Cytotoxicity of Anti-VEGF Agents: An Experimental Study on ARPE-19 Cell Culture
Shergiye Bayramova Emir¹, Nil Irem Ucgun²*, Cenk Zeki Fikret³

Abstract

Purpose: To determine the dose-related cytotoxic and oxidative stress effects of aflibercept, ranibizumab, bevacizumab on human retinal pigment epithelial cell (ARPE-19) culture.

Study Design: Prospective, experimental.

Material and Methods: ARPE-19 cells were inoculated into culture flasks with an appropriate medium mixture. Ranibizumab, Bevacizumab, and Aflibercept were added in clinical doses, twice clinical doses or four times of the clinical doses to the medium. ARPE-19 cell cultures without any anti-VEGF agents were used as the controls. The toxicity was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in ARPE-19 cell cultures. Total antioxidant capacity (TAC) and Malondialdehyde (MDA) levels were measured by spectrophotometric method.

Results: The cell viability was significantly lower than the control group at the clinical doses in Ranibizumab and Aflibercept groups, but not in the Bevacizumab group. Twice- and four times clinical doses resulted in significant cytotoxicity in all three anti-VEGF groups. The TAC levels were similar in all anti-VEGF and control groups. MDA level was significantly higher in all three anti-VEGF groups when compared to the control group.

Conclusions: Aflibercept and ranibizumab, but not Bevacizumab reduced cell viability in ARPE-19 cell cultures at the clinical doses after 24 hours. Higher doses of all three agents caused cytotoxicity. Anti-VEGF agents did not change the TAC levels, however increased the MDA levels, and the cell toxicity after their application may be related to oxidative stress. It is wise to use anti-VEGF agents at minimum doses that provide maximum effectiveness with minimum cytotoxicity.

Keywords: ARPE-19; Aflibercept; Bevacizumab; Ranibizumab; Oxidative Stress

Introduction

Vascular endothelial growth factor (VEGF) is a vasoactive cytokine that controls developmental vasculogenesis and angiogenesis, it also regulates vascular permeability, and promotes cell survival [1,2]. Angiogenesis and increased vascular permeability play important roles in the pathogenesis of intraocular neovascular syndromes including diabetic retinopathy, retinal vascular occlusion and retinopathy of prematurity, and increased VEGF has been shown in the aqueous and vitreous humors in those patients. Age-related macular degeneration (AMD), the leading cause of blindness in the
individuals older than 50 years of age, has also been related to neovascularization and increased vascular permeability [3]. Comprehension of the importance of the VEGF in neovascularization of retina and hence the pathogenesis of retinal vascular disorders popularized use of VEGF inhibitors in treatment of those disorders. Although VEGF’s primary target is vascular endothelial cells, it has been shown to have effects on retinal pigment epithelial cells (RPE) including an autocrine function which enhances cell survival under oxidative stress via the VEGFR2/PI3K/Atk pathway [2]; therefore anti-VEGF therapy may have some possible effects on RPE. However, it is controversial whether administration of anti-VEGF agents results in RPE atrophy or not [4,5]. In addition, it has been claimed that anti-VEGF agents increase reactive oxygen species (ROS) and damage ARPE-19 cells due to oxidative stress [6,7]. In this study, we investigated safety and toxic effects of three anti-VEGF agents, Bevacizumab, Ranibizumab and Aflibercept on ARPE-19 cells in clinical doses and in doses higher than the clinical doses. We employed MTT assay to determine survival of ARPE-19 cells treated with anti-VEGF agents. We also investigated total antioxidant capacity (TAC) and Malondialdehyde (MDA) levels in ARPE-19 cells treated with clinical doses of Bevacizumab, Ranibizumab or Aflibercept to determine whether these agents caused oxidative stress, since oxidative stress may induce/increase the cytotoxic effects of the anti-VEGF agents and/or the abnormalities due to the underlying disorder.

