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Article

Retinal Toxicity of Intravitreal Injection of Ziv-Aflibercept in Albino Rabbits

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Purpose: To evaluate retinal toxicity of ziv-aflibercept, a drug that had been approved for use for patients with colon cancer.

Methods: Twenty-two albino rabbits were injected intravitreally with 0.1 mL of ziv-aflibercept solution into the experimental eye and 0.1-mL saline into the control eye. Twelve were used for electroretinogram (ERG) at 4-weeks follow-up. An additional 10 rabbits were used for testing penetration of ziv-aflibercept into the retina during follow-up. The visual-evoked potential (VEP) was recorded after 4 weeks of ERG follow-up. Glial fibrillary acidic protein (GFAP) immunocytochemistry and retinal histology were performed after the termination of the follow-up period.

Results: The ERG responses of the experimental eyes did not show signs of permanent functional damage. The VEP responses of the experimental eyes were of normal pattern and amplitude, and were similar to those recorded by stimulation of the control eyes. Histologic studies of both experimental and control eyes did not show signs of structural damage. However, GFAP expression was increased in retinal Müller cells of the experimental eyes and not of the control eyes. Retinal penetration of ziv-aflibercept, as indicated by positive antihuman immunoreactivity, was observed 1 day postinjection and was strengthened during the next 7 days. At 14 days postinjection, ziv-aflibercept was not detected.

Conclusions: Ziv-aflibercept was found to be nontoxic to the retina of rabbits based on electrophysiologic testing and histologic examination. However, GFAP immunocytochemistry suggests mild retinal stress caused by the drug.

Translational Relevance: If proven safe, ziv-aflibercept may be a new affordable treatment option in conditions involving neovascularization and macular edema.

Introduction

Age-related macular degeneration (AMD) is the main cause of irreversible blindness among adults, older than 70 years, in the developed world. Tens of millions of patients around the world suffer from AMD of which approximately 1.5 million suffer from the neovascular type (NVAMD), with 600,000 new cases each year.[1] NVAMD is responsible for 80% of cases of severe loss of vision or blindness caused by AMD.[2]

Vascular endothelial growth factor (VEGF) was shown to play a major role in the pathogenesis of neovascularization in NVAMD,[3] and therefore drugs that inhibit the bioactivity of VEGF have become the mainstay therapies of NVAMD. These drugs are also used in the treatment of other retinal conditions involving neovascularization and macular edema.[1] The currently available drugs are Ranibizumab (Lucentis; Genentech, Inc., San Francisco, CA), Bevacizumab (Avastin; Genentech, Inc., San Francisco, CA), and Aflibercept (Eylea; Regeneron Pharmaceuticals, Inc., Tarrytown, NY). While Ranibizumab and Aflibercept are drugs that were originally

developed for intravitreal use, Bevacizumab was developed as an intravenous injection for the treatment of oncologic patients.

The intravitreal use of Bevacizumab, a full-length IgG antibody, is done off-label. This clinical practice has become standard of care following animal-based studies that showed retinal penetrance and nontoxicity,[4] and clinical studies, showing that its efficacy in the treatment of retinal neovascularization was noninferior to that of Ranibizumab.[5] The major advantage of Bevacizumab compared with Ranibizumab is its price for the patients.

Aflibercept, another anti-VEGF agent, is a chimeric protein consisting of extracellular portion of VEGF receptor and Fc segment of human IgG antibody, thus binding VEGF with high affinity.[6] Aflibercept's safety and efficacy was shown to be comparable to those of Ranibizumab.[6]

Ziv-aflibercept (Zaltrap; sanofi-aventis U.S. LLC, Bridgewater, NJ) is the systemic counterpart of aflibercept, indicated for intravenous treatment of oncologic patients. The molecular composition and mechanism of action of the active ingredient in ziv-aflibercept and aflibercept (Eylea) are identical.[7,8] The only difference between the two drugs is in the solvent, being of high osmolarity (~1000 mOsm) in ziv-aflibercept and of iso-osmolarity in aflibercept.[9] Randomized controlled studies for assessing ziv-aflibercept's efficacy and safety in intravitreal treatment of conditions involving retinal neovascularization are yet to be published, although there have been preliminary reports of treatment trials that did not show signs of toxicity.[10]

The aim of this study was to assess the toxicity of intravitreal injection of ziv-aflibercept in albino rabbit eye model and to assess the penetrance of ziv-aflibercept into the retina.

Methods

Animals and Research Plan

Male New-Zealand White (NZW) albino rabbits ($N = 22$), weighing 1.5 to 3 kg were included in the study. The animals were kept in 12/12-hour light/dark cycle and given free access to food and water. Prior to clinical examination, ERG or VEP testing, and intravitreal injection, the animals were anesthetized by an intramuscular injection (0.5-mL/kg body weight) of a mixture containing ketamine hydrochloride (10 mg/mL), acepromazine maleate solution (10%), and xylazine solution (2%) at a ratio of 1:0.2:0.3. Topical anesthesia (benoxinate HCL 0.4%) was administered to prevent any potential animal discomfort. The pupils were fully dilated with cyclopentolate hydrochloride (1%).

Twelve animals (1 died during the follow-up) were included in the electrophysiologic experiments to assess retinal function. Baseline ERG and VEP measurements, and clinical inspection using indirect ophthalmoscope were performed prior to intravitreal injection of 0.1 mL of ziv-aflibercept into the right eye—referred to as the experimental eye—and 0.1-mL saline (0.9% NaCl) into the left eye that served as the control eye. ERG responses were recorded at 3 days, and 1, 2, and 4 weeks postinjection. At termination of the ERG follow-up period (4 weeks postinjection), after final ERG recording, VEP recording was also performed. Then, the animals were euthanized by intravenous injection of an overdose of sodium pentobarbital (80-mg/kg body weight), the eyes were enucleated in order to prepare the retinas for histologic and immunocytochemical studies.

