International Journal of Veterinary and Animal Research 8(3): 64-71, 2025

RESEARCH ARTICLE

IJVAR

International Journal of Veterinary and Animal Research

Journal homepage: https://ijvar.org/index.php/ijvar

Cytotoxicity Profile and Autophagic Activity of Ranibizumab and Aflibercept on Healthy Human Retina Pigment Epithelium Cells: An In Vitro Experimental Study

Eren Ekici[1✉], Ender Yarsan[2], Begüm Yurdakök-Dikmen[2], Büşra Görgün[3], Leyla Didem Kozaci[4], Sinem Pehlivan[5], Recep Uyar [2], Sedat Sevin[2],

[1]*T.R. (Republic of Turkey) Ministry of Health, Ankara Etlik City Hospital, Department of Ophthalmology, Ankara, Türkiye*
[2]*Ankara Faculty of Veterinary Medicine, University, Department of Pharmacology and Toxicology, Ankara, Turkiye*
[3]*Ankara Yildirim Beyazit University, Institute of Health Sciences, Department of Translational Medicine, Ankara, Turkiye*
[4]*Ankara Yildirim Beyazit University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkiye*
[5]*Ankara Medipol University, School of medicine, Department of Pharmacology, Ankara, Turkiye*

ARTICLE INFO

Received: 30/04/2025
Accepted: 21/11/2025

DOI: 10.71336/ijvar.684

✉**Corresponding Author:** opdrerenekici@gmail.com

Keywords

In vitro
Autophagy
Cytotoxicity

Cite this article as: *Ekici E., Yarsan E., Yurdakök-Dikmen B., Görgün B., Kozaci L.D., Pehlivan S., Uyar R., Sevin S. 2025. Cytotoxicity Profile and Autophagic Activity of Ranibizumab and Aflibercept on Healthy Human Retina Pigment Epithelium Cells: An In Vitro Experimental Study. International Journal of Veterinary and Animal Research, 8(2): 64-71. DOI: 110.71336/ijvar.684.*

ABSTRACT

Vascular endothelial growth factor (VEGF) plays a central role in retinal neovascular diseases, and anti-VEGF agents such as ranibizumab and aflibercept are widely used to control these conditions. This study aimed to compare the cytotoxic and autophagic effects of ranibizumab and aflibercept on human retinal pigment epithelium (ARPE-19) cells in vitro. ARPE-19 cells were treated with incremental doses of each drug (1.25× to 40× the clinical concentration). Cell viability was assessed using the MTT assay, and mRNA expressions of autophagy-related genes Beclin1 and ATG4 were quantified by qRT-PCR. Ranibizumab significantly reduced cell viability at 20× and 40× doses compared with the control, whereas aflibercept did not show notable cytotoxicity even at the highest tested concentrations. Both drugs influenced autophagy pathways in different ways: ranibizumab caused less inhibition of Beclin1 expression than aflibercept ($p < 0.05$), while both agents induced a non-significant increase in ATG4 expression. These results suggest that aflibercept may have a lower cytotoxic potential at supratherapeutic concentrations and that both drugs can modulate autophagic activity in retinal pigment epithelium cells. Understanding these cellular effects may help optimize the safety and long-term application of anti-VEGF therapy in retinal diseases.

INTRODUCTION

Ocular neovascularization represents a pathological process characterized by the abnormal proliferation of new blood vessels originating from pre-existing vasculature, typically as a compensatory response to ischemia or hypoxia. Retinal ischemia triggers the activation of hypoxia-inducible factor 1-alpha (HIF-1α), which promotes the expression of vascular endothelial growth factor VEGF-A, major regulator of angiogenesis and vascular permeability. The imbalance between pro-angiogenic and anti-angiogenic signaling disrupts the retinal pigment epithelium and the blood-retinal barrier, resulting in vascular leakage, fibrovascular proliferation, and vision loss in conditions such as diabetic retinopathy, retinal vein occlusion, and neovascular age-related macular degeneration (Campochiaro, 2015). Isolation of vascular endothelial growth factor (VEGF) and increased knowledge about its bioactivities have allowed better understanding of ocular neovascularization pathophysiology. In addition to regulating vascular

permeability and promoting the growth of vascular endothelial cells, in vitro studies have also shown that VEGF stimulates the expression of anti-apoptotic proteins Bcl-2 and A1 in these cells (Ferrara et al., 2003).

The advent of anti-VEGF agents in neovascular eye diseases has been a revolutionary development. It has become an inevitable treatment component of multiple ocular pathologies with VEGF-induced pathological vascular growth as diabetic retinopathy, retinal vein occlusion, iris neovascularization, retinopathy of prematurity, neovascular age-related macular degeneration and corneal neovascularization (Adamis and Shima, 2005).

Among the Food and Drug Administration approved anti-VEGF agents, Ranibizumab (Lucentis®, Novartis Pharma AG, Basel, Switzerland) and Aflibercept (Eylea®, Bayer Pharma AG, Berlin, Germany) are the most commonly used ones in ophthalmic clinical practice. Ranibizumab, a recombinant humanized monoclonal antibody, neutralizes all forms of VEGF-A; while Aflibercept, a recombinant fusion protein, neutralizes all forms of VEGF-A as well as VEGF-B and Placental Growth Factor. The differences in structural features and receptor inhibition may provide distinct efficacy and safety profiles in retinal cells (Gillies et al., 2019). Furthermore, recent studies have concluded that autophagy is strongly associated with VEGF and neovascularization formation. This information has led to the emergence of autophagy as a new therapeutic target (Ye et al., 2016; Miaomiao et al., 2016; Du et al., 2017).

