

# Efficacy of Intravitreal Injections of Antivascular Endothelial Growth Factor Agents in a Rat Model of Anterior Ischemic Optic Neuropathy

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**PURPOSE.** We investigated the efficacy of intravitreal injections of anti-VEGF agents in a rat model of anterior ischemic optic neuropathy.

**METHODS.** We applied laser-induced photoactivation on the optic nerve head after intravenously administered rose bengal (RB). The rats immediately received an intravitreal injection of either ranibizumab or PBS. The density of retinal ganglion cells (RGCs) was calculated using retrograde FluoroGold labeling. Visual function was assessed by flash visual-evoked potentials (FVEP). We investigated TUNEL assays of the retinal sections and ED1 staining of the optic nerve.

**RESULTS.** After treatment, the RGC densities in the anti-VEGF-treated rats were not statistically significant different from those of the PBS-treated rats (57.0% vs. 40.0% in the central retinas; 39.8% vs. 33.6% in midperipheral retinas, both  $P > 0.05$ ). Measurements of FVEP showed no statistically significant differences in preserved latency or amplitude of the P1 wave between anti-VEGF and PBS groups (latency  $131 \pm 15$  ms versus  $142 \pm 14$  ms,  $P = 0.157$ ; amplitude  $34 \pm 12$   $\mu$ v versus  $41 \pm 13$   $\mu$ v,  $P = 0.423$ ). Assays of TUNEL showed that there was no statistical difference in the number of apoptotic cells in the RGC layers between anti-VEGF and PBS groups ( $7.0 \pm 0.8$  cells/high-power field [HPF] versus  $7.8 \pm 1.3$  cells/HPF;  $P = 0.275$ ). In the optic nerves, we did not observe statistically significant differences in ED1-positive cells/HPF between anti-VEGF and PBS groups ( $P = 0.675$ ).

**CONCLUSIONS.** Intravitreal injections of anti-VEGF did not have a protective effect in the rat model of anterior ischemic optic neuropathy.

**Keywords:** rat anterior ischemic optic neuropathy, vascular endothelial growth factor, ranibizumab, anti-VEGF, retinal ganglion cells

Nonarteritic anterior ischemic optic neuropathy (NAION) is the most common type of ischemic optic neuropathy in the elderly. Currently, there is no accepted treatment to rescue vision in patients with NAION. Although a previous study showed that systemic steroid treatment was effective in patients with NAION in whom the initial visual acuity was below 20/70,<sup>1</sup> the complications of systemic steroid use are a concern in clinical practice.<sup>2</sup> Intravitreal steroid injection in patients with NAION has been reported in some small case series with no control group, and local steroid treatment has been shown to improve visual acuity in some cases.<sup>3–6</sup>

Cellular inflammation was shown to play a major early role following infarct in a primate NAION model.<sup>7</sup> In human patients with NAION, venous insufficiency causing initial disc edema associated with the creation of a compartment syndrome has been reported to be a possible pathogenesis.<sup>8</sup> In the retina, VEGF is essential for angiogenesis, and it also

promotes vascular permeability in hypoxic conditions or vascular inflammation.<sup>9</sup> Agents of anti-VEGF such as bevacizumab (Avastin; Genentech, Inc., San Francisco, CA, USA) and ranibizumab (Lucentis; Novartis, Inc., Basel, Switzerland) have been reported to inhibit VEGF signaling and thereby potentially decrease optic disc edema.<sup>10</sup> Agents of anti-VEGF have also been shown to have an anti-inflammatory effect.<sup>11</sup> Therefore, it is reasonable to hypothesize that anti-VEGF agents may have therapeutic effects in NAION. However, the few studies having reported anti-VEGF treatment in patients with NAION show conflicting results. Improvements in visual acuity have been reported in some cases,<sup>12,13</sup> whereas a randomly controlled trial of intravitreal injections of triamcinolone combined with anti-VEGF agents in patients with NAION (15 injected eyes, 17 controls) reported no significant difference between the two groups.<sup>14</sup> Another nonrandomized controlled clinical trial in patients with acute NAION showed no difference between

intravitreal bevacizumab (1.25 mg) and natural history in functional and anatomic improvements.<sup>15</sup> To date, no benefits of anti-VEGF treatment have been reported in patients with NAION.