Ziv-aflibercept's retinal penetration was tested in eyes of additional 10 animals, which were sacrificed and enucleated at 1-, and 3-days, and 1- and 2-weeks postintravitreal injection of ziv-aflibercept, in order to prepare the retinas for immunocytochemical observation for the localization of ziv-aflibercept.

All the experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional committee for experiments on animals.

Intravitreal Injection

Intravitreal injections[11] were performed using a 28-G needle attached to 1-mL tuberculin syringe, under visual control using indirect ophthalmoscope. The needle was inserted approximately 1 mm posterior to the limbus, into the vitreous, and was advanced to the center of the vitreous, above the optic disc. Then, a volume of 0.1 mL was slowly injected. All animals were examined with indirect ophthalmoscope following injection for the exclusion of gross retinal injury or cataract formation.

Ziv-Aflibercept

The right eye of each animal was injected intravitreally with ziv-aflibercept solution having a concentration of 25 mg/mL (original vial concentration).

Ziv-aflibercept's osmolarity was measured and found to be 1018 mOsm.

Electroretinogram (ERG)

The electroretinogram (ERG)[12,13] was recorded after a duration of at least 1 hour in darkness using corneal electrodes (Medical Workshop, Groningen, the Netherlands). Reference and ground electrodes, made of stainless-steel surgical needles, were inserted into the ears.

The ERG responses were recorded with the UTAS 3000 electrophysiology system (LKC Technologies, Gaithersburg, MD), which generated light stimuli from Ganzfeld light source with a maximum strength of 760 cd-s/m^2. Several responses (4–15) elicited by identical flashes that were applied at intervals of 2 to 30 seconds (depending upon stimulus strength) were averaged.

ERG analysis was based on amplitude measurements of the a- and b-waves.[12,13] The a-wave amplitude was measured from the baseline voltage that was recorded prior to the light stimuli, to the trough of the a-wave. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

The b-wave amplitudes that were recorded in the dark-adapted state were plotted as a function of log stimulus strength, and the resulting response—stimulus strength curve was fitted to a hyperbolic function.[13,14]

$$V/Vmax = I/(I + \sigma) \quad (1)$$

Where V and Vmax are the amplitudes of the ERG wave, which are measured in responses that are elicited by a stimulus strength I or by a stimulus of super-saturating strength, respectively. The semisaturation constant, σ, is the stimulus strength that is required to elicit a response of one-half the maximal amplitude.

In some cases, we found a two-limb relationship between the dark-adapted b-wave amplitude and log stimulus strength, in which the b-wave amplitude saturated before growing again for very bright stimuli. This phenomenon that was noticed before, was attributed to destructive interference between the a- and b-wave.[15] In these cases, we eliminated the second limb (usually the data point representing the brightest test stimuli) from the data before fitting the b-wave amplitude to log stimulus strength relationship to Equation 1.

Vmax ratio and logσ difference were calculated for each animal in each time point of ERG recording during follow-up period, by dividing the Vmax value of the experimental eye with that of the control eye, and by subtracting logσ of the control eye from that of the experimental eye, respectively. We showed previously that this method of analysis circumvented variabilities in inter-ERG recording sessions of each animal, and could be used as a reliable indicator for outer retina function in the treated eye.[11,13]

The b-waves amplitudes of the light-adapted (background of 30 cd/m^2) ERG responses that were elicited by a stimulus of 2.5 cd-s/m^2, were used to compare between the eyes of each rabbit, and to assess light-adapted retinal function in the experimental eye compared with the control eye.

We also measured the oscillatory potentials appearing on the ascending part of the ERG b-wave, which reflect neuronal activity in the inner layers of the retina.[12] The measurement of oscillatory potentials of dark-adapted ERG responses elicited by light stimuli of constant intensity (I = 2.5 cd-s/m^2) was done using the built-in filtering procedure (75–300 Hz) of the UTAS- 3000 (LKC Technologies). The sum of the oscillatory potentials (ΣOP) was obtained for the experimental eye and control eye of each animal before injection and during the follow-up period. The ΣOP ratio for each rabbit was calculated by dividing the ΣOP value of the experimental eye by that of the control eye in order to obtain an indicator for inner retina function.

Visual-Evoked Potentials (VEPs)

Visual-evoked potentials (VEPs)[11] were recorded to evaluate the functional integrity of the retina (from photoreceptors to ganglion cells), and the optic pathways from the retina to the visual cortex.

VEPs, elicited by white light stimuli (2.5 cd-s/m^2), were recorded by an active electrode, made of stainless-steel needle, inserted under the skin between the ears above the area of the rabbit's visual cortex. The reference and ground electrodes were inserted into the ears.

The VEP signal was amplified, filtered, and averaged (25 responses delivered at a rate of 1.1 KHz) by the UTAS-3000 electrophysiologic system (LKC Technologies). VEPs were recorded in response to monocular stimulation of each eye before the injection and at the end of the follow-up period.

The analysis of VEPs was based on amplitude and implicit time measurements. VEP amplitude was measured between the trough of the first negative wave and the peak of the following positive wave. VEP timing was defined by the duration from

stimulus onset to the trough of the first negative wave. A paired *t*-test was conducted to compare VEP amplitudes and implicit time between the experimental and control eyes before injection and at termination of the follow-up period (28 days after injection).