In this *in vitro* experimental study, we evaluated the safety profiles of Ranibizumab and Aflibercept in human retina pigment epithelium cell line (RPE) (ARPE-19) at increasing concentrations by employing 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay. Additionally, we investigated the relationship between these anti-VEGF agents and the autophagy-related proteins (Beclin1 and ATG4) at sublethal concentrations.

MATERIALS AND METHODS

Cell Culture

ARPE-19 cell line, was purchased from American Type Cell Culture (ARPE-19, [ATCC], catalog No. CRL-2302, Manassas, Virginia, USA). The manufacturer's instructions were followed during the culturing processes (Dunn et al., 1996). ARPE-19 cells used in the experiments were from the 3rd, 4th, and 5th passages. ARPE-19 cells were grown in T25 flasks using 10% Fetal Bovine Serum (FBS, Biochrom GmbH, Germany); 1% penicillin/streptomycin; and a 1:1 mixture (vol/vol) of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (Biochrom GmbH, Germany) at 37°C, 5%CO2, and 95% relative humidity. The cells were subcultured to the confluency of 70-80%. Cell suspensions (6×103 cells/ml) were seeded onto 96-well tissue culture plates. ARPE-19 cells were incubated in 96-well plates for 48 hours prior to the start of the experiment to ensure optimal cell adherence and growth.

Anti-VEGF Treatments: The commercially available off-the-shelf preparations Ranibizumab (0.5 mg/0.05 ml) and Aflibercept (2 mg/0.05 ml) were employed. The clinically accepted intravitreal doses were therefore defined as 0.5 mg/0.05 mL (equivalent to 125 µg/mL) for Ranibizumab and 2 mg/0.05 mL (equivalent to 500 µg/mL) for Aflibercept, as reported by Malik et al. (2014). ARPE-19 cells were exposed to anti-VEGF agents at concentrations of 1.25, 2.5×, 5×, 10×, 20×, and 40× clinically accepted doses for 24 hours. High-dose exposure (up to 40× the clinical concentration) was applied to simulate potential cumulative or localized effects that may occur following repeated intravitreal injections or reduced vitreous clearance. Such high-concentration in vitro models are used to evaluate the cellular tolerance and safety margins of pharmacological agents beyond therapeutic levels. The clinical concentration was estimated by presuming that the quantity of each anti-VEGF agent used clinically in intravitreal injections was uniformly distributed throughout 4 mL of human vitreous volume (Ranibizumab at a concentration of 125 µg/ml and Aflibercept at a concentration of 500 µg/ml, adjusted for potential dilution by the vitreous humor at the epiretinal site) (Malik et al., 2014). The test concentrations for Ranibizumab (clinical dose: 125 µg/ml) were: 1.25× = 156.25 µg/ml, 2.5× = 312.5 µg/ml, 5× = 625 µg/ml, 10× = 1250 µg/ml, 20× = 2500 µg/ml, and 40× = 5000 µg/ml. For Aflibercept (clinical dose: 500 µg/ml), the test concentrations were: 1.25× = 625 µg/ml, 2.5× = 1250 µg/ml, 5× = 2500 µg/ml, 10× = 5000 µg/ml, 20× = 10000 µg/ml, and 40× = 20000 µg/ml. The IC_{50} values for Ranibizumab and Aflibercept were estimated from dose–response curves generated using GraphPad Prism. To evaluate sublethal cellular responses such as autophagy, half of the IC_{50} concentration ($IC_{50}/2$) was selected, as this dose allows assessment of cellular stress without inducing extensive cytotoxicity. In this study, "$IC_{50}/2$" refers to half of the calculated IC_{50} concentration ("half IC_{50}"), corresponding approximately to a dose that maintains around 70–80% cell viability. This concentration was used to assess sublethal stress and autophagic responses without causing excessive cell death. The control group consisted of two subgroups: A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Cell Viability Assay

The metabolic activity along with the viability of ARPE-19 cells was assessed with MTT assay. This colorimetric assay quantifies the cytotoxicity by measuring absorbance using a multi-well spectrophotometer following a series of reduction reactions. Cell viability (%) was calculated relative to the positive control group (Maximal Viability), and lower absorbance values indicated reduced metabolic activity and higher cytotoxicity. The experiments were conducted in triplicates. Cells incubated for 24 hours at 37°C and 5-6.5% CO2 with different doses of anti-VEGF agents were added 15 µl of the MTT reagent for each well. After 4 hours of incubation of microplates, the Solubilization solution was added. The plate was allowed to stand overnight in the incubator. Finally, the absorbance of the solution with the formazan product was measured. Cytotoxicity was evaluated based on the ratio of dead cells to positive control.

RNA Extraction, Amplification of cDNA, and Quantitative Real-Time PCR (qRT-PCR) Analysis

The ribonucleic acid (RNA) was extracted from ARPE-19 cells using Hybrid-R (GeneAll, Portugal) RNA extraction kit according to the manufacturer's protocol. NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) was used for total RNA

measurements. The concentration and purity of nucleic acid samples were measured using the NanoDrop ND-1000, a fast and convenient device. The absorbance ratio at 260 nm to 280 nm indicates sample purity, with a high ratio indicating a pure nucleic acid sample and a low ratio indicating the presence of contaminants such as proteins. We included samples with an absorbance 260/280 ratio between 2.0 and 2.3 in the study to ensure the purity of the nucleic acid samples. This range is generally considered to indicate high-quality nucleic acid samples that are suitable for downstream applications. If a sample did not meet these parameters, we repeated RNA extraction using spare samples to ensure accuracy and reliability.