In optic neuropathy, there is a concern that inhibition of VEGF function may cause neurodegeneration of retinal neurons. Thaler et al.<sup>16</sup> reported that the retinal ganglion cell (RGC) count in healthy rat eyes was essentially unchanged from those of control animals after the administration of both low and high concentrations of bevacizumab, ranibizumab, or pegaptanib (Macugen; Eyetech Pharmaceuticals, Inc., New York, NY, USA) for 2 months. In addition to inflammation role of VEGF, VEGF-A indicated to be a critical neuroprotectant with evidence of significant loss of RGCs after chronic anti-VEGF treatment in an ischemia/reperfusion model.<sup>17</sup> Vascular endothelial growth factor was reported to protect the neurodegeneration of RGCs via activating ERK-1/2 and Akt pathways in a model of optic nerve (ON) axotomy.<sup>18</sup> However, anti-VEGF agents were reported to contribute to RGC preservation in a mouse model of ON crush.<sup>19</sup> In an animal laser-induced choroidal neovascularization, no significant reduction was found in retinal function, the retinal level of mRNA for ganglion cell-specific genes, and RGC axon count after blockage of VEGF for 7 months in transgenic mice.<sup>20</sup>

To the best of our knowledge, anti-VEGF agents have never been evaluated in a rat model of anterior ischemic optic neuropathy (rAION). Inasmuch as the results of in vivo animal experiments cannot be directly transferred to clinical NAION, physiologic and histologic changes in cellular inflammation in rAION are similar to those in early NAION.<sup>7,21</sup> In this study, we aimed to investigate whether intravitreal anti-VEGF therapy has anti-inflammatory and antiapoptotic effects, and whether it has the ability to rescue RGCs after the induction of rAION.

## MATERIALS AND METHODS

### Animals

Sixty adult male Wistar rats weighing 150 to 180 g (aged 7–8 weeks) were used in this study. Animal care and experimental procedures were performed in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research. In addition, the Institutional Animal Care and Use Committee at Tzu Chi Medical Center approved all animal experiments. All manipulations were performed following the rules of our previous papers.<sup>22,23</sup>

### Study Design

The rats received one intravitreal injection of ranibizumab (2  $\mu$ L per injection, 10 mg/1 mL, 20 rats; Lucentis) or PBS (as the controls; 2  $\mu$ L per injection, 20 rats) in the right eye 1 day after the induction of rAION. The final intraocular concentration used in the rats was no less than that used in routine clinical practice.<sup>16</sup> We used 33-G needles (Hamilton7747-01 with a Gastight syringe, IA2-1701RN 10- $\mu$ L SYR; Hamilton Co., Hamilton, KS, USA) to perform the intravitreal injections. Intraocular pressure (IOP) was measured using a Tono-Pen (Reichert Technologies, Depew, NY, USA) 1 day after the intravitreal injections. The 20 control rats received a sham operation (laser treatment without intravenous rose bengal [RB]; Sigma-Aldrich Corp., St. Louis, MO, USA) injections). The rats were euthanized 4 weeks post infarct by CO<sub>2</sub> insufflation. Density of RGC was measured by retrograde labeling with FluoroGold (Fluorochrome, LLC, Denver, CO, USA), and visual function was assessed by flash visual-evoked potentials (FVEP) 4 weeks post infarct. Assays of TUNEL of the RGC layer and

immunohistochemistry (IHC) of ED1 (markers of macrophage) expression in the ON were also conducted.

We used a sample size of six rats in every test per group that would have 77% probability of achieving a significant result, given the 1.13 effect size of the observed anti-VEGF versus PBS groups in RGC study (Cohen's *d* with  $\alpha = 0.05$  in G\*Power Software; Heinrich-Heine-Universität Düsseldorf, Dusseldorf, Germany). After performing a Mann-Whitney *U* test, an experimental group size of approximately  $n = 16$  has been required to have power of 80% in RGC study. In order to follow-up the principles of the three R's (replacement, reduction, and refinement) for humane animal research, we finally determined the  $n = 6$  in every test per group.

### Induction and Visualization of rAION

The details of rAION induction were the same as in our previous report.<sup>24</sup> Briefly, after general anesthesia, RB (Sigma-Aldrich Corp.) was administered intravenously through the tail vein using a 28-G needle (2.5-mM RB in PBS)/1-mL/kg animal weight). After administration of RB and pupil dilatation, the right optic discs were directly treated with an argon green 532-nm/500- $\mu$ m/80-mW spot laser (MC-500, multicolor laser; Nidek Co., Ltd. Tokyo, Japan) with 12 pulses of 1-second duration. We used an OFA 5.4 laser contact lens (Ocular Instruments, Inc., WA, USA) to perform laser treatment and to check the fundus after rAION. When rose bengal is activated by green laser light, it glows a brilliant golden color, which can prove that photodynamic laser induction of ischemic optic neuropathy has been successfully achieved.<sup>25</sup>

### Retrograde Labeling of RGCs With FluoroGold and Morphometry of RGCs

The detailed procedures of FluoroGold labeling have been described in our previous reports.<sup>22,23</sup> In brief, the retinas were examined for RGCs at a distance of 1 or 3 mm from the center to provide the central and midperipheral RGC densities, respectively. We chose eight areas randomly of 38,250  $\mu$ m<sup>2</sup> (225  $\times$  170  $\mu$ m) each in the central (more than 40% of the central area) and eight areas randomly of 38,250  $\mu$ m<sup>2</sup> each in the midperipheral (more than 30% of the midperiphery) regions of each retina, and their averages were taken as the mean density of RGCs per retina ( $n = 6$  rats in each group). The survival percentage of RGC was defined as the number of RGCs in each treatment group divided by the number of RGCs in the sham-operated retinas, multiplied by 100.