Immunocytochemistry

Following electrophysiologic (ERG and VEP) testing at termination of the follow-up period, the rabbit was euthanized (see above), and the eyes were enucleated. The enucleated eyes were soaked for 10 minutes in a solution of 4% paraformaldehyde in 0.1 M of phosphate buffer solution (PBS; pH 7.4), and then was opened posteriorly to the limbus and fixed for additional 1 hour. The eyecup was cut into two halves along vertical meridian; one-half was used for histology and left in the fixative solution until use, and the other half was used immediately for immunocytochemistry.[16] It was cryoprotected by soaking for 1 hour in 15% sucrose (in PBS), then for 1 hour in 20% sucrose (in PBS), and finally in 30% sucrose (in PBS) overnight in 4°C. The half eyecup was then embedded in optimal cutting temperature compound and cut into 16-µm thick sections on a cryostat.

Glial Fibrillary Acidic Protein (GFAP)

The cryostat sections were soaked in PBS (0.1 M, pH 7.4), and then incubated in 1% triton PBS solution for 10 minutes in room temperature. Slides were incubated for 1 hour in room temperature in 5% fetal bovine serum solution, and washed in PBS. Then, the sections were soaked overnight at 4°C in a moist chamber with primary antibody to GFAP (Chemicon, Temecula, CA) at 1:400 dilution in PBS 0.1 M+3% serum+0.1% TritonX-100 at 1:100. For secondary antibody, we used Alexa Fluor donkey anti-mouse (Invitrogen; Thermo Fisher Scientific, Waltham, MA) diluted to 1:500.

Retinal Penetrance Study

The frozen retina slices were washed three times in PBS, and then treated with 1% triton solution for 10 minutes in room temperature. Following three washes with PBS, the slices were incubated in 5% fetal bovine serum for 1 hour in room temperature. Anti-human IgG (Jackson ImmunoResearch Labs, West Grove PA), diluted to 1:500, was added and the slices were incubated at 4°C overnight in order to stain the ziv-aflibercept.

All retinal section studied for either GFAP or ziv-aflibercept were also treated with 4′,6-Diamidino-2-phenylindole (DAPI; 1:2000) in order to stain cell nuclei and reveal retinal structure in order to localize the proteins.

Histology

One-half of the eyecup was rinsed in PBS, dehydrated in alcohol, soaked in a solution of resin and catalyst without the hardener overnight, and embedded in resin (JB-4; Bio-Rad, Watford City, UK). The tissue was cut into 2-µm sections (Reichert-Jung, Nussloch, Germany) and mounted on slides. The sections were stained with Richardson's solution for examination with the light microscope.[16]

Statistical Analysis

In order to estimate the sample size of rabbits that was needed for reliable significance, we used data that were published in a previous study from our laboratory on retinal toxicity of rituximab.[17] The most important variable for assessing toxicity using ERG is a change of Vmax. The average Vmax ratio (right eye/left eye) in rabbit ERG that we measured in the above study was 1.03 (SD = 0.11). The desired effect is a 10% (at least) reduction in the average of Vmax ($0.9 \times 1.03 = 0.927$). We postulated α error = 0.05, power = 0.8. Using statistical power analysis program GPower 3.1 (Heinrich-Heine-Universität, Düsseldorf, Germany), we calculated that the needed minimum sample size was nine animals.

Data were analyzed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA). The ERG parameters were tested for statistical significance by using ANOVA with repeated measures. The VEP parameters and histologic measurements were tested for statistical significance using paired *t*-test. All tests were two-tailed, and the threshold for statistical significance was defined as a $P \leq 0.05$.

Results

Clinical Examination

Throughout the follow-up period, the rabbits gained weight and showed no signs of adverse systemic effects of the treatment, such as apathy, weight loss, or behavioral abnormalities. Clinical eye examinations with indirect ophthalmoscope showed no signs of cataract formation, inflammation, or retinal detachment.

