn stimulates neovascularization. Vascular Endothelial Growth Factor (VEGF), normally present in healthy eyes, becomes significantly more pronounced in proliferative disease, triggering the neovascularization process.



Retinal vasculature. A – normal state, B. choroidal neovascularization with subretinal infiltration C. superior retinal neovascularization with infiltration into the vitreous space. Adapted from [4]

During the process of neovascularization, new blood vessels are formed to compensate for the lack of oxygen and nutrients, yet they exacerbate the pathological condition due to their abnormal structure compared to the vessels in a healthy retinal vasculature. Unlike normal retinal vessels, neovascularization gives rise to thinner vessels lacking tight junctions, which are crucial components of the retinal/blood barrier. Under these circumstances, these new blood vessels are susceptible to blood or plasma leakage into surrounding tissues, including the

vitreous. This can result in vitreous degeneration, leading to severe complications such as vitreous hemorrhages or retinal detachment [2].

In diagnosing retinal neovascularization, fundus examination reveals smaller and thin vessels (appearing as delicate tufts or fronds), accompanied by connective or fibrotic tissues that intensify over time. These structures are found near the optic disc or grow superficially toward the vitreous or beneath the retina. Fluorescein angiography helps identify dye leakage from these vessels into the extravascular space. The newly formed vessels are often located close to areas with poor capillary perfusion, attempting to compensate for the deficient perfusion in those regions. When using OCT angiography, abnormal vascular proliferation or viguros growth of small blood vessels at the periphery of the newly formed vessels can be observed.

After validating VEGF as a central element in the neovascularization process, including proliferative eye diseases, the subsequent step in translational medicine involved the development of antiangiogenic therapies aimed to block and neutralize VEGF within affected tissues, later recognized as anti-VEGF therapies. Anti-VEGF therapy entails the use of drugs that specifically target VEGF, aiming to inhibit its activity and curtail pathological angiogenesis in the retina. These drugs are typically administered through intravitreal injections, directly into the vitreous cavity of the eye. Their effect is to obstruct VEGF's action, to inhibit the formation of new blood vessels, diminish vascular leakage, and encourage the regression of blood vessel irregularities in the retina.

The mechanism of action of anti-VEGF therapy comprises several steps. Initially, the anti-VEGF drug binds to VEGF, preventing it from attaching to specific receptors on endothelial cell membranes. This process hampers intracellular signaling pathways stimulated by VEGF, which plays a role in stimulating angiogenesis and increasing vascular permeability. Subsequently, anti-VEGF therapy enhances vascular stability, curbing leakage of fluids and proteins into surrounding tissues. This stabilization is particularly beneficial in reducing retinal edema, a common characteristic of numerous eye conditions such as age related macular degeneration (AMD) and diabetic retinopathy (DR). Moreover, anti-VEGF therapy disturbs the growth of abnormal blood vessels, a distinct feature of pathological angiogenesis in eye diseases. By preventing the formation of fragile and leaky blood vessels, the therapy contributes to averting vision loss.

Anti-VEGF therapy has demonstrated remarkable efficacy in enhancing visual acuity and reducing retinal edema in eye conditions like neovascular age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinal vascular occlusions (RVO). Clinical trials have convincingly shown that anti-VEGF drugs considerably decrease the risk of severe vision loss and enhance the quality of life for patients affected by these conditions [3,5].