### Flash Visual-Evoked Potentials

The detailed procedures of FVEP have been described in our previous reports.<sup>22–24</sup> In brief, we used a visual electrodiagnostic system (UTAS-E3000; LKC Technologies, Inc., Gaithersburg, MD, USA) to measure the FVEP. After 10 minutes of light adaptation, we performed photopic FVEP based on the report showing no significant differences of latency between photopic and scotopic visual-evoked potentials (VEP) in Wistar rats.<sup>26</sup> The settings were background illumination off, a flash intensity of ganzfeld at 0 db, single flash with the flashlight set to 3.0 scot cd/m<sup>2</sup>, flash rate of 1.9 Hz, test average at 80 sweeps, threshold for rejecting artifacts at 150  $\mu$ V, and a sample rate of 2000 Hz. To exclude the possibility that the contralateral fellow eye contaminated the FVEP test in the other eye during stimulation, we performed the same procedures in both eyes of the rats that received FVEP examinations. This specific consideration was approved by the Institutional Animal Care and Use Committee of Tzu Chi Medical Center. We covered the fellow eye as performing test.

We compared the latency and amplitude of the first positive going wavelet (P1) of FVEP between the two groups ( $n = 6$  rats in each group).

### In Situ TdT-dUTP Nick End Labeling (TUNEL) Assay

To ensure the use of equivalent fields for comparisons, all retina paraffin or frozen sections were prepared with retinas at 1- to 2-mm distance from the ON head. Reactions of TUNEL (DeadEnd Fluorometric TUNEL System; Promega Corp., Madison, WI, USA) were performed to detect apoptotic cells. The cells that were TUNEL positive in the RGC layer of each sample were counted in 10 HPFs ( $\times 400$ ), and three sections per retina were averaged ( $n = 6$  rats in each group).

### Immunohistochemistry of ED1 in the ONs

Antibody ED1 reacts against both extrinsic macrophages and intrinsic microglia.<sup>27</sup> Briefly, immunohistochemistry of ED1 (CD68) using a monoclonal antibody (1:50; AbD Serotec, Oxford, UK) was performed. A secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was then applied at room temperature for 1 hour. Counterstaining was performed using 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma-Aldrich Corp.). For comparison, the ED1-positive cells were counted in 10 HPFs at the ON lesion site ( $n = 6$  rats in each group).

### Western Blotting for VEGF

Retinal protein samples containing 30  $\mu$ g protein were separated on 12% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated in Tris-buffered saline and Tween 20 ([TBST], 0.02-M Tris base, pH 7.6, 0.8% NaCl, 0.1% Tween 20) supplemented with 5% dry skimmed milk for 60 minutes to block nonspecific binding. After rinsing with TBST buffer, the samples were incubated with primary antibodies to VEGF (1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:1000; Sigma-Aldrich Corp.) at 4°C overnight. The membranes were washed twice with TBST buffer followed by incubation with Biotin horse-radish peroxidase-conjugated appropriate rabbit anti-mouse IgG secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories) at room temperature for 1 hour. The blots were then washed with TBST. The specific immune complexes were detected by ECL plus Western blotting reagents (GE; RPN2232;  $n = 3$  in each group).

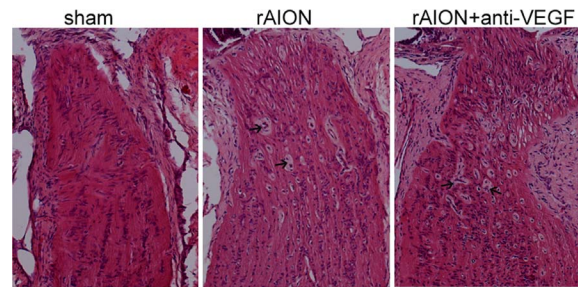
### Statistical Analysis

All measurements were performed in a masked fashion. Statistical analysis was performed with commercial software (IBM SPSS Statistics 19; International Business Machine Corp., Armonk, NY, USA). We used Kruskal-Wallis test and Mann-Whitney  $U$  test to evaluate differences between the groups in terms of the number of cells. A value of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Histology of ON Photographs After rAION

In the sections of ON, rAION-induced disruption of the regular cellular columns, vacuolation of myelinated axons, and accumulation of inflammatory cells in the PBS-treated rats were noted 4 weeks after rAION induction. However, these



**FIGURE 1.** Representative images of the histology of optic nerve sections at 4 weeks after rAION induction and anti-VEGF treatment. In the ONs, rAION-induced disruption of the cellular columns, vacuolation of myelinated axons (*arrows*), and accumulation of inflammatory cells in the PBS-treated rats were noted 4 weeks after rAION induction; however, these changes were not improved after anti-VEGF treatment (hematoxylin-eosin staining;  $n = 6$  in each group).

effects were not improved in the anti-VEGF-treated rats ( $n = 6$  in each group; Fig. 1).