Total RNA was used to analyze Beclin1 and ATG4 mRNA expression by real-time PCR (qPCR). From the isolated RNA samples, cDNA was obtained with the NG dART RT kit (Cat No: E0801-02; EURx). Reagents were added and cDNA was synthesized for each sample. Reactions were performed in a PCR device (Applied Biosystems) at 50°C for 40 minutes and 85°C for 5 minutes. BrightGreen 2X qPCR MasterMix-No Dye kit (Cat No: MasterMix-S; Applied Biological Materials Inc. ABM) was used for real-time PCR (qPCR) analysis according to the manufacturer's instructions. Reactions with reagents were performed using a real-time PCR analyzer (Rotor-Gene Q, Qiagen, Germany) at 95°C for 10 minutes (1 cycle), 95°C for 15 seconds, and 60°C for 60 seconds (40 cycles). Each sample was run in duplicate 2 times and mean values were recorded. The expression of target genes according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated using the 2-ΔΔCT method.

Statistical Analysis

SPSS (version 28.0, Statistical Package for Social Sciences Inc., Chicago, IL, USA) and GraphPad Prism V.7.0 version (GraphPad Software, San Diego, California, USA) were used for the statistical analysis of the data. Each experiment was performed in triplicate. The Mann-Whitney U tests were used to compare the control group with the two anti-VEGF agent groups. In-group comparisons, including the clinical and higher concentrations of the anti-VEGF agents, were also performed using Mann-Whitney U tests. The one-way analysis of variance (ANOVA) was used to determine whether there was a difference between more than two independent groups in qPCR results. The level of significance was set at 0.05. Data were reported as the mean ± standard deviation.

RESULTS

Effects of Ranibizumab and Aflibercept on Cytotoxicity Assay

Based on MTT absorbance values, Ranibizumab and Aflibercept had no significant cytotoxicity on the ARPE cells at the doses applied in this study (Figure 1) ($p>0.05$). These findings indicate that both anti-VEGF agents are generally well tolerated by RPE cells at or below clinical dose ranges, suggesting minimal acute toxicity under short-term exposure conditions.

IC50 values of active pharmaceutical preparations of Ranibizumab and Aflibercept used in our study were much higher than the concentrations used in clinical practice, therefore estimated IC50 values obtained from the graph of dose dilutions calculated with the GraphPad Prism program were utilized in our study. Since the concentrations were within the dilution range used for cytotoxicity analysis, doses corresponding to approximately IC50/2 values were used in the calculations made according to in question IC50 values. The dose corresponding to IC50/2 for Ranibizumab was 428.12 µg/ml, while 1260.88 µg/ml for Aflibercept. Karagöz et al., after examining nanoparticle-loaded anti-VEGF and performing an MTT cytotoxicity test, stated that the existing literature contained no toxicity study reporting an IC50 value. (Karagoz et al., 2021).

Ranibizumab demonstrated a statistically significant decrease in cell viability compared to the positive control group at 20× and 40× clinical doses. Whereas no statistically significant difference was found among positive control and Aflibercept-treated groups in cell viability at 1.25, 2.5×, 5×,10×, 20×, and 40× clinical doses after 24 hours of exposure. There was no statistically significant difference in cytotoxicity when Ranibizumab and Aflibercept were compared separately for each dose range. This suggests that Ranibizumab exerts mild cytotoxic effects only at supratherapeutic levels, while Aflibercept maintains cellular viability even at higher concentrations, consistent with a more favorable safety profile.

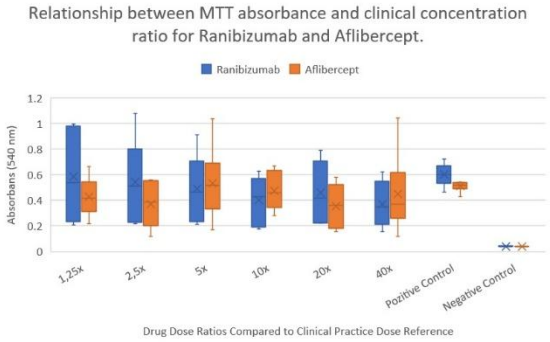

Figure 1. Concentration-dependent change in ARPE-19 cell viability after Ranibizumab and Aflibercept treatments using MTT assay. Three independent experiments were performed in triplicate and all data are expressed as means±SD. The control group consisted of two subgroups: A positive control group was considered 100% viable, and no drug was administered. A negative control group was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Effects of Ranibizumab and Aflibercept on Beclin1 and ATG4 Expressions

Statistical analysis of Beclin1 mRNA expression levels in ARPE-19 cells showed a significant difference between the control and Aflibercept groups (*$P\leq0.05$). Compared to the control group, the Ranibizumab-treated group showed an 8% decrease in Beclin1 mRNA expression levels, while the Aflibercept-treated group showed a 54% decrease. These data imply that Aflibercept may more strongly suppress Beclin1-related autophagic activity compared with Ranibizumab, indicating distinct effects on autophagy regulation. Statistical analysis of ATG4 mRNA expression levels showed no significant difference among the groups ($P\geq0.05$). However, both the Ranibizumab-treated group (95%) and the Aflibercept-treated group (81%) had higher levels of ATG4 mRNA expression compared to the control group. This elevation, although not statistically significant, may represent a compensatory cellular response to anti-VEGF exposure, reflecting mild activation of autophagic pathways.