Material and Methods

Cell Culture

Human RPE cell line (ARPE-19, American Type Cell Culture [ATCC], catalog No. CRL-2302, Manassas, Virginia, USA) which stored in a cryogenic tube frozen in liquid nitrogen tank was rapidly dissolved in a 37°C water bath. The cells were planted in T25 flasks using 10% Fetal Bovine Serum (FBS, Biochrom GmbH, Germany), 1% penicillin / streptomycin, Dulbecco’s modified Eagle’s medium (DMEM) / Ham’s F-12 1:1 (Biochrom GmbH, Germany) as the medium. The cells were subcultured with a confluency of 70-80% in trypsin EDTA 0.05% (Ethylene diamine tetra acetic acid-BM-EdB01-01, Turkey) solution. The liquid cell line was placed in the DMEM cell culture medium in accordance with the sterility rules. DMEM cell culture medium was mixed with 10% FBS, 1% glutamine, 1% Penicillin / Streptomycin, and made ready for use. The prepared medium was stored in a refrigerator at +4 °C. ARPE-19 cells have an adherent character. The fact that the adhering cells are 80% coated (confluent) indicates that passage (division) time has come. The cells were subcultured according to the rules.

Exposure of ARPE-19 Cells to Anti-VEGF Agents

The tested anti-VEGF agents were Bevacizumab (100 mg/4 ml; Avastin; Genentech/Roche, San Francisco, California, USA), Ranibizumab (0.23 mg/0.23 ml; Lucentis; Genentech, San Francisco, California, USA), and Aflibercept (4 mg/0.1 ml; Eylea; Bayer/Regeneron, Tarrytown, New York, USA).

ARPE-19 cells were divided into groups in relation with the anti-VEGF agents tested:

1. Control group (ARPE-19 cells and medium)
2. Bevacizumab group (ARPE-19 cells + medium + Bevacizumab)
3. Ranibizumab group (ARPE-19 cells + medium + Ranibizumab)
4. Aflibercept group (ARPE-19 cells + medium + Aflibercept)

The volume of the intraocular vitreous was accepted as 4 ml, and the anti-VEGF agents were applied to the ARPE-19 cell media in the following amounts:

- **Bevacizumab**: Three doses as,
  - Clinical dose, 1.25 mg/0.05 ml,
  - 2 x clinical dose (2.5 mg/0.1 ml),
  - 4 x clinical dose (5 mg/0.2 ml).

- **Ranibizumab**: Three doses as,
  - Clinical dose, 0.5 mg/0.05 ml,
  - 2 x clinical dose (1 mg/0.1 ml),
  - 4 x clinical dose (2 mg/0.2 ml).

- **Aflibercept**: Three doses as,
  - Clinical dose, 2 mg/0.05 ml,
  - 2 x clinical dose (4 mg/0.1 ml),
  - 4 x clinical dose (8 mg/0.2 ml).

Cell Viability Test (MTT Assay)

MTT measures cell metabolic activity and cell viability. It is a sensitive, repeatable, easy-to-apply test, and does not need the use of radioactive isotopes. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. Cells with dead or impaired mitochondria do not stain. In our study, the morphology of the cells was examined with phase contrast microscopy. RPE cells up to passage 2 with characteristic RPE morphologies were used for the experiment. ARPE-19 cells were cultured in the presence of the anti-VEGF agents for 24 h. After 24-hour incubation, 20 µl MTT solution was put into each well and then left in the incubator for 4 hours. After 4 hours, the orange-colored supernatant was gently suctioned without contacting the cells. 100 µl isopropyl
alcohol was added to each well, the plate was wrapped with foil, and incubated at room temperature for 3-4 hours in the dark. The plate was shaken slightly at the end of the process, and read at 570 nm in the ELISA plate reader.

The cell viability rate of anti-VEGF-added ARPE-19 cells was calculated as follows:

\[
\text{(Absorbance of the anti-VEGF-added ARPE-19 cells / absorbance of the control) × 100 = Cell viability percent}
\]

**Measurement of Total Antioxidant Capacity**

Real Assay Diagnostics® test was used to measure TAC.

The study groups were designed as follows:

1. Control group: ARPE-19 cells + culture medium,
2. Bevacizumab group: ARPE-19 cells + 4 mM (1.25 mg) Bevacizumab + culture medium,
3. Ranibizumab group: ARPE-19 cells + 12 mM (0.5 mg) Ranibizumab + culture medium,
4. Aflibercept group: ARPE-19 cells + 5 mM (2 mg) Aflibercept + culture medium.