### Morphometry of the RGCs

The densities of RGCs in the laser-controlled eyes (sham group) showed no significant difference with the normal rats ( $P > 0.05$ , data not shown). The densities of RGCs in the central and the midperipheral retinas in the sham-operated rats were  $2230 \pm 230/\text{mm}^2$  (95% CI [1990, 2480]) and  $1280 \pm 150/\text{mm}^2$  (95% CI [1110, 1440]), respectively. Four weeks after induction of rAION, the RGC densities of the central and midperipheral retinas in the PBS-treated group were  $890 \pm 250/\text{mm}^2$  (95% CI [660, 1150]) and  $430 \pm 90/\text{mm}^2$  (95% CI [340, 530]);  $P = 0.004$  and  $0.004$ , respectively;  $n = 6$  in each group). In the anti-VEGF-treated group, the RGC densities of the central and midperipheral retinas were  $1260 \pm 380/\text{mm}^2$  (95% CI [870, 1620]) and  $510 \pm 320/\text{mm}^2$  (95% CI [240, 770]), respectively ( $P = 0.115$  with Cohen's  $d$  effect size 1.13 and  $P = 0.606$  with Cohen's  $d$  effect size of 0.34, respectively, compared with the PBS-treated group;  $n = 6$  in each group; Fig. 2). The survival rate of RGCs in the central and midperipheral retinas failed to show statistically significant differences between anti-VEGF-treated and PBS-treated rats after rAION (57.0% vs. 40.0% in the central retina; 39.8% vs. 33.6% in midperipheral retina, respectively; both  $P > 0.05$ ).

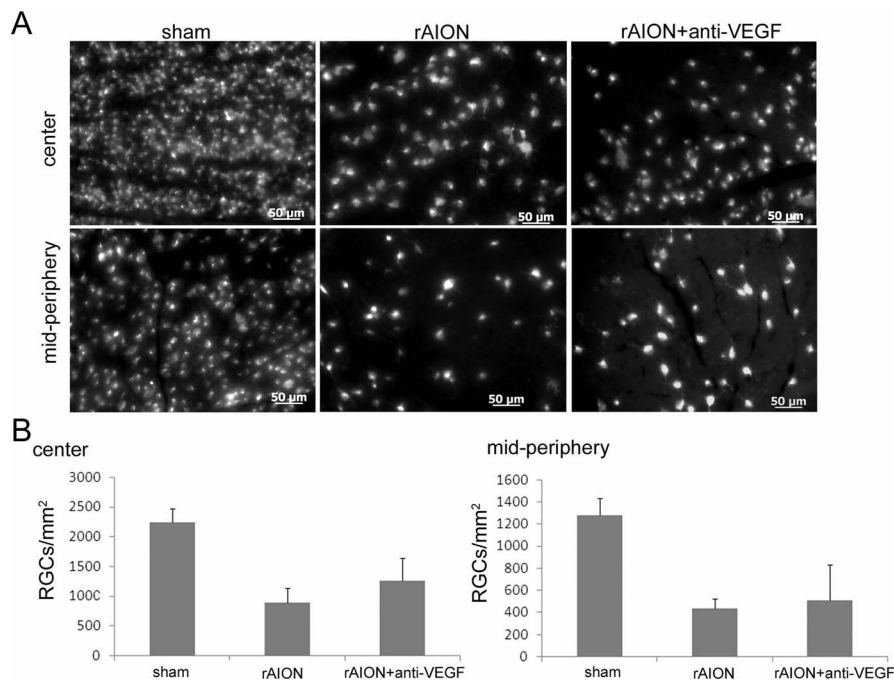
### FVEP

The latencies of the P1 wave 4 weeks after the induction of rAION were  $73 \pm 4$  ms (95% CI [69, 77]),  $142 \pm 14$  ms (95% CI [131, 151]), and  $131 \pm 15$  ms (95% CI [113, 141]) for the sham, PBS-treated, and anti-VEGF-treated rats, respectively ( $P = 0.157$  with Cohen's  $d$  effect size 1.19 versus PBS-treated rats;  $P = 0.004$  versus sham group). The amplitudes of the P1 wave were  $70 \pm 19$   $\mu$ V (95% CI [58, 82]);  $41 \pm 13$   $\mu$ V (95% CI [30, 52]); and  $34 \pm 12$   $\mu$ V (95% CI [14, 49]) for the sham, PBS-treated, and anti-VEGF-treated rats, respectively ( $P = 0.423$  with Cohen's  $d$  effect size of 0.64 versus PBS-treated rats;  $P = 0.001$  versus sham group). Our data failed to show statistically significant differences on FVEP results between PBS-treated and anti-VEGF-treated groups ( $n = 6$  in each group; Fig. 3).