DISCUSSION AND CONCLUSION

Assessing cell viability is crucial for understanding the safety profile of Ranibizumab and Aflibercept, especially at higher concentrations that may induce cytotoxic effects not observed at clinical doses. This study extends previous research by evaluating concentrations up to 40× clinical doses and exploring autophagic pathways through key markers like Beclin1 and ATG4, providing new insights into the impact of these agents on both cell viability and autophagy, which has been minimally addressed in the literature.

In this *in vitro* study, the effects of commercially available anti-VEGF agents on human retina pigment epithelium cells in different concentrations were compared.

Spitzer et al. reported that Ranibizumab did not have any cytotoxicity of at clinical doses on ARPE-19 cell line, parallel to our results (Spitzer et al., 2007). Malik et al. investigated the safety profiles of anti-VEGFs including Ranibizumab and Aflibercept on the ARPE-19 cell line. They exposed the cells to anti-VEGF agents for 24 hours at 0.5×, 1×, 2×, and 10× clinical concentrations. While they found Ranibizumab to be safe on cell viability in all concentrations, they observed decreased cell viability in the Aflibercept group at 10× concentration. They monitored no statistically significant differences in cell numbers at 1/2×, 1×, and 2× therapeutical doses of Ranibizumab and Aflibercept, compared to the untreated cells (Malik et al., 2014). However, there was a dissimilarity in results at 10× doses which may be due to the difference in the cell viability assay method. While an automated trypan blue dye exclusion assay was used in their study, an MTT assay was employed in ours. Ammar et al. investigated the effects of Aflibercept on various ocular cells including ARPE-19. For comparison, they used Ranibizumab as an active control. Their MTT assay revealed that Aflibercept had no in vitro detrimental effect on ARPE-19 cell viability even at up to twice of the recommended dosage (Ammar et al., 2013). Schnichels et al. applied the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) – 2 - (4 - sulfophenyl) - 2H - tetrazolium] assay (a 'one step' MTT assay) and suggested that cell viability of ARPE-19 cells treated with varying concentrations of Ranibizumab and Aflibercept did not show any relevant decrease at different time intervals (1st, 24th, 48th, and 72nd hours) (Schnichels et al., 2013). Puddu et al. have achieved comparable results after the MTS assay that Ranibizumab and Aflibercept displayed no significant difference in viability (Puddu et al., 2016). Saenz-de-Viteri et al. brought a different approach to the in vitro ARPE-19 studies and examined the cumulative effects of anti-VEGF agents including Ranibizumab and Aflibercept. They applied repeated clinical doses of anti-VEGFs. After grouping the cell lines, the medication was performed every other day (days of 0, 2, and 4 days) in Group 1 while daily (days of 0, 1, 2, 3, and 4 days) in Group 2. Both groups tested with MTT assay on the 5th day. There were no statistically significant measurable cytotoxic effects after single or repeated doses of anti-VEGFs under both normal and oxidative stress conditions (Saenz-de-Viteri et al., 2016). Recently, in contrast to previous studies, Emir et al. concluded that even the clinical doses of Ranibizumab and Aflibercept caused reduced cell viability after 24 hours in the MTT analysis of ARPE-19 cells (Emir et al., 2022).

Differences or similarities between our results and previous studies may be attributed to several experimental and biological factors. First, variations in the choice of assay (e.g., MTT, MTS, or trypan blue exclusion) and exposure duration can significantly influence cytotoxicity outcomes, as metabolic assays tend to be more sensitive to early mitochondrial dysfunction. Second, intrinsic physicochemical differences between Ranibizumab and Aflibercept—including molecular weight, VEGF-binding domains, and receptor affinity—may result in distinct cellular responses and autophagic modulation. Third, variations in culture conditions such as cell seeding density, serum percentage, passage number, and medium composition can affect cellular metabolism and VEGF receptor expression, thereby altering the observed responses to anti-VEGF exposure. In addition, potential assay interference caused by protein-rich media or differences in incubation time may contribute to discrepancies in measured viability between studies such as Malik et al. (2014), Schnichels et al. (2013), and Emir et al. (2022). Finally, differences in statistical power and dose scaling beyond clinical relevance can further explain why some studies report minimal toxicity, whereas others find decreased viability or altered autophagy profiles following anti-VEGF exposure. Collectively, these factors provide plausible reasons for the discrepancies observed among reports evaluating anti-VEGF cytotoxicity on retinal pigment epithelial cells

In the present study, no statistically significant difference in cell viability between Ranibizumab and Aflibercept was present, compared to the positive control group at 1.25×, 2.5×, 5× and 10× clinical doses after 24 hours of exposure (Table 1).

On the other hand, Ranibizumab demonstrated decreased cell viability compared to the positive control group at 20× and 40× clinical doses, while Aflibercept did not (Table 2).

In addition, when Ranibizumab was compared to Aflibercept in terms of cell viability for each incremental dose range, no statistically significant difference was found between them (Table 3).