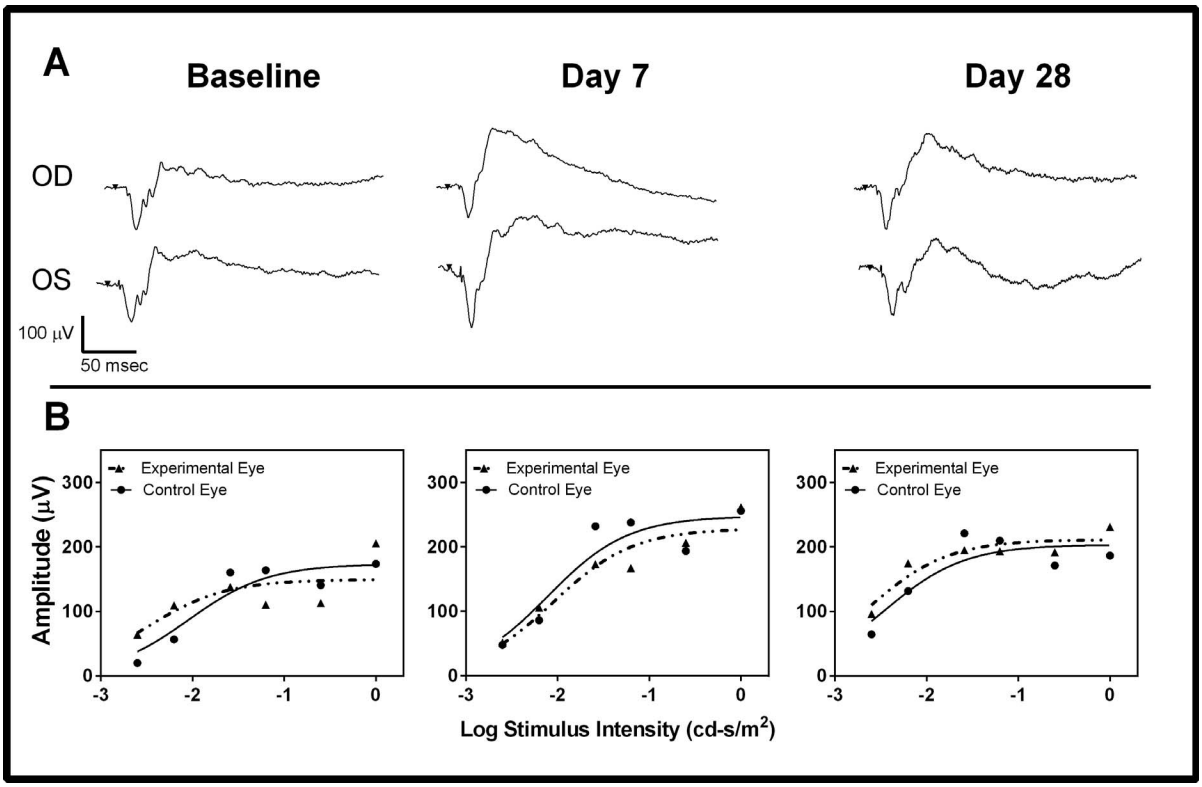

Figure 1. ERG follow-up data from a single rabbit. (A) Dark-adapted ERG responses from the experimental (OD) and the control (OS) eyes, that were elicited by white-light stimuli of Intensity = 2.5 cd-s/m^2. Stimulus timings are noted by *arrowheads*. (B) Response-stimulus strength relationships for the dark-adapted ERG b-wave for each ERG recording session. The relationships were fitted to a hyperbolic function (Equation 1) in order to derive the maximal amplitude (Vmax) and the semisaturation constant (σ).

Electroretinogram

Dark-adapted ERG responses from the control and experimental eyes were similar in pattern and amplitude in baseline measurements before injection, and throughout the follow-up period, as illustrated for one rabbit in Figure 1. Examples of ERG responses that were elicited by bright ($I = 2.5$ cd-s/m^2) white–light stimuli, and recorded at baseline; 7 and 28 days after intravitreal injection of ziv-aflibercept are shown in the upper part of Figure 1. During the recording sessions, we used stimuli of different strengths in order to derive the response-log stimulus strength relationships (Fig. 1, lower part). These relationships were fitted to hyperbolic function (Equation 1) to derive the maximal response amplitude (Vmax) and the semisaturation constant (σ) for the dark-adapted ERG b-wave.

All rabbits underwent similar analysis for the dark-adapted ERG at all recording sessions. The ERG parameters that were derived, as described above, were used to calculate the Vmax ratio (experimental eye/control eye), and the difference in logσ (experimental eye – control eye) of the dark-adapted ERG b-wave. Figure 2 shows time-depended changes in Vmax ratio (B) and logσ difference (C) of the dark-adapted ERG b-wave (mean ± SD) of all the rabbits ($N = 11$).

Because the dark-adapted a-wave of rabbits was too small and did not saturate by the stimulus strength range we used here, we measured the maximal a-wave amplitude from the ERG responses, elicited by the brightest stimulus used here ($I = 2.5$ cd-s/m^2), to calculate the mean ± SD a-wave maximal amplitude ratio (Fig. 2A). The ratio of the maximal amplitudes of a-waves fluctuated around 1 throughout the follow-up period, showing no statistically significant interaction between time and treatment ($P = 0.811$). Vmax ratio of the dark-adapted b-wave (Fig. 2B) also fluctuated around 1 throughout the follow-up period. However, statistical analysis showed significant interaction between time and treatment ($P = 0.0029$), most probably due to a transient peak in the Vmax ratio, indicating larger Vmax of the experimental eye compared with the control eye at day 7 postinjection (Fig. 3B).

The differences of semisaturation constants (logσ)