### TUNEL Assay in the Retinas

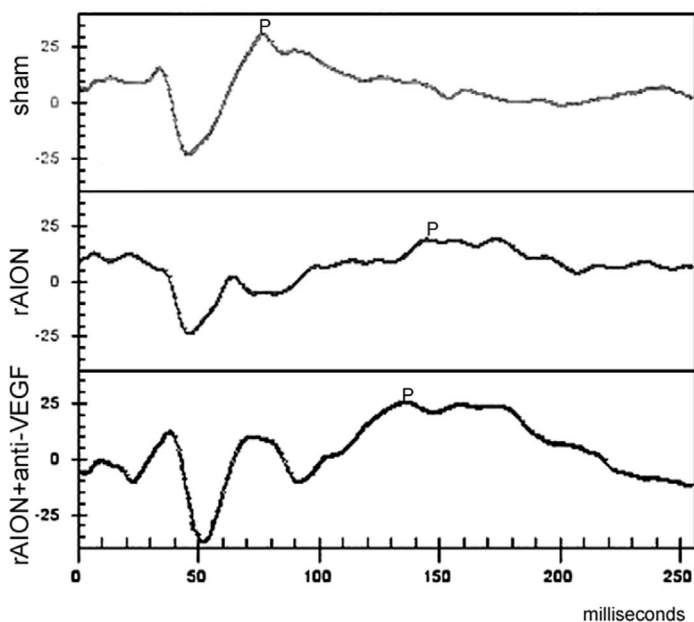
Assays of TUNEL of the frozen sections of the retinas demonstrated that there were  $1.1 \pm 1.0$  positive cells/HPF (95% CI [0, 1.9]) in the RGC layer of retinas in the sham-operated rats,  $7.8 \pm 1.3$  positive cells/HPF (95% CI [6.9, 9.1])



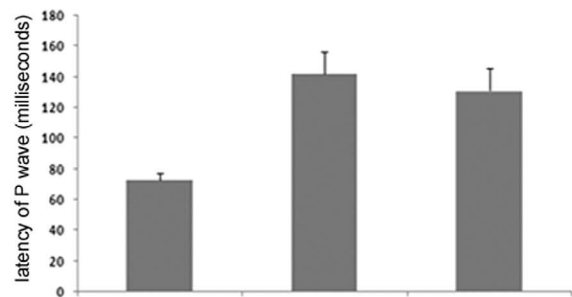


**FIGURE 2.** Flat preparations of retinas and morphometry of RGCs by FluoroGold retrograde labeling at 4 weeks after rAION induction. **(A)** Representative flat preparation of central and midperipheral retinas in normal rats (*left*), rAION with PBS (*middle*), and rAION with anti-VEGF-treated rats. **(B)** Morphometry of RGCs in the central and midperipheral retinas. Densities of RGC in the central and midperipheral retinas of the sham group were  $2230 \pm 230/\text{mm}^2$  and  $1280 \pm 150/\text{mm}^2$ , respectively. Four weeks after rAION induction, the RGC densities in the PBS-treated group were  $890 \pm 250/\text{mm}^2$  and  $430 \pm 90/\text{mm}^2$ , respectively ( $P = 0.004$  and  $0.004$ , respectively). The densities of RGC of the central and midperipheral retinas in the anti-VEGF-treated group were  $1260 \pm 380/\text{mm}^2$  and  $510 \pm 320/\text{mm}^2$ , respectively ( $P = 0.003$  and  $0.002$ , respectively, compared with the sham group;  $P = 0.115$  and  $0.606$ , respectively, compared with the PBS-treated group;  $n = 6$  in each group).

