

Efficacy of Intravitreal Injections of Triamcinolone Acetonide in a Rodent Model of Nonarteritic Anterior Ischemic Optic Neuropathy

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PURPOSE. To investigate effects of intravitreal injections of triamcinolone acetonide (IVI-TA) at different times in a rodent model of nonarteritic anterior ischemic optic neuropathy (rAION).

METHODS. After inducing ischemic optic neuropathy, the rats received either IVI-TA (0.32 mg/2 μ L) at 1 day (1d-TA), 1 week (7d-TA), 2 weeks (14d-TA), or PBS. The density of retinal ganglion cells (RGCs) was calculated using retrograde Fluorogold labeling. Electrophysiological visual function was assessed by flash visual evoked potentials (FVEPs). Apoptosis assays of the retinal sections and immunohistochemistry of ED1 staining of the optic nerves were performed.

RESULTS. Four weeks postinfarct, the 1d- and 7d-TA groups had significantly rescued RGCs in the central (2160 ± 250 mm², $P = 0.004$; 2050 ± 660 , $P = 0.008$, respectively) and midperipheral retinas (1240 ± 130 ; 1250 ± 220 , respectively, both $P = 0.004$) compared with those of the PBS-treated rats. Flash visual evoked potentials demonstrated improvements in P1 amplitude (μ V) in the 1d- and 7d-TA groups (both $P < 0.05$). Assays of TUNEL showed a decrease in the number of apoptotic cells in the RGC layers of 1d- and 7d-TA-treated retinas compared with the PBS-treated group (both $P = 0.004$). Cells ED1-positive were significantly decreased in the optic nerve (ON) of the 1d- and 7d-TA groups compared with the PBS group ($P = 0.004$ and 0.02 , respectively).

CONCLUSIONS. Within 1 week postinfarct of rAION, IVI-TA had neuroprotective effects on RGC survival with an increase in the electrophysiological amplitude of VEPs and a decrease in microglial infiltration in the ONs.

Keywords: rodent model of nonarteritic anterior ischemic optic neuropathy, triamcinolone acetonide, retinal ganglion cell, visual-evoked potentials, neuroprotection

Nonarteritic anterior ischemic optic neuropathy (NA-AION) is a multifactorial disease and the most common acute optic neuropathy in people aged older than 50 years.¹ In rat models of anterior ischemic optic neuropathy (rAION), superoxide radicals generated by a photochemical reaction have been shown to result in nonthermal optic nerve (ON) damage and secondary loss of retinal ganglion cells (RGCs).^{2,3} Although a large retrospective histopathologic review of 193 eyes with presumed ischemic optic neuropathy was performed by Knox et al.,⁴ no specific immunohistochemical staining methods were used to assess inflammation. However, an important study on a patient who died shortly after developing NA-AION showed that inflammation was a prominent feature of early human NA-AION.^{5,6} In addition, Bernstein and Miller⁷ reported that different methods of inducing AION and variations in vascular anatomy of the ON among different

models may result in variable speeds of resolution of disc edema postinfarct.

Similar to other central nerve system ischemic infarcts, ON ischemia results in the early recruitment of extrinsic macrophages to the core of the ischemic infarct.⁸ Manipulation of the inflammatory response has been suggested to improve visual outcomes in rAION.⁸ Systemic methylprednisolone administration was shown to be effective in quickly reducing disc edema instead of increasing RGC survival and the amplitude of visual evoked potentials (VEPs) compared with control groups in a rAION model,⁹ and we previously reported that systemic methylprednisolone administration has protective effects on RGC survival in rAION.¹⁰ Systemic glucocorticoid treatment decreases tissue edema by increasing the expressions of tight junction genes (occludin and cadherin-9) in retinal endothelium as well as reducing VEGF and TNF- α .¹¹⁻¹³ A prospective study showed that systemic steroid treatment was effective in



patients with acute NA-AION in whom the initial visual acuity was 20/70 or worse.¹⁴ The complications of systemic steroid use in this large study were minor or easily manageable if precautionary measures were taken.¹⁵ On the other hand, a report of 10 acute NA-AION eyes showed that systemic steroid administration did not have a rescue effect but caused serious complications in 3 of the 10 treated patients.¹⁶ The complications of systemic steroid use are a concern in clinical practice and especially when treating NA-AION due to reports of comorbidities.¹⁷⁻¹⁹ Local treatment such as intravitreal injection of triamcinolone acetonide (IVI-TA) delivers higher concentrations of the drug to the targeted tissue and thereby potentially increases the therapeutic response without causing serious complications compared with systemic administration.