Although several *in vivo* and *in vitro* studies in various dose ranges have been carried out on safety profiles of anti-VEGFs, the present study is the first work that assesses the Ranibizumab and Aflibercept at rates up to 40 × of the clinical doses. The estimated IC50/2 for Ranibizumab in our study was calculated as 428.12 µg/mL, indicating the concentration needed to reduce cell viability by 50% under our experimental conditions. However, significant reductions in cell viability were observed at 20× and 40× clinical doses of Ranibizumab (2500 µg/mL and 5000 µg/mL, respectively), which are much higher than the IC50. This can be explained by the fact that IC50 represents a midpoint in the dose-response curve, and at concentrations far exceeding this threshold, the cytotoxic effects of Ranibizumab accumulate, resulting in more substantial reductions in cell viability. Thus, the cytotoxicity observed at 20× and 40× doses align with the predicted dose-response relationship beyond the IC50 point. Additionally, the IC50 was determined under specific experimental conditions (such as controlled pH, temperature, and assay settings), and it's important to recognize that IC50 values can shift depending on the biological system and environmental factors.

Another aim of the current study was to reveal whether a relationship exists between anti-VEGF agents and autophagy pathways in the ARPE-19 cell line.

Table 1. Statistical comparison of Aflibercept and positive control for each concentration.

	Group	n	Mean Rank	Total Rank	U	p
40× (20000 µg/ml)	Aflibercept	6	4.67	28.00	7.000	0.078
	Positive Control	6	8.17	50.00		
20× (10000 µg/ml)	Aflibercept	6	4.83	29.00	8.000	0.109
	Positive Control	6	8.17	49.00		
10× (5000 µg/ml)	Aflibercept	6	4.83	32.00	8.000	0.179
	Positive Control	6	8.71	49.00		
5× (2500 µg/ml)	Aflibercept	6	6.50	39.00	18.000	1.000
	Positive Control	6	6.50	39.00		
2.5× (1250 µg/ml)	Aflibercept	6	6.67	40.00	17.000	0.873
	Positive Control	6	6.33	38.00		
	Aflibercept	6	5.67	34.00	13.000	0.423
	Positive Control	6	7.33	44.00		

*p<.05

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration, µg/ml: micrograms per milliliter.

A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Table 2. Statistical comparison of Ranibizumab and positive control for each concentration.

	Group	n	Mean Rank	Total Rank	U	p
40× (5000 µg/ml)	Ranibizumab	6	4.17	25.00	4.000	0.025*
	Positive Control	6	8.83	53.00		
20× (2500 µg/ml)	Ranibizumab	6	4.33	26.00	5.000	0.037*
	Positive Control	6	8.67	52.00		
10× (1250 µg/ml)	Ranibizumab	6	5.83	35.00	14.000	0.522
	Positive Control	6	7.17	43.00		
5× (625 µg/ml)	Ranibizumab	6	5.34	32.00	13.000	0.237
	Positive Control	6	8.67	52.00		
2.5× (312.5 µg/ml)	Ranibizumab	6	5.50	33.00	12.000	0.337
	Positive Control	6	7.50	45.00		
1.25× (156.25 µg/ml)	Ranibizumab	6	6.17	37.00	16.000	0.749
	Positive Control	6	6.83	41.00		

*p<.05

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration, µg/ml: micrograms per milliliter.

A positive control group (Maximal Viability, Max V) was considered 100% viable, and no drug was administered. A negative control group (Minimal Viability, Min V) was treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) Triton-X and considered 0% viable.

Table 3. Statistical comparison of Aflibercept and Ranibizumab for each concentration.

	Active Substance	n	Mean Rank	Total Rank	U	p
40×	Aflibercept	6	6.67	40.00	17.000	0.873
	Ranibizumab	6	6.33	38.00		
20×	Aflibercept	6	5.50	33.00	12.000	0.337
	Ranibizumab	6	7.50	45.00		
10×	Aflibercept	6	5.83	35.00	14.000	0.552
	Ranibizumab	6	7.17	43.00		
5×	Aflibercept	6	6.50	39.00	18.000	1.000
	Ranibizumab	6	6.50	39.00		
2.5×	Aflibercept	6	7.00	42.00	15.000	0.631
	Ranibizumab	6	6.00	36.00		
1.25×	Aflibercept	6	6.17	37.00	16.000	0.749
	Ranibizumab	6	6.83	41.00		

n: Sample size.; u: The difference between the two rank totals.; p: Probability value.; ×: multiples of clinical concentration.

Angiogenesis is a complex process that involves stages such as endothelial cell proliferation, matrix degradation, migration, tube formation, and vessel maturation. Pathological angiogenesis is linked to several diseases, including cancer, macular degeneration, and diabetic retinopathy. In each of these conditions, inhibition of angiogenesis has been shown to inhibit disease progression (Folkman, 2006; Reddy et al., 2003). VEGF is a transcriptionally regulated survival factor for endothelial cells. Blocking VEGF receptor-mediated signaling via the phosphatidylinositol 3-kinase (PI3K) / protein kinase B (Akt) pathway leads to cell cycle arrest and programmed cell death (Folkman, 2007). Autophagy and apoptosis usually occur in the same cell, often in a sequence where autophagy precedes apoptosis. This is because stress often stimulates an autophagic response, especially when the stress level is not fatal. In most cases, autophagy constitutes a strategy for adapting and coping with stress (Kroemer et al., 2010). However, if the cell initiates apoptosis, autophagy can be inactivated partially due to the caspase-mediated cleavage of essential autophagy proteins. Beyond this general scenario, essential proteins involved in autophagic processes may promote cellular death either by catabolizing vital cell components or facilitating the activation of apoptotic or necrotic programs, respectively (Galluzzi et al., 2009).