Anti-VEGF therapies encompass several variants, among which the following are the most commonly used:

1. Monoclonal anti-VEGF antibodies that hinder members of the VEGF family, with the most significant being the VEGF-A isoform variant 165. Examples include Ranibizumab (Lucentis), Bevacizumab (Avastin), Brolucizumab (Beovu), and Faricimab (Vabysmo).
2. Monoclonal antibodies that obstruct VEGF receptors (VEGFR), preventing the binding of VEGF molecules to these receptors and subsequently thwarting the activation of vascular proliferation signal pathways.
3. Soluble variants of VEGF receptors, which, when present in the bloodstream, attach to VEGF molecules before they can bind to cellular VEGF receptors. For instance, Aflibercept (Eylea) and Conbercept (Lumitin).
4. Small molecules that selectively obstruct the binding sites on VEGFR membrane receptors for VEGF molecules.
5. Inhibitors of tyrosine kinases - small molecules capable of penetrating cell membranes and specifically attaching to the tyrosine kinase domain of VEGFR receptors. This action impedes the further activation of signaling pathways involved in the promotion of vascular growth and proliferation.
6. Gene and cell therapies.

The special part

Nearly two decades after the approval of the first therapeutic agent in antiangiogenic therapy (anti-VEGF), administered via intravitreal injection for eye diseases with neovascularization, a remarkable success has been achieved in treating the primary diseases responsible for vision loss.

However, this success is coupled with the persistent occurrence of some related issues. These include challenges like the standardization of administration protocols, the presence of resistance phenomena or inadequate response to therapy, the emergence of adverse effects due to retinal ischemia over time, the underutilization of the therapeutic potential of each employed anti-VEGF agent, the dearth of comparative studies among various anti-VEGF agents, and the lack of research on combined eye therapies incorporating therapeutic agents with complementary and synergistic effects.

The existence of these issues is influenced not only by the passage of time but also by the 'off-label' application of certain anti-VEGF agents originally approved for different types of

therapies. For instance, bevacizumab (Avastin), the pioneering VEGF inhibitor introduced to the market, received FDA approval in February 2004 for treating metastatic colorectal cancer. In 2005, Rosenfeld P. [7] presented a case study involving a patient with neovascular age-related macular degeneration who was treated with intravitreal bevacizumab. Remarkably, after just one week, a normal macular contour and stable visual acuity were observed. The conclusion was that intravitreal administration of bevacizumab might serve as an effective, affordable, and safe option for patients with age-related macular degeneration experiencing vision loss due to macular neovascularization. Subsequently, the applications of Avastin diversified, leading to an increased number of ophthalmological indications. Currently, Avastin is utilized for various pathologies including neovascular age-related macular degeneration, proliferative diabetic retinopathy, chorio-retinal inflammation, and macular edema refractory to other treatments [6].

In efforts to enhance antiangiogenic therapy, several optimization strategies have been explored [10,11].

One approach involved quantifying the therapy. Studies have revealed that patients in real-world treatments receive fewer anti-VEGF injections compared to clinical trials. Consequently, the insufficiency of anti-VEGF treatment may be associated with poorer visual outcomes [9]. Higher doses (2 mg) of the anti-VEGF agent ranibizumab did not yield additional visual acuity benefits beyond conventional dosing (0.5 mg), indicating a plateau had been reached. Interestingly, the group of AMD patients receiving the higher dose required fewer injections compared to the lower-dose group [9].

There is ongoing interest in sustained-release anti-VEGF formulations or delivery systems that could potentially validate the hypothesis that continuous VEGF inhibition leads to improved long-term visual outcomes. A promising approach involves a slow-release rechargeable device system designed for continuous drug delivery into the vitreous cavity of the eye. Numerous attempts have been made to develop extended drug delivery systems for anti-VEGF agents. Genentech's Port Delivery System (PDS), designed to administer ranibizumab (Lucentis, Genentech), may potentially become the first device of its kind to receive FDA approval in the US. The PDS represents an innovative device that could replace injectable administration, effectively eliminating the need for frequent intravitreal injections and follow-up appointments. The PDS is conceived as a permanent, reusable reservoir of the anti-VEGF agent. It is initially implanted in a surgical setting, with subsequent refills conducted in an office setting.

Another approach involved assessing the potential for qualitative optimization, particularly analyzing resistance to anti-VEGF therapy. This phenomenon is prominent in both cancers and eye diseases.