In our previous retrospective case series study, we reviewed six patients with NA-AION who were treated with a single IVI-TA, and found that 50% of the patients had better final visual acuity and 15% had an improved visual field. The average time of treatment was 4.6 weeks after the onset of symptoms.¹⁸ Better improvements in visual acuity and visual field after IVI-TA in acute NA-AION were also reported in a randomized, controlled study.¹⁹ On the other hand, one case series showed that IVI-TA was not markedly effective in increasing visual acuity in three patients with acute NA-AION.¹⁷ Because of confounding factors such as the severity of initial ischemia of the optic nerve, comorbidities of the patients and timing of treatment, the benefits of IVI-TA for acute NA-AION are inconclusive and controversial. The therapeutic window of IVI-TA may be one of the factors determining the therapeutic effects in clinical practice.¹⁹⁻²¹ Therefore, in this study, we investigated the effect and potential therapeutic window of IVI-TA in a rAION model.

MATERIALS AND METHODS

Animals

A total of 80 adult male Wistar rats weighing 150 to 180 g (aged 7-8 weeks) were used in this study. The rats were obtained from the breeding colony of BioLASCO Co. (Taiwan, China). 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 of Tzu Chi Medical Center approved all animal experiments.

Study Design

The details of rAION induction were the same as in our previous reports.^{10,22,23} All inductions were performed after anesthetization. Following AION induction, 64 animals were randomized into those receiving treatment in the right eyes with one injection of TA (triamcinolone suspended in 40 mg/mL/amp, Taiyu Pharmaceutical Co., Taiwan, China; $n = 48$) or PBS alone ($n = 16$). The other 16 rats received sham laser treatment without photosensitizing agents. All of the animals tolerated this treatment without any complications, and all of the rats survived to the end of treatment. We separated the TA-treated group into three different treatment times: 1, 7, and 14 days after infarct (1d-, 7d-, and 14d-TA groups) and gave the right eye of each rat 2 μ L of TA at a concentration of 0.16 mg/ μ L (40 mg/250 μ L, after centrifugation with the supernatant being discarded). Based upon a previous report that the vitreous volume is approximately 56 μ L, the chosen dosage of 0.32 mg of TA for each eye was equal to 5.7 mg/mL.²⁴ Such a dose has been shown to prevent insufficient dosing without toxicity.^{24,25} In the control group, the right eye of each rat was

injected intravitreally with PBS (2 μ L per injection) after the AION experiments. All rats had normal intraocular pressure after intravitreal treatment. The rats were euthanized at 4 weeks postinfarct by CO₂ insufflation. The details of the intravitreal injections were reported in our previous study.²⁶

Neuroprotection Studies

1. Retrograde Labeling of RGCs With Fluorogold. We described the detailed procedures of Fluorogold labeling in our previous reports.^{10,22,23} We masked the groups in counting number of RGCs. The percentage of RGCs that survived 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.

2. Flash Visual Evoked Potentials (FVEPs). All induction was performed after anesthetization. We also masked the groups in assessing FVEP. The settings of FVEPs were based on previous reports with some modifications,^{2,27,28} including no background illumination, a flash intensity of Ganzfeld 0 dB, a single flash with a flash rate of 1.9 Hz, flash intensity of 3 scot cd/m², test average at 80 sweeps, threshold for rejecting artifacts at 50 mV, and a sample rate of 2000 Hz. When the wave was nonrecordable, the latency of P₁ was set at 200 ms for comparison. The amplitudes of P₁ for each VEP wave within the initial 100-ms interval were determined and used for amplitude analysis (amplitude of P₁ = amplitude of P₁ - amplitude of N₂).²⁹ The general characteristic of the FVEPs in rAION is a reduction in amplitude.² Therefore, we recorded the amplitude of P₁ only in FVEPs after the induction of rAION. The results were associated with the effects of axon preservation of visual pathways without or without treatment.