In the present study, the decrease in Beclin-1 gene expression levels after anti-VEGF exposure is consistent with this basic information in the literature. There is still no consensus in the literature (whether induction or inhibition) on the relationship between anti-VEGF and autophagy. Lytvynchuk et al. showed in a mouse fibroblast cell line that anti-VEGFs dose-dependently inhibit survival, mitotic activity, and proliferation while increasing cellular heterogeneity, apoptosis, and autophagy. Authors reported that Ranibizumab showed lower antiproliferative and apoptotic activity than Aflibercept and other anti-VEGFs. This result for Ranibizumab, not having a significant effect on proliferation and apoptotic activity despite increasing doses, was explained by the different mechanisms of action (more induction) of Ranibizumab on autophagy (Lytvynchuk et al., 2015). In our results, the effect of Ranibizumab on the autophagy gene Beclin1 (lower inhibitory effect) was significantly dissimilar to Aflibercept (Figure 2).

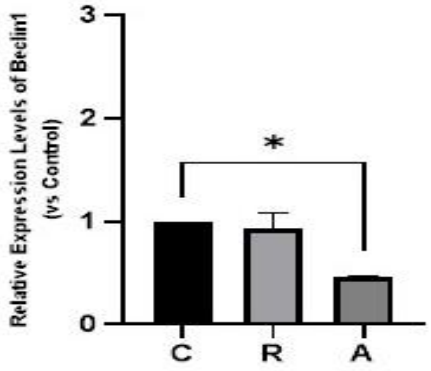

Figure 2. Beclin1 mRNA expression in ARPE-19 cells (n=3). Control (C), Ranibizumab at 50% viability inhibition concentration half (IC50/2) (R), and Aflibercept at 50% viability inhibition concentration half (IC50/2) (A). *P≤0.05(n=3).

Liang et al. investigated the relationship between the anti-VEGF agent Bevacizumab and autophagy in a glioma tumor cell line, concluding that the agent induced autophagy. They attributed this to the fact that Bevacizumab (and other antiangiogenic agents) may cause nutrient deprivation and oxygen stress in the tumor microenvironment by inhibiting the tumor vasculature and induction of the autophagy process (Liang et al., 2015). Liu et al. claimed that autophagy kills tumor cells during tumorigenesis and inhibits tumor growth, but once tumor formation occurs, autophagy protects the survival of tumor cells against various environmental conditions. It has even been hypothesized that autophagy plays a crucial role in suppressing tumorigenesis that as many human cancers, such as breast and prostate cancers, often involve the loss of two core autophagy genes, Beclin1 and/or ATG4. The hypoxia induced autophagy, caused by anti-angiogenesis therapies, is now recognized as an essential contributor to resistance to the drugs (Liu et al., 2016). While autophagy is well-studied in cancer cells, it is less understood in retinal cells. However, insights from tumor biology help us see how VEGF inhibition might influence similar autophagic processes in the retina, especially in diseases with neovascularization. Research on tumor cells shows that blocking VEGF can trigger autophagy, offering clues about how these pathways might work in RPE cells. Indeed, Wang et al.'s study on rhesus monkey choroid/retinal endothelial cell line showed that Ranibizumab and Conbercept triggered autophagy in cells under hypoxia conditions, but Aflibercept has a different effect on autophagy and inhibits the expression of Beclin1. Wang et al. proposed that this autophagy activation could, in turn, promote angiogenesis and thereby weaken the role of anti-angiogenic drugs, so induced autophagy may be the reason for the poor therapeutic effect of anti-angiogenic drugs. They suggested this induced autophagy may be a primary reason for the observed poor therapeutic outcomes associated with certain anti-VEGF treatments. Ultimately, their findings support the conclusions of various clinical trials indicating the superiority of Aflibercept over other currently available anti-VEGF agents. (Wang et al., 2021). Although our study utilized a different cell line than that investigated by Wang et al., we similarly observed Aflibercept's distinct effect, noting its inhibition of Beclin1 expression.

In this study, an increase (statistically not significant) in ATG4 expression was determined in ARPE-19 cell line treated with Ranibizumab and Aflibercept compared to the control group (Figure 3).

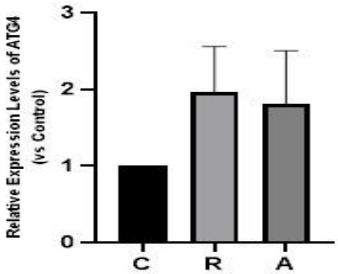

Figure 3. ATG4 mRNA expression in ARPE-19 cells. Control (C), Ranibizumab at 50% viability inhibition concentration half (IC50/2) (R), and Aflibercept at 50% viability inhibition concentration half (IC50/2) (A). *P≤0.05(n=3).