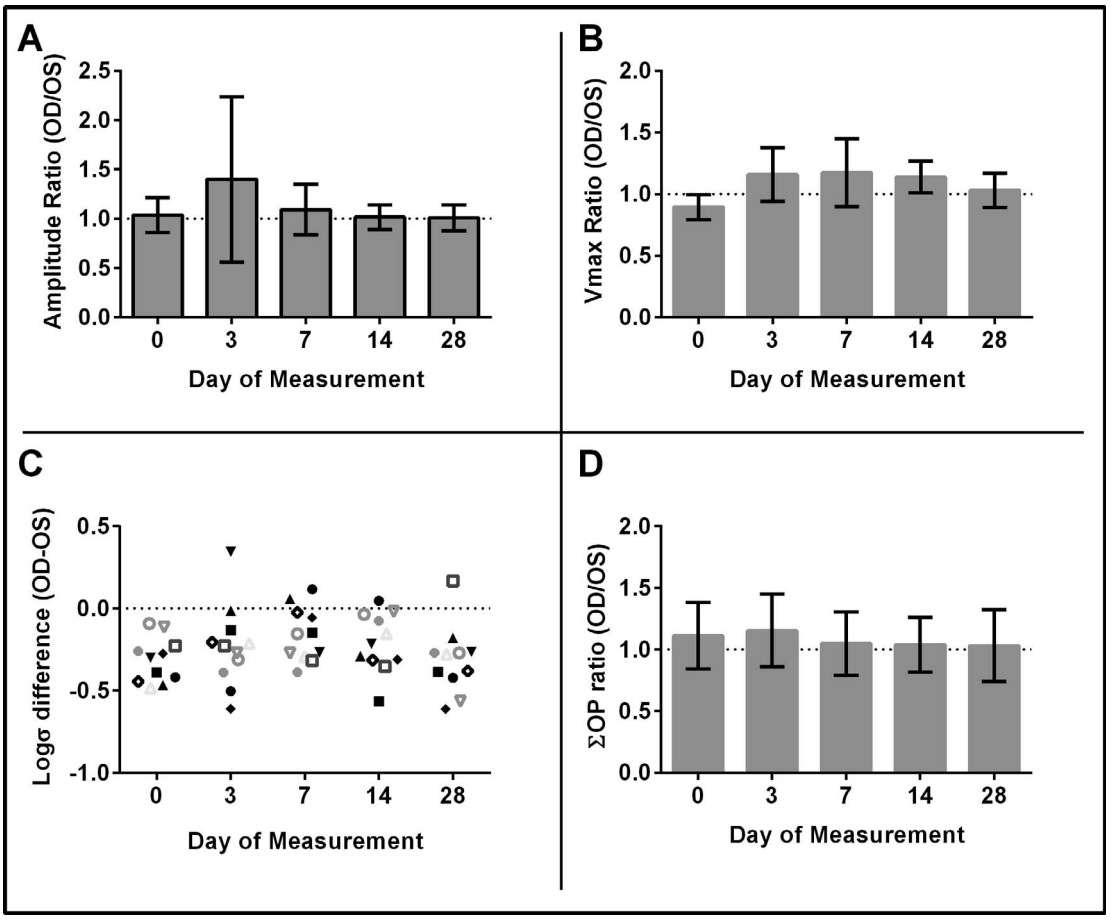

Figure 2. ERG analysis of all rabbits as a function of time after injection of ziv-aflibercept (A) Mean ± SD of maximal ERG a-wave ratio (experimental eye/control eye). Data from baseline measurements (Day 0) and throughout the follow-up period. (B) Mean ± SD of b-wave Vmax ratio (experimental eye/control eye) at each time point from baseline (Day 0) throughout the follow-up period. (C) Scattergram for logσ differences (experimental eye-control eye) of each rabbit throughout the follow-up period. Each *dot* represents a single rabbit. (D) Mean ± SD sum of oscillatory potentials (ΣOP) ratios (experimental eye/control eye) for ERG responses throughout the entire follow-up period.

of the dark-adapted ERG b-waves were negligible in the baseline measurements and follow-up period (Fig. 3C). No statistically significant interaction was found between time and treatment for $\Delta\log\sigma$ ($P = 0.29$).

The maximal amplitude of the a- and b-wave and the semisaturation constant of the b-wave serve as indicators for the functional integrity of the distal retina. The oscillatory potentials are generated in the inner plexiform layer, and therefore can serve as an objective indicator of inner retinal function. We obtained the sum of the oscillatory potentials (ΣOP) from the dark-adapted ERG responses that were elicited by bright-light ($I = 2.5$ cd-s/m^2) stimuli and calculated the ratio of the sum of the oscillatory potentials (experimental eye/control eye; Fig. 2D). There was no statistically significant difference between the eyes throughout the follow-up period ($P = 0.7604$).

Visual-Evoked Potentials

The VEP that is recorded from the scalp, reflects visual information processing in the distal retina, the proximal retina and conductance in optic pathways from the ganglion cells to the primary visual cortex. Therefore, we recorded VEPs, evoked by monocular stimulations of the control and experimental eyes in each rabbit, to test potential toxic effects on ganglion cells and their axons (nerve fiber layer). Recording from one rabbit (Fig. 3, upper panel), show a typical pattern containing a negative wave, appearing approximately 30 ms after onset of light stimuli, followed by a positive wave, appearing approximately 80 ms, that was easily identified in the recordings

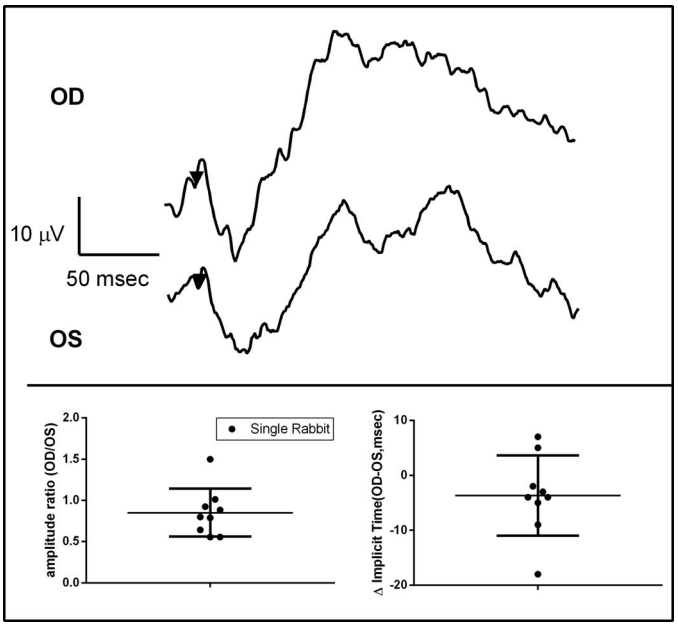

Figure 3. Analysis of VEPs that were measured at termination of follow-up period. *Upper pane*: VEP recording from one rabbit, that were evoked by light stimuli to the experimental eye (OD) and the control eye (OS). Timings of light stimuli are indicated by *arrowheads*. *Left lower pane:* VEP amplitude ratios (experimental eye/control eye). Each *dot* represents the VEP ratio for a single rabbit. *Horizontal lines* represent mean VEP Ratio ± SD. *Right lower pane:* VEP implicit times difference (experimental eye – control eye). Each *dot* represents the VEP implicit time difference for a single rabbit. *Horizontal lines* represent mean VEP implicit time difference ± SD.

from 9 of 11 rabbits. In two rabbits, nonmeasurable VEP responses were obtained from both eyes, probably due to technical reasons, such as depth of anesthesia or placement of recording electrodes. For each rabbit, we calculated the VEP amplitude ratio (experimental eye/control eye) and the VEP implicit time difference (experimental eye – control eye shown in Figure 3, lower pane) as scatter-gram. The mean ± SD amplitude ratio was 0.855 ± 0.287 and mean implicit time difference of the first negative wave was –3.67 ± 7.314 ms. A paired *t*-test for the VEP amplitudes and implicit times that were recorded in the final recording session, at day 28 after injection, showed no significant differences between the experimental eyes and the control eyes ($P = 0.09$, and 0.17, respectively).