3. In Situ TUNEL Assay. To ensure the use of equivalent fields for comparison, all retinal sections (either in paraffin or frozen) were prepared with the 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 TUNEL-positive cells in the RGC layer of each sample were counted in 10 high-powered fields (HPF, $\times 400$), and three sections per retina were averaged.

4. Immunohistochemistry of ED1 (CD68) in the ONs. Longitudinal sections of ONs were stained with hematoxylin-eosin for morphologic evaluation. Antibodies ED1 react against extrinsic macrophages and intrinsic microglia.³⁰ Immunohistochemistry of ED1 using a monoclonal antibody (1:50; AbD Serotec, Oxford, UK) following the protocol of the manufacturer was performed.²⁶ For comparison, ED1-positive cells were counted in six HPF at the ON lesion site.

Statistical Analysis

All measurements were performed in a masked fashion. Statistical analysis was performed with commercial software (IBM SPSS Statistics, version 19; IBM Corp., Armonk, NY, USA). Data are presented as means \pm SD. We used the 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

Protection of RGCs Within 1 Week of IVI-TA Treatment

The densities of RGCs in the central and midperipheral retinas in the laser-controlled eyes (sham) were $2230 \pm 250/$

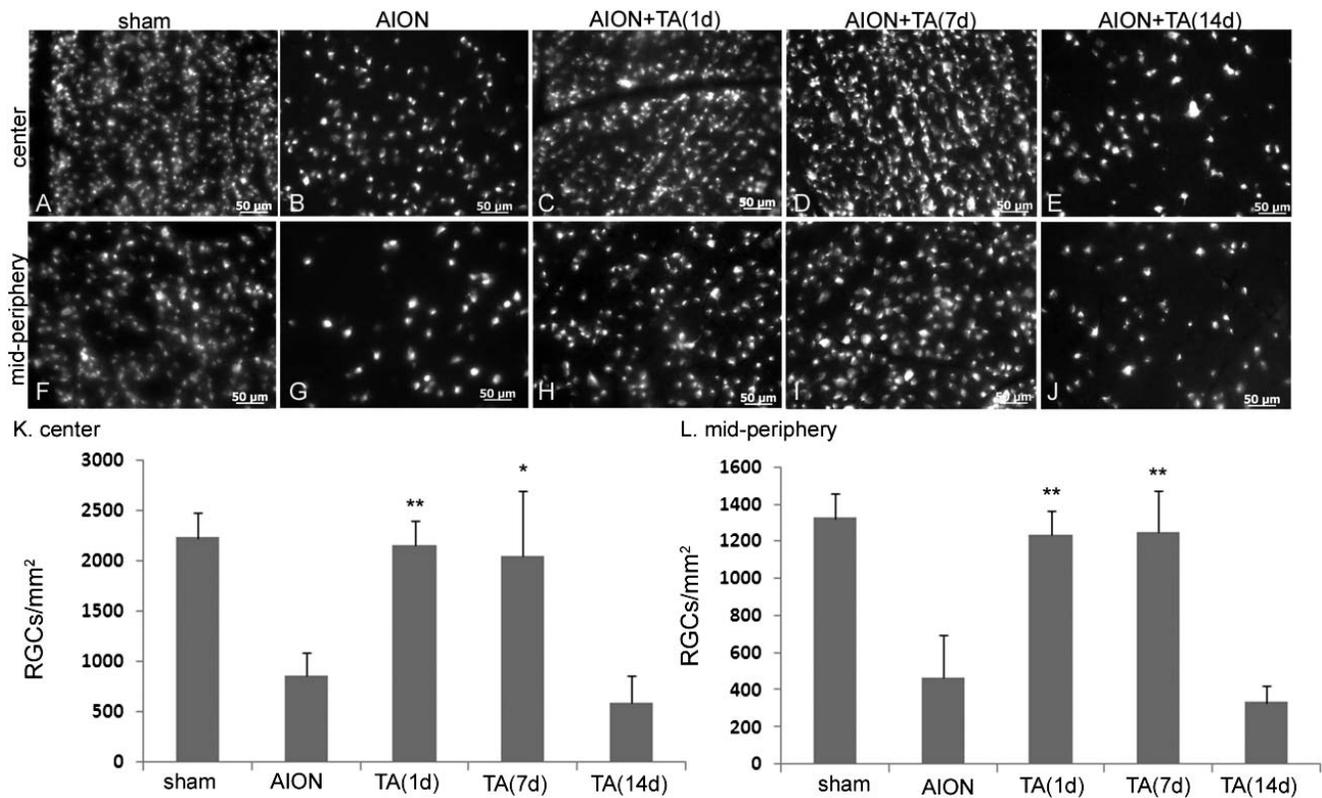


FIGURE 1. Improvement in RGC density (mm^2 ; mean \pm SD) in the retinas after IVI-TA treatment within 1 week. (A, F) In the sham group, the densities of RGCs were 2230 ± 250 and 1330 ± 140 in the central and midperipheral retinas, respectively. (B, G) In both central and midperipheral retinas, RGC densities were significantly decreased in the PBS-treated group (center 860 ± 230 and midperiphery 470 ± 230 , respectively). (C, H) In the 1d-TA group, the densities of RGCs in the central and midperipheral retina increased to 2150 ± 250 and 1240 ± 130 , respectively (both $**P < 0.005$) compared with the PBS-treated group. (D, I) In the 7d-TA group, the RGC densities increased to 2050 ± 660 and 1250 ± 220 , respectively, in the central and midperipheral retinas ($*P < 0.05$ and $**P < 0.005$) compared with the PBS-treated group. (E, J) In the 14d-TA group, the RGC densities decreased to 600 ± 260 and 330 ± 90 , respectively, in the central and midperipheral retinas (both $P > 0.05$) compared with the PBS-treated group ($n = 6$ in each group; Scale bar: $50 \mu\text{m}$).

mm^2 and $1330 \pm 140/\text{mm}^2$, respectively (Figs. 1A, 1F). The densities of the RGCs in the central and midperipheral retinas in the laser-controlled eyes with IVI-TA were $2210 \pm 290/\text{mm}^2$ and $1320 \pm 190/\text{mm}^2$, respectively. These results showed that there was no toxicity to the RGCs after IVI-TA treatment ($P > 0.05$).

Four weeks postinfarct, the central and midperipheral RGC densities in the PBS-treated group decreased to $860 \pm 230/\text{mm}^2$ and $470 \pm 320/\text{mm}^2$, respectively (Figs. 1B, 1G). In the 1d-TA group, the RGC densities increased to $2160 \pm 250/\text{mm}^2$ and $1240 \pm 130/\text{mm}^2$ in the central and midperipheral retinas, respectively (Figs. 1C, 1H). In the 7d-TA group, the RGC densities increased to $2050 \pm 660/\text{mm}^2$ and $1250 \pm 220/\text{mm}^2$ in the central and midperipheral retinas, respectively (Figs. 1D, 1I), compared with $600 \pm 260/\text{mm}^2$ and $330 \pm 90/\text{mm}^2$ in the 14d-TA group, respectively (Figs. 1E, 1J). There were significant differences in RGC densities in the TA-treated groups in both central and midperipheral retinas within 1 week of treatment compared with the PBS-treated group ($n = 6$ in each group, all $P < 0.05$). However, there were no significant differences in RGC densities after 2 weeks of treatment compared with the PBS-treated group ($n = 6$ in each group, $P = 0.15$ and 0.36 , respectively). In the central retinas, the survival rates in the 1d-, 7d-, and 14d-TA and PBS groups were 96.9%, 91.9%, 26.9%, and 38.6%, respectively, compared with 3.2%, 94.0%, 24.8%, and 35.3%, respectively, in the midperipheral retinas.