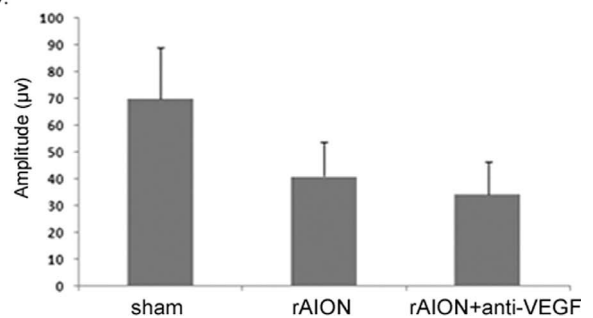
**A. Flash VEP**



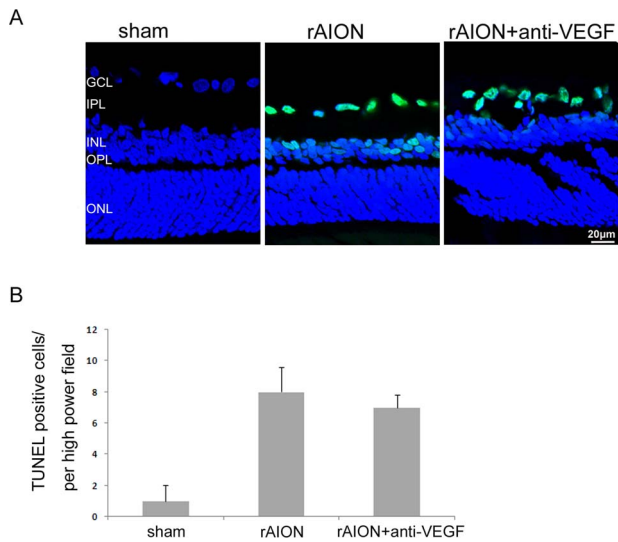
**B.**



**C.**

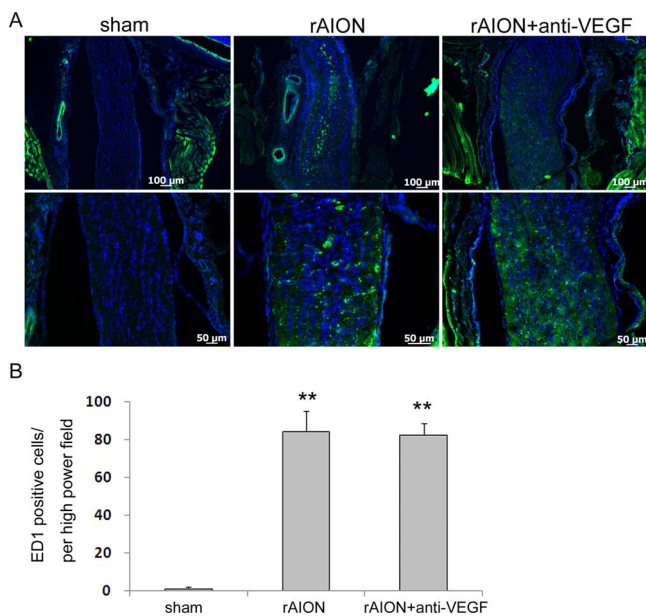


**FIGURE 3.** Photopic FVEPs. **(A)** Representative FVEP tracings at 4 weeks after rAION induction. **(B)** The latencies of the P1 wave were  $73 \pm 4$  ms,  $142 \pm 14$  ms, and  $131 \pm 15$  ms in the sham, PBS-treated, and anti-VEGF-treated rats, respectively ( $P = 0.157$  versus PBS-treated rats;  $P = 0.004$  versus sham group). **(C)** The amplitudes of the P1 wave were  $70 \pm 19$  μV,  $41 \pm 13$  μV, and  $34 \pm 12$  μV in the sham, PBS-treated, and anti-VEGF-treated rats, respectively ( $P = 0.423$  versus PBS-treated rats,  $P = 0.001$  versus sham group;  $n = 6$  in each group).