Histology

Histologic examinations of retinal preparations with a light microscope show normal retinal structure, and no signs of retinal detachment or atrophy of retinal nuclear layers in the experimental eye and control eye as shown in Figure 4A. In order to assess quantitatively changes in retinal structure due to ziv-aflibercept, we measured the thickness of outer and inner nuclear layers (ONL and INL, respectively), and show the averages for all rabbits in Figure 4B. The mean (±SD) thickness of the ONL in the experimental and control eyes was 57.2 ± 6.3 and 57.9 ± 8.2 µm, respectively. The mean (±SD) thickness of the INL in the experimental and control eyes was 30.2 ± 6.6 and 27 ± 4.5 µm, respectively. A paired *t*-test that was conducted for the ONL and INL thickness no significant difference between the eyes ($P = 0.6764$ and 0.1063, respectively).

Glial Fibrillary Acidic Protein

GFAP is commonly expressed only in astrocytes located in close to the surface of the retina, but not in retinal Müller cells. Because GFAP expression is increased in retinal Müller cells following gliosis due to retinal trauma,[18–21] GFAP expression is used as a sensitive biological marker for retinal trauma. Ten of 11 rabbits (91%), tested for the effects of ziv-aflibercept, showed positive GFAP immunoreactivity in retinal Müller cells of the experimental eyes, 28 days after ziv-aflibercept injection as shown for two rabbits in Figure 5 (left column). No GFAP expression was found in the retinas of the control eyes (Fig. 5, right column).

Ziv-Aflibercept Retinal Penetrance

Retinal penetration of ziv-aflibercept, as indicated by positive antihuman immunoreactivity, was observed 1 day postinjection and was strengthened during the following 7 days (Fig. 6). Strong immunoreactivity was shown around bipolar retinal cells. At 14 days postinjection, ziv-aflibercept was not detected in the retina (Fig. 6).

Discussion

This study shows that intravitreal injection of ziv-aflibercept is overall a safe procedure for the treatment of choroidal neovascularization in retinal diseases, such as AMD, as suggested by the absence of signs of toxicity in retinal function and retinal structure in the albino rabbit model. Electrophysiologic testing did not show signs of functional impairment in the distal retina (Figs. 1, 2), inner retina (Fig. 2D), and retinal output (Fig. 3). Furthermore, retinal histology showed no signs of