The mechanisms underpinning resistance to anti-VEGF treatment in cancer differ from those seen in response to traditional cytostatic treatments. Notably, there is no evidence of alterations in the tumor genome related to VEGF or the signaling pathways it influences [12].

In retinal diseases, certain clinical trials have documented intriguing positive therapeutic responses achieved by switching from one anti-VEGF agent to another following a clinical determination of resistance to the initial agent used in therapy. An example of this is the transition from bevacizumab therapy (for which resistance developed) to aflibercept therapy, yielding promising outcomes.

Consequently, the alteration in anti-VEGF angiogenic therapy significantly improved anatomical results, while visual function remained stable. This change had a comparable effect to other anti-VEGF agents in preserving vision. These patients had chronic, poorly responsive conditions with limited potential for visual recovery. Switching to aflibercept with frequent monitoring emerged as a viable option for patients who developed resistance to bevacizumab treatment [13].

Our study is embedded within the intricate theme of optimizing and personalizing antiangiogenic therapy through intravitreal administration of anti-VEGF agents. Its objective is to conduct a comparative analysis of the therapeutic effects of various anti-VEGF agents in ocular pathology. Simultaneously, the study aims to identify potential mechanisms of their antiangiogenic action and investigate a natural antiangiogenic agent through in vitro and in ovo studies.

The study objectives were structured as follows:

- Multiparametric evaluation of bevacizumab therapy in neovascular glaucoma.
- Comparisons between the main anti-VEGF agents used in ocular pathology, specifically bevacizumab and aflibercept. This includes evaluating their efficiency in treating exudative age-related macular degeneration, as well as investigating the antiproliferative mechanism through in vitro and in vivo assessments.
- The isolation, characterization, and exploration of a phytotherapeutic agent from mistletoe as a potential component in a combined antiproliferative therapy. This would complement classic synthetic compounds with established anti-tumor properties.

For the multiparametric assessment of bevacizumab's action in treating neovascular glaucoma, a retrospective study was conducted on 67 patients. The primary objective was to evaluate the effectiveness of intravitreal treatment with bevacizumab (Avastin) in addressing visual impairment and pain. The study encompassed medical histories, ophthalmological examinations, assessments of visual acuity, tonometry, fundus examinations, gonioscopy, and visual field tests.

Neovascular glaucoma, situated in the anterior eye segment, represents the advanced stage of retinal ischemic complications originating in the posterior eye segment. Thus, the administered therapy was comprehensive, aiming to lower intraocular pressure through the administration of local combinations of beta-blockers, prostaglandin analogs, and carbonic anhydrase inhibitors. Simultaneously, systemic carbonic anhydrase inhibitors were administered to further mitigate intraocular pressure. Additionally, efforts were directed towards improving visual acuity by arresting and eliminating neovascularization near the visual field. This involved initiating treatment with intravitreal injections of bevacizumab (Avastin) followed by pan-retinal photocoagulation.

The multiparametric analysis conducted at the end of the treatment period indicated that neovessels regressed within the first 4-7 days following Avastin injection. Subsequent to the commencement of pan photocoagulation, neovessels vanished in 63.88% of the eyes. Furthermore, intraocular pressure was reduced, normalizing in approximately 60% of cases three months after treatment initiation. It is noteworthy that therapeutic success was achieved across diverse etiological backgrounds and stages of patients' pathologies.

The study's conclusion underscores that therapy for neovascular glaucoma must consider inflammation reduction, the decrease in vitreous fluid synthesis, and the inhibition and elimination of neovascularization affecting both the visual field and the optic nerve.

Regarding the comparative analysis of the effects of Aflibercept versus Bevacizumab, two therapeutic agents with anti-VEGF action, the study aimed to clinically assess the effectiveness of these agents as first-line therapy for exudative age-related macular degeneration. Additionally, the study aimed to analyze the in vitro effects of the two agents on cell culture and in ovo conditions.