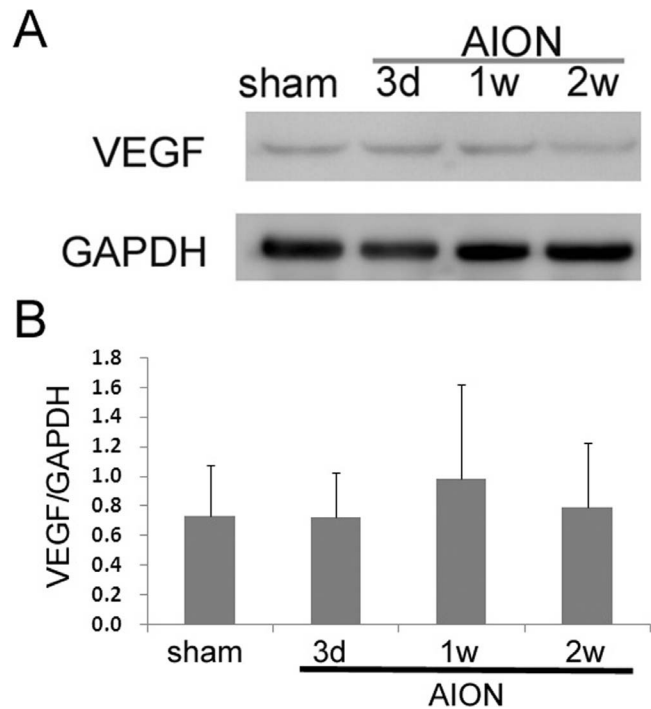


**FIGURE 4.** Retinal Section TUNEL Assays. **(A)** The TUNEL assay of the retinas among the three groups. **(B)** There were  $1.1 \pm 1.0$  positive cells/HPF in the RGC layer of the retinas in the sham-operated rats,  $7.8 \pm 1.3$  positive cells/HPF in the PBS-treated group, and  $7.0 \pm 0.8$  in the anti-VEGF-treated rats ( $P = 0.275$  versus PBS-treated group,  $P = 0.013$  versus sham group;  $n = 6$  in each group). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

in the PBS-treated group, and  $7.0 \pm 0.8$  (95% CI [5.9, 8.1]) in the anti-VEGF-treated rats ( $P = 0.275$  with Cohen's  $d$  effect size = 0.75 versus PBS-treated group;  $P = 0.013$  versus sham group;  $n = 6$  in each group; Fig. 4). Our data failed to show statistically significant differences on antiapoptotic effect on RGCs after the induction of rAION between PBS-treated group and anti-VEGF-treated groups.



**FIGURE 5.** (A) Persistent infiltration of ED1-positive-stained cells in the ONs after anti-VEGF treatment. **(B)** At the fourth week post rAION, ED1+ cells were prominent at the ON lesion sites in the PBS-treated group ( $84 \pm 11$  cells/HPF). The number of ED1+ cells was not statistically significantly decreased at the ON lesion sites in the anti-VEGF treated groups ( $82 \pm 6$  cells/HPF,  $P = 0.675$  versus PBS-treated group;  $n = 6$  in each group; \*\* $P < 0.01$  versus sham group). Scale bars: 100  $\mu$ m ([A], top row), 50  $\mu$ m ([A], bottom row).



**FIGURE 6.** Western blot analysis of VEGF expression of the rat retinas after AION. **(A)** Representative photograph of the Western blot analysis. **(B)** The ratio of VEGF/GAPDH was  $0.73 \pm 0.34$  in sham group and  $0.72 \pm 0.31$ ,  $0.98 \pm 0.64$ , and  $0.79 \pm 0.44$  in the AION-treated rats at 3 days, 1 week, and 2 weeks, respectively. We did not observe statistically significant changes in VEGF between the sham and AION groups at 3 days, 1 week, and 2 weeks (all  $P > 0.5$ ;  $n = 3$  in each group).

### ED1 in the ONs

Immunohistochemistry of ED1 showed few ED1+ cells in the sham group  $1 \pm 1$  cells/HPF (95% CI [0, 1]). However, a prominent number of ED1+ cells infiltrating into the ONs were observed in the PBS-treated group  $84 \pm 11$  cells/HPF (95% CI [73, 95]) at the fourth week post infarct. In the anti-VEGF treatment group,  $82 \pm 6$  cells/HPF (95% CI [75, 85]) were noted in the ONs (Fig. 5). We did not observe statistically significant differences of the number of ED1+ cells between anti-VEGF and PBS groups ( $n = 6$  in each group;  $P = 0.675$  with Cohen's  $d$  effect size of 0.47).

### Western Blot Analysis of VEGF

Retinal VEGF expression was not altered in the sham-operated retinas or the retinas of the AION rats at 3 days, 1 week, and 2 weeks. The ratio of VEGF/GAPDH was  $0.73 \pm 0.34$  (95% CI [0.19, 1.28]) in the sham group; and  $0.72 \pm 0.31$  (95% CI [0.23, 1.21]),  $0.98 \pm 0.64$  (95% CI [-0.03, 1.99]), and  $0.79 \pm 0.44$  (95% CI [0.10, 1.48]) in the AION-treated rats at 3 days, 1 week, and 2 weeks, respectively (all  $P > 0.5$ , Cohen's  $d$  effect size = 0.1–0.5; Fig. 6). We did not observe statistically significant differences of VEGF expression in the retinas between anti-VEGF and PBS groups after the induction of rAION.

### DISCUSSION

The results of the current study demonstrate that intravitreal injections of anti-VEGF did not have an anti-inflammatory effect

on ONs or a rescuing effect on RGCs after the induction of rAION.

It has been reported that some patients develop NAION within 4 to 60 days after intravitreal injections of anti-VEGF agents, and a transient rise in intraocular pressure after the injection has been postulated to be a possible mechanism.<sup>28</sup> In our model, we used a 33-G needle with a 10- $\mu$ L syringe (Hamilton Co.) to prevent trauma or IOP fluctuations from the injections. The average IOP the day after the injection of the anti-VEGF agent was 9.8 mm Hg, which was not significantly different to the IOP of the sham group (9.3 mm Hg) in our study. However, we did not note secondary IOP elevations after the intravitreal injections.