It has been previously reported that anti-VEGF agents induce autophagy (Liang et al., 2015; Liu et al., 2016; Lytvynchuk et al., 2015). In more recent studies, the relationship between anti-VEGF therapies and autophagy has remained the center of attention of researchers. For example, Segatto et al. applied Ranibizumab and Aflibercept to New Zealand white rabbit eyes and evaluated their retinas *in vitro* after enucleation. They found that VEGF inhibition led to changes in neurotrophin signaling and strongly stimulated apoptosis and autophagy. They also noted that the effects caused by Aflibercept at earlier time-points were more pronounced compared to Ranibizumab (Segatto et al., 2019). Schottler et al. found that the autophagosome area increased more using Ranibizumab compared to Aflibercept in a study which they applied Ranibizumab and Aflibercept long-term (12 weeks), and repeated doses to porcine RPE cell line (Schottler et al., 2018). In our study, the increase caused by Ranibizumab in ATG4 gene expression was higher than Aflibercept and in parallel with this study.

Although ATG4, similar to Beclin1, is an essential autophagy protein, its mechanism of action is quite different. Beclin1 initiates the formation of an isolation membrane (also called phagophore) in the first stage of autophagy, the nuclear autophagy pathway (nuclear complex formation), mainly at the contact sites between mitochondria and endoplasmic reticulum (Marino et al., 2014). Unlike other known BH3-only proteins, Beclin1 does not function as a proapoptotic molecule (He and Levine, 2010). Besides this exception, it is noteworthy that most Beclin1-interacting proteins modulate autophagy and apoptosis in the same direction; that is to say, they inhibit or promote both processes (Marino et al., 2014). Unlike Beclin1, ATG4 has a crucial role in autophagosome formation, a more advanced stage of autophagy. Since both the processing and delipidation reactions of ATG8 by ATG4 are essential for autophagosome formation, inhibition of ATG4 leads to the inhibition of autophagy at the autophagosome formation stage (Maruyama and Noda, 2018). In our study, although both are associated with autophagy; the different directions of progress in Beclin1 and ATG4 compared to the control group as decrease and increase respectively, may be explained by this difference in their mechanisms of functioning. The findings from our study regarding Beclin1 and ATG4 expression in ARPE-19 cell line should be interpreted cautiously. While autophagy was influenced by the treatments, the absence of statistically significant changes for ATG4 and the modest decrease in Beclin1 suggest that these results are preliminary. This highlights the need for more targeted autophagy studies in the future, particularly in primary retinal cells or in vivo models of retinal disease.

The current study has several limitations. First, our primary focus was examining the effects of Ranibizumab and Aflibercept on the ARPE-19 cell line. As a result, the generalizability of the findings to other cell types or patient populations may be limited. Second, while the ARPE-19 cell line is commonly used in *in vitro* studies, it may not fully represent the complexity and heterogeneity of the human retina. Therefore, caution is necessary when extrapolating these findings to the *in vivo* settings. Third, we only focused on the short-term (24-hour) effects of anti-VEGF agents. Therefore, we may speculate about their long-term or potential cumulative effects, which requires further investigation.

In conclusion, our study highlights that Ranibizumab reduced cell viability in ARPE-19 cells only at 20× and 40× clinical doses, whereas no significant cytotoxicity was observed at clinical doses. Aflibercept demonstrated less cytotoxicity, maintaining cell viability even at higher doses. Additionally, distinct autophagic responses were observed, with Ranibizumab inhibiting Beclin1 less than Aflibercept. Both drugs increase ATG4 expression suggesting a potential autophagic response. However, this result is preliminary, and further studies are needed to confirm the role of autophagy in retinal cells, using additional markers and assays.

From a clinical perspective, these findings suggest that both Ranibizumab and Aflibercept are safe at standard intravitreal doses, supporting their continued clinical use. The observed cytotoxicity at supratherapeutic concentrations underscores the importance of strict adherence to recommended dosing and monitoring cumulative exposure in patients receiving long-term anti-VEGF therapy. Moreover, the differential autophagic responses between agents may provide a rationale for individualized treatment selection or potential combination strategies targeting both VEGF and autophagy pathways in refractory retinal diseases.

Acknowledgement

-

Ethical Declaration

This study does not involve human or animal participants. Ethical approval and informed consent were not required for this study.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: E.E., E.Y., Design: E.E., E.Y., L.D.K. Data Collection or Processing: E.E., B.YD., S.S., M.Y., Analysis or Interpretation: B.G., L.D.K., S.P., R.U., M.Y., Literature Search: E.E., L.D.K., B.G., Writing: E.E.

Financial Support

This research received no grant from any funding agency/sector.

REFERENCES

Adamis AP, Shima DT. 2005. The role of vascular endothelial growth factor in ocular health and disease. Retina, 25(2), 111-118.

Ammar DA, Mandava N, Kahook MY. 2013. The effects of aflibercept on the viability and metabolism of ocular cells in vitro. Retina, 33(5), 1056-1061.

Campochiaro PA. Molecular pathogenesis of retinal and choroidal vascular diseases. Progress in retinal and eye research. 2015 Nov 1;49:67-81.

Du JH, Li X, Li R, Cheng BX, Kuerbanjiang M, Ma L. 2017. Role of autophagy in angiogenesis induced by a high-glucose condition in RF/6A cells. Ophthalmologica, 237(2), 85-95.

Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. 1996. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Experimental eye research, 62(2), 155-170.

Emir SB, Ucgun NI, Fikret CZ. 2022. Cytotoxicity of Anti-VEGF Agents: An Experimental Study on ARPE-19 Cell Culture. Archives of Clinical and Biomedical Research, 6: 800-806.

Ferrara N, Gerber HP, LeCouter J. 2003. The biology of VEGF and its receptors. Nature medicine, 9(6), 669-676.

Folkman J. 2006. Angiogenesis. Annu. Rev. Med., 57(1), 1-18.