Improvement in P₁ Amplitude Within 1 Day of IVI-TA Treatment

Changes in FVEP after the induction of rAION were measured 4 weeks after infarct. In the sham group, the amplitude of the P₁ wavelet was $81 \pm 11 \mu\text{V}$. In the PBS-treated group, the amplitude of the P₁ decreased to $8 \pm 1 \mu\text{V}$. The amplitudes of P₁ in the 1d-, 7d-, and 14d-TA groups were $44 \pm 12 \mu\text{V}$, $14 \pm 3 \mu\text{V}$, and $8 \pm 4 \mu\text{V}$, respectively (Fig. 2). There were significant increases in the amplitudes of the P₁ wavelet in the 1d- and 7d-TA groups compared with the PBS-treated group ($P = 0.014$ and 0.009 , respectively, $n = 6$ in each group). However, there was no significant difference in the amplitude of P₁ between the 14d-TA and PBS-treated groups ($P = 0.9$, $n = 6$ in each group).

Decreased Number of Apoptotic Cells in the RGC Layer Within 1 Week of IVI-TA Treatment

Assays of TUNEL demonstrated that there were 11.5 ± 1.9 positive cells/HPF in the RGC layer in the PBS-treated group, compared with 2.5 ± 1.4 cells, 3.0 ± 0.9 , and 12.0 ± 3.4 cells, respectively, in the 1d-, 7d-, and 14d-TA groups (Fig. 3). The number of apoptotic cells decreased in the 1d- and 7d-TA groups compared with the PBS-treated group ($n = 6$ in each group, all $P = 0.004$). There was no significant difference in the percentage of apoptotic cells between the 14d-TA and PBS-treated groups ($P = 0.8$, $n = 6$ in each group).