The standard kit procedures were followed and cell lysates were obtained. In each group, the absorbance was measured at 660 nm to determine the TAC.

**Lipid Peroxidation Analysis**

Malondialdehyde (MDA) (Elabscience Catalog No: E-EL-0060) kit was used. MDA levels were measured by ELISA reader and with spectrophotometric method in the control, Bevacizumab, Ranibizumab and Aflibercept groups.

**Statistical Analysis**

SPSS (Statistical Package for Social Sciences) for Windows v.11.5 software was used for the statistical analysis of the data. The comparisons of control and three anti-VEGF groups were done with One Way Analysis of Variance and Bonferroni test. In-group comparison of clinical and higher doses of the anti-VEGF agents were done with paired T-test. Descriptive statistics were presented as mean ± standard deviation. Statistical significance was set at p <0.05.

**Results**

**Effects of Anti-VEGF Agents on the Viability of ARPE-19 Cells**

The viability results of ARPE-19 cells after 24-h application of anti-VEGF agents at clinical doses (Ranibizumab 0.5 mg/0.05 ml, Aflibercept 2 mg/0.05 ml, and Bevacizumab 1.25 mg/0.05 ml) are shown in Table 1. Paired comparisons showed that the ARPE-19 cell viabilities were significantly lower in Aflibercept and Ranibizumab groups compared to the control group. However, cell viabilities were similar in Bevacizumab and control groups. In addition, the cell viabilities were significantly lower in Ranibizumab and Aflibercept groups compared to the Bevacizumab group. Therefore, at clinical doses, cell viability was higher with Bevacizumab compared to other anti-VEGF agents.

The viability results of ARPE-19 cells after 24-h application of anti-VEGF agents at twice clinical doses (Ranibizumab 1 mg/0.1ml, Aflibercept 4 mg/0.1ml and Bevacizumab 2.5 mg/0.1ml) are shown in Table 2. Two Aflibercept and two Bevacizumab wells were excluded from the study due to insignificant and incompatible results. The cell viabilities were significantly lower in the Aflibercept, Bevacizumab and Ranibizumab groups compared to the control group. The cell viability was significantly worse in the Ranibizumab group compared to Bevacizumab group.

Table 3 shows the viability of ARPE-19 cells after administration of four times of the clinical doses of the anti-VEGF agents (Ranibizumab 2 mg/0.2 ml, Aflibercept 8 mg/0.2 ml, and Bevacizumab 5 mg/0.2 ml). The results of 1 Aflibercept, 1 Bevacizumab and 2 Ranibizumab wells were excluded from the study due to pipetting errors. The cell survival was worse in all three anti-VEGF agent groups.

### Table 1: MTT results of the study groups after administration of clinical doses of anti-VEGF agents.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>p</th>
<th>Binary comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>100.00 ± 0.00</td>
<td>0.001</td>
<td>C-A, C-R, C-B, B-R</td>
</tr>
<tr>
<td>Aflibercept, 2 mg/0.05 ml</td>
<td>8</td>
<td>89.87 ± 6.578</td>
<td>0.002</td>
<td>C-A, C-R, C-B, B-R</td>
</tr>
<tr>
<td>Bevacizumab, 1.25 mg/0.05 ml</td>
<td>8</td>
<td>89.25 ± 11.247</td>
<td>0.001</td>
<td>C-A, C-B, C-R, B-R</td>
</tr>
</tbody>
</table>

### Table 2: MTT results of the study groups after administration of twice the clinical doses of anti-VEGF agents.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>p</th>
<th>Binary comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>58.00 ± 3.16</td>
<td>0.001</td>
<td>C-A, C-B, C-R, B-R</td>
</tr>
<tr>
<td>Aflibercept, 4 mg/0.1 ml</td>
<td>5</td>
<td>62.40 ± 6.35</td>
<td>0.001</td>
<td>C-A, C-B, C-R, B-R</td>
</tr>
<tr>
<td>Bevacizumab, 2.5 mg/0.1 ml</td>
<td>7</td>
<td>54.29 ± 4.50</td>
<td>0.001</td>
<td>C-A, C-B, C-R, B-R</td>
</tr>
</tbody>
</table>