In the clinical study, the wet form of AMD treatment was evaluated by comparing ocular and systemic effects following intravitreal injections of the two anti-VEGF agents, Aflibercept and Bevacizumab. These agents were administered in comparable doses and regimens.

Both agents yielded favorable results:

- Average visual acuity exhibited significant improvement starting from the third month of treatment.
- Retinal thickness reduction was observed on OCT scans.
- No ocular complications (endophthalmitis, vitreous hemorrhage, retinal detachment) or systemic adverse effects were documented.

The comparative analysis of the effects of Aflibercept versus Bevacizumab highlighted Aflibercept's slight superiority, demonstrated by:

- Aflibercept's higher therapeutic efficacy from the first injection.

- Greater visual acuity improvement achieved with aflibercept compared to bevacizumab after the initial three injections.
- Superior effectiveness of aflibercept in clearing subretinal fluid following the first three injections.
- Aflibercept's advantage in allowing maintenance injections every two months, compared to bevacizumab's requirement for monthly injections.

From the perspective of adverse effects, some cases exhibited retinal ischemia leading to retinal atrophy, a phenomenon arising from decreased blood flow due to VEGF inhibition. Interestingly, retinal ischemia appeared to manifest more rapidly in patients treated with aflibercept.

The conclusion drawn from the comparative clinical trial is that while aflibercept demonstrates slightly superior therapeutic effects compared to bevacizumab, these advantages are tempered by bevacizumab's lower cost and reduced incidence of retinal ischemia side effects. This discrepancy can be attributed to the structural distinction between the two agents. aflibercept possesses a high affinity for VEGF-A, VEGF-B, and placenta growth factor (PIGF) forms, whereas bevacizumab only exhibits affinity for VEGF-A isoforms.

In the context of the in vitro and in ovo comparative study, the investigation focused on the cytotoxic effects of aflibercept versus bevacizumab. This analysis was conducted in vitro on two human melanoma cell lines (A375 and SK-Mel-28), as well as on a healthy cell line (human HaCaT keratinocytes). Subsequently, the study characterized the effects of both anti-VEGF agents on the chorioallantoic membrane (CAM) in an in ovo setting. The choice of melanoma cell lines stemmed from the ocular malignant variant, uveal melanoma, which holds the foremost position among eye-related cancers. Despite its relatively low incidence, uveal melanoma's high mortality rate ensures its significance [14,15].

Results from the in vitro study, involving human melanoma cell lines A375 and SK-Mel-28, showcased both agents' significant antitumor effects. These agents effectively reduced the viability of human melanoma cells in a dose-dependent manner, with aflibercept exhibiting a more pronounced effect.

In evaluating the safety profile of the tested compounds, encompassing the assessment of potential harm to healthy tissues, three parameters were monitored:
- The ability to impact the healthy cell line (HaCaT human keratinocytes).
- Calculation of potential vascular irritation using the HET-CAM test.
- Evaluation of chicken embryo viability.

Results from the study indicated a slight reduction in the viability of healthy cells (HaCaT keratinocytes), with aflibercept demonstrating a smaller decrease compared to the effect observed with bevacizumab.

Regarding the potential vascular irritant effects as determined by the HET-CAM assay for the tested active compounds, both bevacizumab and aflibercept yielded an irritation score (SI) of 1.64 and 2.13, respectively. Both scores fall within the low irritation category. Furthermore, no distinctive effects on vascular capillaries were observed in the same experiment. The viability of embryos, following the application of bevacizumab and aflibercept using the CAM method, proved positive as they survived more than 24 hours after administration of these compounds to the fertilized egg embryo.

In conclusion, the tested compounds, bevacizumab and aflibercept, exhibit no detrimental impact on healthy cells and do not induce vascular irritation. This suggests the safety of their ophthalmic application. Notably, although the analysis of cytotoxic and irritant effects of bevacizumab and aflibercept was conducted in vitro, it's worth highlighting that their impact was not tested in ovo at the CAM level, making this aspect an original contribution of our study.