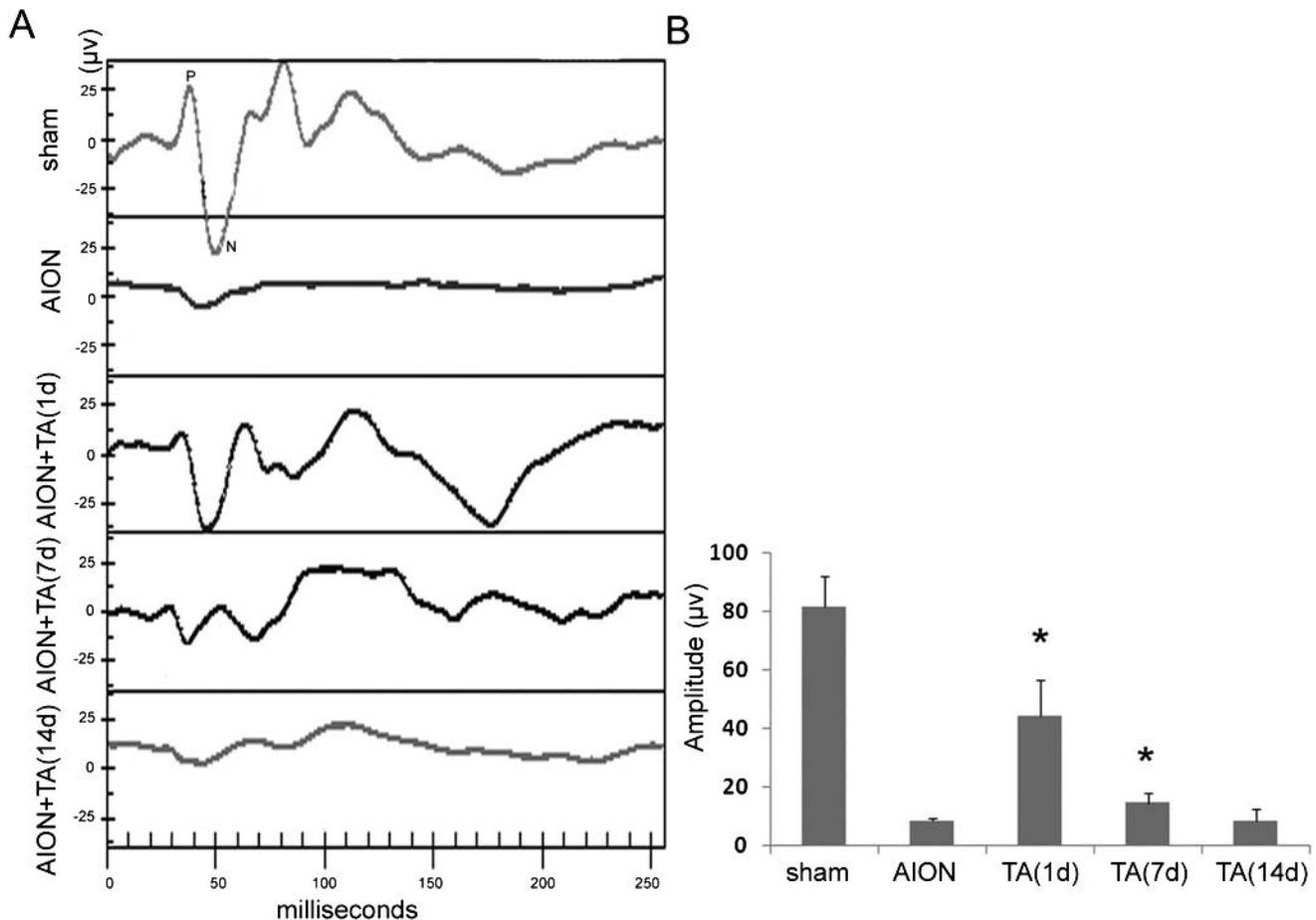


FIGURE 2. (A) Improvement in the amplitude of P₁ (mean \pm SD) in FVEPs after TA treatment within 1 week compared with later treatment. All amplitudes of FVEP were a composite from the mean of waves in testing rats. (B) Four weeks post rAION, the amplitudes of P₁ in the 1d-, 7d-, and 14d-TA groups were 44 ± 12 μ V, 14 ± 3 μ V, and 8 ± 4 μ V, respectively ($^*P < 0.05$ in the 1d- and 7d-TA groups compared with the PBS-treated group, $n = 6$ in each group).

Decreasing ED1-Positive Cells in the ONs Within 1 Week of IVI-TA Treatment

Immunohistochemistry of ED1 showed few ED1+ cells in the sham group (3 ± 2 cells/HPF), compared with a prominent number of ED1+ cells in the ONs in the PBS-treated group (52 ± 14 cells/HPF) 4 weeks postinfarct. After TA treatment, the numbers of ED1+ cells in the ONs were 15 ± 5 , 28 ± 11 , and 41 ± 8 cells/HPF in the 1d-, 7d-, and 14d-TA groups, respectively (Fig. 4). The differences in the number of ED1+ cells were statistically significant in the 1d- and 7d-TA groups compared with the PBS-treated group ($n = 6$ in each group, $P = 0.004$ and 0.02 , respectively). There was no significant difference in microglial infiltration in the ONs between the 14d-TA and PBS-treated groups ($P = 0.26$, $n = 6$ in each group).

DISCUSSION

In this study, we demonstrated that IVI-TA within 1 week postinfarct in rats had the potential to rescue secondary RGC death, improve electrophysiological visual function and decrease the number of apoptotic cells in the RGC layer and macrophage/microglial infiltration in the ONs. Our results also demonstrate that a therapeutic window for IVI-TA treatment for rAION does exist, and that later treatment (14 days) postinfarct showed no benefits.

Our megadose of local IVI-TA is a safe treatment in rAION. Gao et al.²⁴ used a dose of IVI-TA in a rat model (5.7 mg/mL), and his data indicated that 5.7 mg/mL final concentration of TA in eye does not affect basal VEGF mRNA expression in normal adult rat retina. In addition, we used crystalline corticosteroid instead of vehicle with triamcinolone particle. The evidence of retinal toxicity of triamcinolone's vehicle (benzyl alcohol) was coming from the vehicle, not the crystalline corticosteroid itself.³¹ Other albino rabbit studies using 16.7 to 20 mg/mL of IVI-TA showed no statistically significant differences in rabbits 28 days after the injection both in ERG and immunohistochemistry.^{32,33} We previously reported the results of systemic methylprednisolone treatment in rAION.¹⁰ Comparing the effects of systemic and intravitreal treatment of corticosteroids in rAION, we noted a better protective effect on the number of RGCs with intravitreal treatment and a better antiapoptosis effect on the RGC layer and the amplitude of FVEP recovery. On the other hand, based on the results of ED₁ staining of the ONs, systemic corticosteroid treatment seems to inhibit macrophage infiltration into the ON to a greater extent, and that this is associated with barrier disruption compared with the effect of IVI-TA.