**Citation:** Shergiyev Bayramova Emir, Nil Irem Ucgun, Cenk Zeki Fikret. Cytotoxicity of Anti-VEGF Agents: An Experimental Study on ARPE-19 Cell Culture. Archives of Clinical and Biomedical Research 6 (2022): 800-806.
compared to the control group. Cell survival was better in Ranibizumab group compared to Bevacizumab group, and better in Ranibizumab group compared to Aflibercept group (p=0.001). At this dose, Ranibizumab showed the lowest cytotoxic effect on ARPE-19 cells compared to the other two anti-VEGF agents.

Table 4 shows the cell viability comparisons of anti-VEGF agents when applied at higher-than-clinical doses. It is evident that four times the clinical dose application of Aflibercept and Bevacizumab decreased cell viability more than twice clinical dose application (p = 0.018 and p = 0.012, respectively). However, application of four times the clinical dose of Ranibizumab resulted in a higher cell viability compared to the twice-clinical dose application (p=0.034).

**TAC Levels in the Study Groups**

Administration of anti-VEGF agents at the clinical doses did not result in significantly increased TAC levels when compared to the control group. Binary comparisons of the study groups did not show any significant differences between the groups (Table 5).

### Lipid Peroxidation Analysis Results of the Study Groups

MDA levels were significantly lower in the control group when compared to all three anti-VEGF agents applied in clinical doses (p = 0.001). Binary comparisons of the anti-VEGF groups did not show any significant differences between the groups.

**Discussion**

The MTT analysis results of our study indicated that, except for Bevacizumab applied at the clinical dose, all other doses of Bevacizumab and all tested doses of Aflibercept and Ranibizumab resulted in cytotoxicity in ARPE-19 cells after exposure for 24 hours. The highest cell viability was observed at the clinical dose of Bevacizumab, without any difference between Bevacizumab and control groups for cell survival. Whether inhibition of VEGF isoforms increases apoptotic effect in the ischemic retina and, if so, for how long, is not yet known although a number of studies have investigated the effect of single inhibition or repeated doses on cellular toxicity [8,9]. The results of the animal studies on cytotoxicity of anti-

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**Table 3:** MTT results of the study groups after administration of four times of the clinical doses of anti-VEGF agents.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>p</th>
<th>Binary comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-Control</td>
<td>7</td>
<td>100.00 ± 0.00</td>
<td>0.001</td>
<td>C-A, C-B, C-R, A-R, B-R</td>
</tr>
<tr>
<td>Aflibercept 8mg/0.2 ml-Aflibercept 8mg</td>
<td>6</td>
<td>51.83 ± 2.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bevacizumab 5mg/0.2 ml-Bevacizumab 5mg</td>
<td>6</td>
<td>48.67 ± 2.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranizibizumab 2 mg/0.2 ml-Ranizibizumab 2 mg</td>
<td>5</td>
<td>58.00 ± 3.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4:** Comparison of MTT results of anti-VEGF agents when applied above the clinical doses.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Twice the clinical dose (mean ± SD)</th>
<th>Four times the clinical dose (mean ± SD)</th>
<th>Difference (mean ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflibercept</td>
<td>5</td>
<td>58.00 ± 3.16</td>
<td>51.20 ± 1.79</td>
<td>6.80 ± 3.96</td>
<td>0.018</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>5</td>
<td>62.40 ± 6.35</td>
<td>48.40 ± 3.13</td>
<td>14.00 ± 7.18</td>
<td>0.012</td>
</tr>
<tr>
<td>Ranizibizumab</td>
<td>5</td>
<td>52.20 ± 2.86</td>
<td>58.00 ± 3.16</td>
<td>-5.80 ± 1.83</td>
<td>0.034</td>
</tr>
</tbody>
</table>