In an ischemia/reperfusion brain model, reduced tissue oxygen tension was reported to trigger VEGF expression and increase protein and mRNA levels of VEGF and its receptors (Flt-1, Flk-1/KDR), resulting in brain edema.<sup>29</sup> Antagonism of VEGF has been shown to reduce ischemia/reperfusion-related brain edema and injury, implicating VEGF in the pathogenesis of stroke and related disorders.<sup>30,31</sup> However, in a rat model of focal cerebral embolic ischemia, the systemic administration of VEGF after a longer period (after 48 hours) was found to markedly enhance angiogenesis in ischemic brains and reduce neurologic deficits during stroke recovery.<sup>32</sup> Vascular endothelial growth factor is known to have protective and antiapoptotic effects on brain neurons in hypoxic conditions through lessening caspase-3 activation and antiexcitotoxic effects, and it has been implicated to be an endogenous neuroprotective factor.<sup>33-36</sup>

In our rAION model, immediate anti-VEGF intravitreal injections did not significantly rescue RGCs after ischemic optic neuropathy insults compared with the results of an ON crush model.<sup>18</sup> The dose and type of anti-VEGF agent were different between the two studies (the present study: ranibizumab 20  $\mu$ g/2  $\mu$ L; Rappoport's study: bevacizumab 75  $\mu$ g/3  $\mu$ L). In addition, the time course of RGC death has been reported to be longer in an rAION model (2-3 weeks) than in a crush model (7 days), and this delay in RGC death in rAION suggests that a potential treatment window does exist for anti-VEGF therapy.<sup>37,38</sup> In retina ischemia/reperfusion models, anti-VEGF treatment has been proven to inhibit vascular permeability without effecting apoptosis or cell degeneration.<sup>39</sup> In a model of ON axotomy, VEGF was found to protect neurodegeneration of RGCs via activating ERK-1/2 and Akt pathways.<sup>14</sup> To date, the therapeutic role of anti-VEGF agents in an ischemic ON model is uncertain. In the Western blot analysis of retinas after rAION, no significant changes of VEGF level were noted in our results, which is similar to a study that reported minimal changes in VEGF mRNA levels in the retinas of mice at 1, 3, and 21 days following rAION induction.<sup>40</sup> These observations imply that an rAION model may not involve angiogenesis and that VEGF plays a minor role in rAION.

We previously reported that recombinant human granulocyte colony-stimulating factor has dual actions of antiapoptosis for RGC survival and anti-inflammation in ONs in an rAION model.<sup>24</sup> Granulocyte colony-stimulating factor has been shown to stimulate neurogenesis in adult rat brains via the actions of VEGF and signal transducer and activator of transcription activation.<sup>41</sup> Vascular endothelial growth factor-A165a (VEGF-A165a) has been shown to modulate inflammatory pathways, resulting in upregulation of intercellular adhesion molecule 1 (ICAM-1) in retinal vascular endothelial cells.<sup>42</sup> Vascular endothelial growth factor-A165b has also been shown to inhibit tumor necrosis factor- $\alpha$ -mediated upregulation of ICAM-1 expression and increase monocyte-retinal pigment epithelium adhesion, suggesting an anti-inflammatory property of VEGF-A165b in the eye.<sup>43</sup> Extrinsic macrophages (blood-borne macrophages, which invade the damaged tissue)

have been reported to be identifiable 3 days post induction in ONs by the presence of ED1 (CD68)-positive macrophages/microglia, indicating significant blood-brain barrier disruption.<sup>25</sup> In the current study, inflammatory activity in the ONs did not show statistically significant decrease after anti-VEGF treatment as evidenced by persistent infiltration of ED1+ cells, suggesting that intravitreal injections of anti-VEGF agents have no anti-inflammatory effect on ONs in rAION. The histologic studies of the ONs also demonstrated that inflammatory cell infiltration and vacuolization of axons were not changed by anti-VEGF treatment. To ensure that the dose of anti-VEGF agent in our model was sufficient, it was no less than used in clinical practice.<sup>16</sup> Visual functional evaluation by FVEP did not show statistically significant improvements in the amplitude and latency of the P1 wavelet after intravitreal injection of anti-VEGF agents in our rAION model.

In conclusion, we demonstrated that early intravitreal injections of an anti-VEGF agent did not have statistically significant protective effects on RGCs and ONs in our rAION model, as evidenced by RGC morphometry, TUNEL apoptotic assay, inflammation of the ONs, and functional assessment of FVEP.

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