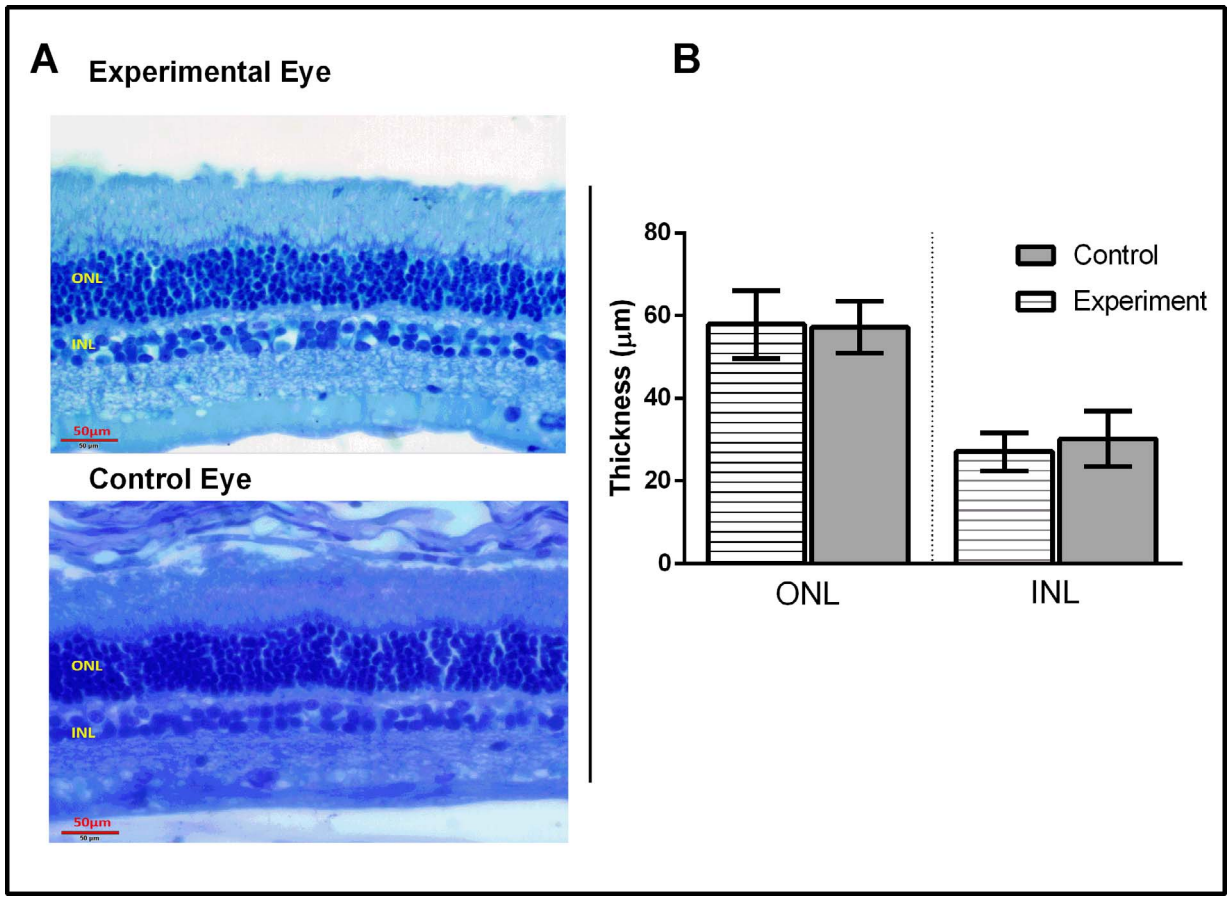

Figure 4. Effect of ziv-aflibercept on retinal structure of albino rabbits. (A) Micrographs from the experimental eye and control eye of a single rabbit. (B) Thickness measurements of ONL and INL of all rabbits. Mean ± SD of INL and ONL thickness are compared between experimental eyes and control eyes.

structural damage in the ONL and INL in the experimental eyes as compared with the control eyes (Fig. 4). The only sign for retinal trauma in the experimental eyes, injected intravitreally with ziv-aflibercept, is the increased expression of GFAP in retinal Müller cells (Fig. 5).

Ziv-aflibercept was approved by the Food and Drug Administration (FDA) for intravenous treatment of cancer patients, but not for intravitreal injection in ocular diseases. Therefore, the drug is sold with a printed warning on its package against intravitreal injection, probably for fear of ocular toxicity.

The active molecule in ziv-aflibercept, is identical to the active molecule in aflibercept (Eylea), an FDA-approved drug for intravitreal injection. Thus, it cannot be the source of ziv-aflibercept's alleged toxicity. None of the inactive components in ziv-aflibercept (sucrose, sodium citrate, sodium phosphate, sodium chloride)[22] is known to be inherently toxic to the retina. Therefore the possible source for retinal toxicity of intravitreal ziv-aflibercept may be the high osmolarity of the drug preparation, 1018 mOsm, as measured with a freezing point depression osmometer or 1021 mOsm as calculated from information provided by the manufacturer of the drug.[22] In contrast, aflibercept (Eylea), is iso-osmotic containing in addition to the active molecules also sodium phosphate (10 mM), sodium chloride (40 mM), 0.03% polysorbate 20, and 5% sucrose as taken from the drug brochure.

A detailed study on the effects of the osmolarity of intravitreally injected solutions showed severe damaging effects depending upon the solutes, and upon the level of osmolarity.[23] The solutions studied were sodium chloride, sodium-aspartate, Ethylenediaminetetraacetic acid, mannitol, sucrose, and penicillin. According to this study, injection of 0.05-mL hyperosmolar solution into the vitreous of albino rabbit's eye can cause almost immediate retinal

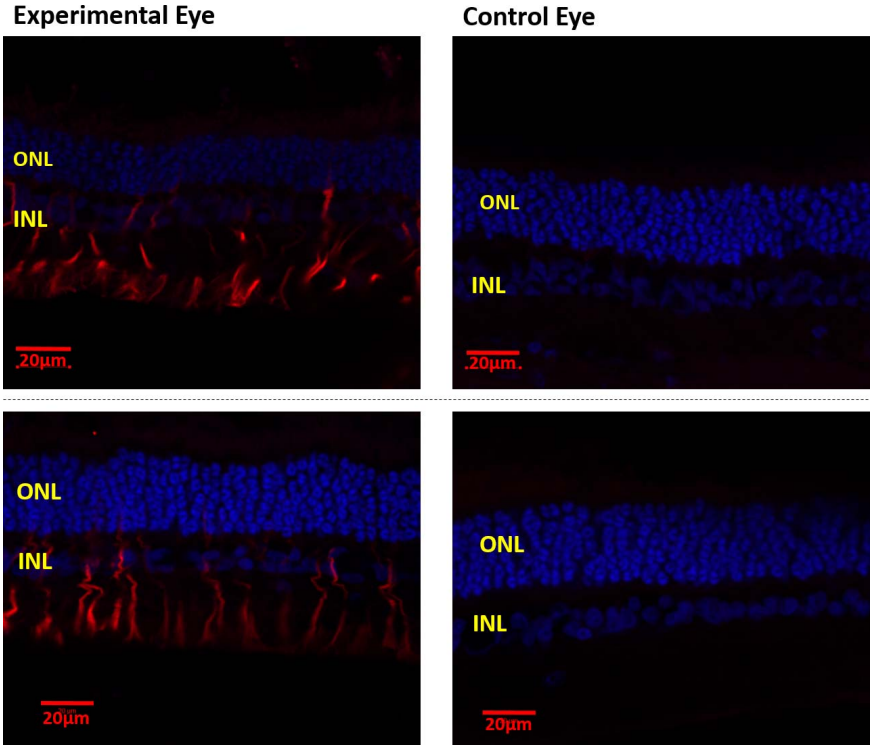

Figure 5. GFAP immunostaining. Retinal preparations from two rabbits (*upper* and *lower rows*). For each rabbit, GFAP immunostaining is compared between the experimental eye and control eye (*left and right columns*, respectively. *Blue*, DAPI staining of cell nuclei; *Red*, positive anti-GFAP immunostaining).

opacification, and later widespread retinal detachment, and degeneration associated with a significant amplitude reduction of the ERG b- and a-wave, reflecting functional impairment.[23] The minimal osmolarity to cause these effects depended upon the type of solvent compromising the major osmolarity increase, and could be as low as 500 mOsm for sodium chloride, and higher than 1000 mOsm for sucrose.[23]

In contrast to the above findings, we did not see any clinical, structural, or ERG signs for retinal impairment even though the osmolarity (1018 mOsm) of ziv-aflibercept is in the range that was reported to cause severe retinal damage, and the volume (0.1 mL) injected was twice larger. The lack of hyperosmolarity-induced retinal damage following injection of ziv-aflibercept in our study can be explained by the following several options:

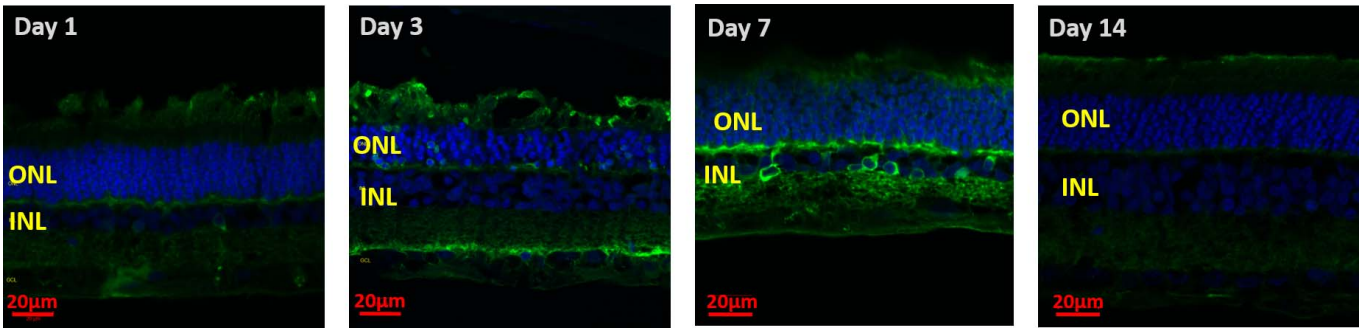

Figure 6. Penetrance of ziv-aflibercept into the retinas of albino rabbits. Retinal preparations from albino rabbit eyes injected intravitreally with ziv-aflibercept, 1, 3, 7, and 14 days after the injections. *Blue*, DAPI staining of cell nuclei; *Green*, positive anti-human IgG immunostaining, indicating presence of ziv-aflibercept in the tissue. Retinal penetrance of ziv-aflibercept was observed 1, 3, and 7 days postinjection. At 14 days postinjection, ziv-aflibercept is not detected in the retina.

1. Marmor et al.[23] injected solutions with only one solute, while the osmolarity of ziv-aflibercept is composed of five different solutes. It is possible that some of these solutes are quickly absorbed into blood vessels of the retina, and removed from the vitreous, before a steep osmolarity gradient that can cause retinal damage is established;
2. Sucrose solution had a mild effect and caused retinal damage only with osmolarity of approximately 1000 mOsm, and even then, only incipient retinal detachment was observed.[23] The major (~50%) osmolarity increase in ziv-aflibercept is due to sucrose (584 mOsm), raising the possibility that the total hyperosmolarity in ziv-aflibercept was insufficient to cause retinal damage; and
3. A potential mechanism for hyperosmolarity-induced retinal damage assumes that vitreal hyperosmolarity can induce fluid efflux from retinal blood vessels that can cause retinal edema and loss of function. Permeability of retinal blood vessels depends upon VEGF, and if ziv-aflibercept molecules trap efficiently extracellular VEGF molecules in the retina hyperosmolarity-induced fluid efflux can be significantly reduced.

Although ERG recordings did not show retinal toxicity, we observed a transiently increase of b-wave Vmax in the experimental eyes compared with the control eyes at day 7 postinjection. In most cases, retinal toxicity causes a decrease in b-wave amplitudes and Vmax[13]; however, some toxic substances, such as barium, can cause an increase in ERG amplitudes, but in these cases the increase was followed by a decrease to subnormal values and severe morphologic damage to the retina.[24] Transient increase in b-wave amplitude has been previously noticed in early stages of retinal ischemia.[25,26] While ziv-aflibercept probably affects retinal blood vessels and thus may potentially cause ischemia, in such case we would expect an apparent prolongation of the ERG b-wave implicit time as was described during retinal ischemia.[25] We observed no significant prolongation of the implicit time of the dark-adapted ERG b-wave on day 7 recording, or on any other ERG recording session during the follow-up period.

Although no functional or structural damage was detected in physiologic tests and histology, we observed upregulation in GFAP expression in retinal Müller cells in 91% of the experimental eyes, while none was observed in the control eyes. GFAP is an intermediate filament protein that is normally expressed in retinal astrocytes, but not in retinal Müller cells.[18] Retinal stress of any kind, such as retinal detachment,[19] ischemia,[20] and high intraocular pressure,[21] is correlated with expression of GFAP in retinal Müller cells. Therefore, GFAP has been used as an accepted biological marker for retinal stress. The increased GFAP expression can reflect minor retinal trauma by the high osmolarity of the solvent in the ziv-aflibercept preparation and/or effect of the active molecules. In a recent study,[27] rat in vitro retinal preparations were exposed to aflibercept, the FDA-approved drug for treatment of AMD, and exhibited increase expression of GFAP in Müller cells. Because aflibercept and ziv-aflibercept contain the same active molecules, these findings suggest that increased GFAP expression in Müller cells was caused by the active drug and not by the osmolarity of the solvent. Therefore, if ziv-aflibercept and aflibercept cause increase in GFAP expression by the same mechanism, the retinal trauma, causing the GFAP increased expression, may be of no clinical consequences.

To summarize, our findings indicate no functional or structural damage to the retina by intravitreal ziv-aflibercept and mild retinal trauma of probably no clinical concern, as indicted by the increase in GFAP expression. Thus, our observations, may pave the way to future use of intravitreal injection of ziv-aflibercept in the ophthalmic clinic. Yet, larger studies assessing the safety and efficacy of intravitreal ziv-aflibercept need to be conducted.

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