Folkman J. 2007. Angiogenesis: an organizing principle for drug discovery?. Nature reviews Drug discovery, 6(4), 273-286.

Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Kroemer G. 2009. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death & Differentiation, 16(8), 1093-1107.

Gillies MC, Hunyor AP, Arnold JJ, Guymer RH, Wolf S, Ng P, McAllister IL. 2019. Effect of ranibizumab and aflibercept on best-corrected visual acuity in treat-and-extend for neovascular age-related macular degeneration: a randomized clinical trial. JAMA ophthalmology, 137(4), 372-379.

He C, Levine B. 2010. The beclin 1 interactome. Current opinion in cell biology, 22(2), 140-149.

Karagoz IK, Allahverdiyev A, Demircioglu A, Abamor ES, Dinparvar S, Bagirova M. 2021. Producing Aflibercept Loaded Poly (Lactic Co Glycolic Acid)[Plga] Nanoparticles As A New Ocular Drug Delivery System and Its Challenges. Feb Fresenıus Envıronmental Bulletın, 1481.

Kroemer G, Mariño G, Levine B. 2010. Autophagy and the integrated stress response. Molecular cell, 40(2), 280-293.

Liang J, Piao Y, Henry V, Tiao N, de Groot JF. 2015. Interferon-regulatory factor-1 (IRF1) regulates bevacizumab induced autophagy. Oncotarget, 6(31), 31479.

Liu J, Fan L, Wang H, Sun G. 2016. Autophagy, a double-edged sword in anti-angiogenesis therapy. Medical oncology, 33(1), 10.

Lytvynchuk L, Sergienko A, Lavrenchuk G, Petrovski G. 2015. Antiproliferative, apoptotic, and autophagic activity of ranibizumab, bevacizumab, pegaptanib, and aflibercept on fibroblasts: implication for choroidal neovascularization. Journal of Ophthalmology, 2015(1), 934963.

Malik D, Tarek M, del Carpio JC, Ramirez C, Boyer D, Kenney MC, Kuppermann BD. 2014. Safety profiles of anti-VEGF drugs: bevacizumab, ranibizumab, aflibercept and ziv-aflibercept on human retinal pigment epithelium cells in culture. British Journal of Ophthalmology, 98(Suppl 1), i11-i16.

Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. 2014. Self-consumption: the interplay of autophagy and apoptosis. Nature reviews Molecular cell biology, 15(2), 81-94.

Maruyama T, Noda NN. 2018. Autophagy-regulating protease Atg4: structure, function, regulation and inhibition. The Journal of antibiotics, 71(1), 72-78.

Miaomiao W, Chunhua L, Xiaochen Z, Xiaoniao C, Hongli L, Zhuo Y. 2016. Autophagy is involved in regulating VEGF during high-glucose-induced podocyte injury. Molecular biosystems, 12(7), 2202-2212.

Puddu A, Sanguineti R, Traverso CE, Viviani GL, Nicolò M. 2016. Response to anti-VEGF-A treatment of retinal pigment epithelial cells in vitro. European Journal of Ophthalmology, 26(5), 425-430.

Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. 2003. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. Journal of Biological Chemistry, 278(23), 20915-20924.

Saenz-de-Viteri M, Fernández-Robredo P, Hernández M, Bezunartea J, Reiter N, Recalde S, García-Layana A. 2016. Single-and repeated-dose toxicity study of bevacizumab, ranibizumab, and aflibercept in ARPE-19 cells under normal and oxidative stress conditions. Biochemical pharmacology, 103, 129-139.

Schnichels S, Hagemann U, Januschowski K, Hofmann J, Bartz-Schmidt KU, Szurman P, Aisenbrey S. 2013. Comparative toxicity and proliferation testing of aflibercept, bevacizumab and ranibizumab on different ocular cells. British Journal of Ophthalmology, 97(7), 917-923.

Schottler J, Randoll N, Lucius R, Caliebe A, Roider J, Klettner A. 2018. Long-term treatment with anti-VEGF does not induce cell aging in primary retinal pigment epithelium. Experimental eye research, 171, 1-11.

Segatto M, Fico E, Gharbiya M, Rosso P, Carito V, Tirassa P, Lambiase A. 2019. VEGF inhibition alters neurotrophin signalling pathways and induces caspase-3 activation and autophagy in rabbit retina. Journal of Cellular Physiology, 234(10), 18297-18307.

Spitzer MS, Yoeruek E, Sierra A, Wallenfels-Thilo B, Schraermeyer U, Spitzer B, Szurman P. 2007. Comparative antiproliferative and cytotoxic profile of bevacizumab (Avastin), pegaptanib (Macugen) and ranibizumab (Lucentis) on different ocular cells. Graefe's Archive for Clinical and Experimental Ophthalmology, 245(12), 1837-1842.

Wang Y, Yao Y, Li R, Wu B, Lu H, Cheng J, Du J. 2021. Different effects of anti-VEGF drugs (Ranibizumab, Aflibercept, Conbercept) on autophagy and its effect on neovascularization in RF/6A cells. Microvascular research, 138, 104207.

Ye F, Kaneko H, Hayashi Y, Takayama K, Hwang SJ, Nishizawa Y, Terasaki H. 2016. Malondialdehyde induces autophagy dysfunction and VEGF secretion in the retinal pigment epithelium in age-related macular degeneration. Free Radical Biology and Medicine, 94, 121-134.