**Table 5:** TAC levels in ARPE-19 cell groups after administration of clinical doses of anti-VEGF agents.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>TAC levels (mean ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.808 ± 0.055</td>
<td>0.169</td>
</tr>
<tr>
<td>Aflibercept 2 mg/0.05 ml</td>
<td>6</td>
<td>0.761 ± 0.077</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab 1.25 mg/0.05 ml</td>
<td>6</td>
<td>0.886 ± 0.431</td>
<td></td>
</tr>
<tr>
<td>Ranizibizumab 0.5 mg/0.05 ml</td>
<td>6</td>
<td>0.557 ± 0.243</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6:** Lipid peroxidation (MDA) levels in ARPE-19 cell groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>MDA levels (mean ± SD)</th>
<th>p</th>
<th>Binary comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.890 ± 0.383</td>
<td>0.001</td>
<td>C-A, C-B, C-R</td>
</tr>
<tr>
<td>Aflibercept, 2 mg/0.05 ml</td>
<td>6</td>
<td>1.498 ± 0.176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bevacizumab, 1.25 mg/0.05 ml</td>
<td>6</td>
<td>1.467 ± 0.204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranizibizumab, 0.5 mg/0.05 ml</td>
<td>6</td>
<td>1.550 ± 0.094</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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VEGF agents are conflicting. Sahiner et al. applied Aflibercept (0.5 mg/ml), Bevacizumab (0.3125 mg/ml), Ranibizumab (0.125 mg/ml) for 72 hours to the RPE cell culture obtained from enucleated eyes of New Zealand white rabbits. In Aflibercept group, less apoptosis and higher viability were found compared to the Bevacizumab, Ranibizumab and control groups. On the other hand, Bevacizumab and Ranibizumab groups had higher apoptosis and less viability when compared to the control group [10]. On the other hand, Feiner et al. and Inan et al. did not demonstrate any toxicity on intraocular structures after intravitreal Bevacizumab injection on electrophysiological and light microscopic examinations of rabbit eyes; however mitochondrial damage was seen in the inner segments of photoreceptors on electron microscopy [11,12]. Avci et al. demonstrated toxicity using immunohistochemistry and DNA fragmentation methods in rabbits treated with intravitreal bevacizumab, but they did not detect any morphological changes on light microscopy [13]. In fact, in vitro experimental rabbit studies may not constitute a complete model for humans due to factors such as smaller vitreous cavity, less vascular retina, different pharmacokinetics in rabbits, and low affinity of anti-VEGF agents for rabbit VEGF [14]. Toxicity of anti-VEGF agents have also been studied on human cell lines yielding conflicting results. Schnichels et al. used three different human cell line cultures and reported that Aflibercept did not change cell morphology and did not induce apoptosis in RPE, ganglion cells or photoreceptor cells at different doses (0.125 mg, 0.5 mg, 2 mg) at 1st, 24th, 48th and 72nd hours [15]. Malik et al. studied cell viability and mitochondrial membrane potentials after 24-hour incubation of ARPE-19 cell cultures with Bevacizumab, Ranibizumab, Aflibercept and Ziv-aflibercept at 1/2x, 1x, 2x, 10x clinical doses. Ranibizumab was reported to be a safer agent in terms of cell viability, and no difference was found between Ranibizumab and control groups for all doses of Ranibizumab. Although serious cytotoxic effects were detected at 10x doses of Aflibercept, Ziv-aflibercept and Bevacizumab, no cytotoxic effects were observed at 1/2x, 1x and 2x doses. In the aforementioned study, Malik et al. used a non-vital method with ViCell XR cell viability analyzer, which showed dead cells with trypan blue [16]. Luthra et al. evaluated cell viability in ARPE-19 cell culture and neurosensory retinal cell line (R28) of rats using trypan blue dye exclusion assay after application of 1x, 2x, 5x clinical doses of Bevacizumab and Ranibizumab, and reported that Bevacizumab was not toxic to human retinal pigment epithelial, rat neurosensory retinal, or human microvascular endothelial cells in vitro at the concentrations normally used in clinical practice or at higher concentrations [17]. In contrast to Malik et al. and Luthra et al.’s results, we determined cytotoxic effect of Bevacizumab at 2 x clinical dose (2.5 mg) on MTT assay. This difference may be related to different cell viability tests employed in our and in the aforementioned studies. Similar to our study, Spitzer et al. employed MTT analysis to investigate