In animal models of AION, a qualitative temporal map of inflammatory cellular expressions of the early invasion of polymorphonuclear leukocytes into the infarct region within 1 week after injury has been reported.^{5,8} In the acute phase,

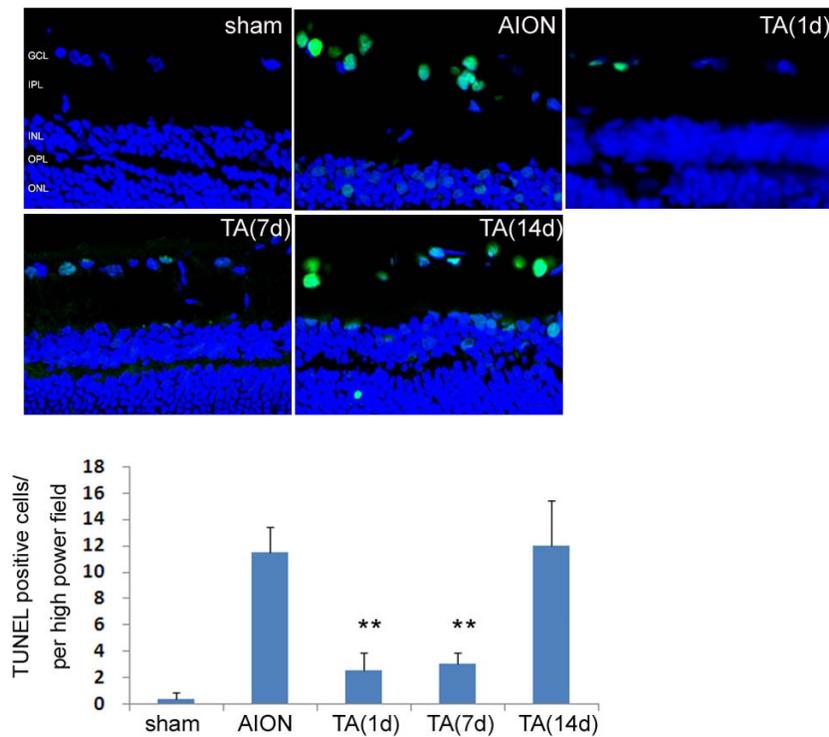


FIGURE 3. Assays of TUNEL revealed a decreased number of apoptotic cell (mean ± SD) after IVI-TA treatment within 1 week. The number of apoptotic cells decreased to 2.5 ± 1.4 cells and 3.0 ± 0.9 cells in the 1d- and 7d-TA groups, respectively, compared with the PBS-treated group (11.5 ± 1.9 cells; both $**P < 0.005$, $n = 6$ in each group; Scale bar: 20 μm). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

cytotoxic edema is rapidly followed by vasogenic edema, which can further damage the ON and surrounding retina.³ Microglial activation has been shown to occur as early as 1 day after ischemia, with a high peak breakdown of the blood-retinal barrier on day 3 in rAION.³⁴ Extrinsic macrophages

(ED1+) have been reported to begin to appear by 3 days after the induction of rAION and continue to accumulate (~35 days), indicating that a long-term inflammatory response continues in rAION.^{5,35} Our results demonstrated that IVI-TA treatment within 1 week postinfarct could decrease ED1