Shifting to the in vitro experimental study concerning the antitumor effect of a natural antiangiogenic agent, the mistletoe hydroalcoholic extract, as an alternative or complementary therapeutic option in cases of resistance development to conventional antitumor therapy, the study encompassed the following objectives: isolation, characterization, capacity testing, and elucidation of the antitumor mechanism of the phytoagent derived from mistletoe.

Analyzing the hydroalcoholic extract from mistletoe through the LC-MS method unveiled an array of polyphenolic compounds, chiefly epicatechin and kaempferol. These compounds are recognized for their anti-proliferative and anti-inflammatory properties [16,17].

The assessment of the hydroalcoholic mistletoe extract's cytotoxic activity encompassed the utilization of two skin cancer cell lines: A431, representing cutaneous epidermoid carcinoma with squamous cells, and B164A5, a murine melanoma tumor cell line. As a comparative control, a healthy human keratinocyte cell line was employed.

To study the mechanism by which the phytoextract exerts its antitumor influence, a staining technique was utilized to accentuate the impact exerted by VAex on the nucleus's structure. This methodology illuminated the nature of cell death induced by the extract. All three cell lines were exposed to mistletoe extract at five concentrations of VAex (50, 100, 250, 500, and 1000 µg/mL) and monitored for viability over a 24-hour period.

The results of the present study point to the potent cytotoxic effect of VAex on both pigmented (B164A5) and non-pigmented (A431) cells. Remarkably, the squamous cell carcinoma line exhibited the most pronounced response, with VAex triggering a dose-dependent reduction in cell viability while also instigating alterations in nuclear shape and structure, indicative of an effect akin to apoptosis.

The findings demonstrated that, at low concentrations (< 500 µg/mL), mistletoe extract exerts a selective cytotoxic influence, leaving healthy cells unaffected. In the case of tumor cells, mistletoe extracts displayed a concentration-dependent cytotoxic effect. This led to morphological and structural changes within the nucleus, resembling an apoptotic process. Particularly, the non-pigmented cells (A431) showed the most significant impact, implying a protective role of melanin against the cytotoxic effect of the polyphenols present in the mistletoe hydroalcoholic extract.

In conclusion, the polyphenolic components, particularly epicatechin and kaempferol, demonstrated significant dose-dependent antitumor activity, with the most robust effect observed on squamous cell carcinoma cells. This antiproliferative action exhibited selectivity for tumor cells, with non-pigmented cells being more affected, likely due to the absence of melanin's protective influence.

The present study has made noteworthy contributions by shedding light on the nuances of the actions of the primary antiVEGF agents, bevacizumab and aflibercept, both in clinical contexts for treating proliferative eye diseases and in vitro and in ovo experiments where antiproliferative capabilities and therapeutic safety were assessed in healthy tissues. The outcomes obtained possess direct applicability in optimizing personalized antiVEGF therapy via the intravitreal administration of antiVEGF agents. Additionally, a phytotherapeutic agent from mistletoe was extracted, characterized, and tested, offering an alternative or complementary therapeutic avenue for cases involving resistance to conventional antitumor treatment.

Achieving these objectives necessitated a collaborative and transdisciplinary effort involving the resources and expertise of the Ophthalmology Disciplines at UMF Victor Babeș in Timișoara and UMF Carol Davila in Bucharest, as well as the Biochemistry Discipline at UMF Victor Babeș in Timișoara, the Discipline of Toxicology and Medicine Industry at the Faculty of Pharmacy of UMF Victor Babeș in Timișoara, and the Research Center for Pharmaco-toxicological Evaluation at the Faculty of Pharmacy of UMF Victor Babeș in Timișoara. Lastly, the Doctoral School of Medicine-Pharmacy at UMF Victor Babeș in Timișoara provided invaluable support and guidance throughout this interdisciplinary research effort.

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