cytotoxic and anti-proliferative effects of Bevacizumab (0.008-2.5 mg/ml) on ARPE-19, rat retinal ganglion (RGC5) and pig choroidal endothelial cells (CEC). Toxic effects were not observed in any of the cell lines after 1 day, however 2.5 mg/ml Bevacizumab caused a moderate decrease in ARPE-19 cell numbers and cell viability after two days. The safe dose of Bevacizumab was reported as 0.25 mg/ml [18]. In our study, we did not observe any cytotoxicity with the clinical dose of Bevacizumab (1.25 mg) after 24 hours, however we did not continue the experiment after 24 hours, therefore we do not know if this dose resulted in any cytotoxicity thereafter. This is a limitation of our study. Differences in the molecular structures of anti-VEGF agents may explain the differences concerning their adverse effects. The half-life of Ranibizumab is shorter in the vitreous compared to Bevacizumab since its molecular weight is smaller than bevacizumab. Bevacizumab accumulates in RPE due its sugar and Fc components, and diffuses into the choroidal and inner layers of the retina [19]. Oxidative stress plays an important role in the pathogenesis and progression of ocular diseases such as AMD, cataracts, glaucoma, pseudoxfoliation syndrome, autoimmune uveitis and diabetic retinopathy. Hypoxia, hyperglycemia, oxidative stress and cytokines increase VEGF release and affect the progression of angiogenesis, which is the cause of some disorders. Oxidative stress causes degeneration in RPE and photoreceptor cells [20]. Wu et al. found that ARPE-19 cells and retinal pericytes produce excessive ROS in the in vitro hyperglycemia environment compared to the control normoglycemic environment [21]. Our study is one of the first studies in the literature that investigated oxidative stress with TAC and MDA levels in ARPE-19 cell lysates exposed to anti-VEGF agents for 24 hours. Zhou et al. reported that Bevacizumab stimulated the expression of ROS.6 Lin et al. reported that there was no statistically significant increase in ROS production at all tested doses of Ranibizumab including at 10x the clinical dose, Bevacizumab caused a significant increase in ROS level at 2x and 10x the clinical dose, and Aflibercept resulted in a significantly increased ROS level at 10x the clinical dose, but not in the other studied concentrations [7]. In our study, there was no statistically significant difference in TAC levels among the control group and three anti-VEGF groups. TAC levels might have been reduced after 24 hours, however we did not continue the experiment after 24 hours, which is a limitation of our study. On the other hand, MDA level was found significantly higher in all three anti-VEGF groups compared to the control group. Since a high MDA level reflects oxidative stress, we may say that all three anti-VEGF agents we studied caused oxidative stress in ARPE-19 cells. Our study has some limitations. As we mentioned previously, we stopped the experiment at 24 hours, and continuing the study after 24 hours might have given additional results. We performed our study on one human cell line, and including other human cell lines could
have been give more detailed results. We employed MTT assay as the cell viability measure, however it determines the metabolic status of the cell, which could reflect cell death, changes in cell proliferation, or changes in mitochondrial function. Inclusion of flow cytometry to differentiate apoptosis, necrosis, growth arrest, etc. and assays such as lactate dehydrogenase measurement in the supernatant, Annexin-V labeling, etc. might have better defined the fate of cells after treatment with anti-VEGF agents.

**Conclusion**

Anti-VEGF agents are widely used for therapeutic purposes, but side effects such as incomplete visual acuity, RPE atrophy and inflammation may occur, even with anatomical improvement. We believe that it is wise to use anti-VEGF agents at a minimum dose to provide maximum benefit and minimum cytotoxicity since the toxic effects of intravitreal anti-VEGF agents are dose-dependent. We suppose that different cell viability markers will be discovered in the future, we will be able to perform more sensitive experiments, and further enlighten the cellular mechanisms related to cytotoxicity of anti-VEGF agents.

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**Disclosure Statement**

No potential conflict of interest was reported by the author(s).

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**References**