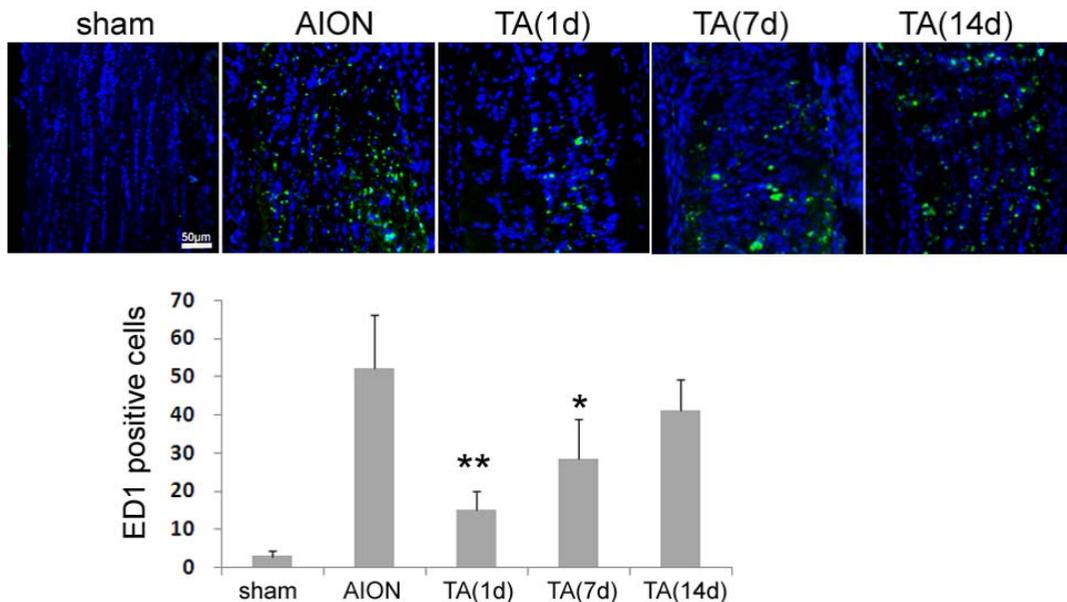


FIGURE 4. Less infiltration of ED1-positive cells (mean ± SD) in the ONs after IVI-TA treatment within 1 week. Cells that were ED1 positive were prominent at the ON lesion sites in the PBS-treated group (52 ± 14 cells/HPF). The number of ED1+ cells was significantly decreased at the ON lesion sites in both the 1d- and 7d-TA groups (15 ± 5 cells/HPF and 28 ± 11 cells/HPF, respectively, $**P < 0.005$ and $*P < 0.05$ compared with the PBS-treated group ($n = 6$ in each group; Scale bar: 50 μm).

extrinsic macrophage infiltration in ONs. Triamcinolone treatment has also been reported to stabilize the blood-retinal barrier and prevent osmotic swelling of Müller cells in diabetic retinas.^{36,37} It is possible that treatment with IVI-TA can stabilize the blood-retinal barrier at the ischemic ONs, and further decrease infiltration of the extrinsic macrophages nearby the insult area.

However, these results should be interpreted with caution before clinical application because animal models of ischemic optic neuropathy are different from clinical NA-IION.⁷ In ischemic optic neuropathy in humans, the risk of disease is multifactorial unlike the simple photodynamic thrombosis in rAION. With treatment of IVI-TA (as well as systemic treatment) in human NA-AION, the treatment time is often late; variable comorbidities are often present in affected individuals; and the amount of medication injected (or given systemically) may be insufficient for the severity of damage. With treatment of IVI-TA in human NA-AION, the treatment time is often late, and variable comorbidities are often present in individuals with NA-AION. Furthermore, the side effects of IVI-TA such as secondary glaucoma or treatment-related infection and the different effects on individual clinical application should be considered.

Previous studies have reported that other injected intravitreal agents exhibit neuroprotective effects on decreasing microglial activation and preventing the subsequent loss of RGCs in rAION, such as ciliary neurotrophic factor³⁸ and prostaglandin J2.³⁹

In conclusion, early doses of IVI-TA played a role in rescuing RGC survival and improving electrophysiologic visual function in a rAION model. The rescue effects may be through multiple actions including an antiapoptosis effect on RGCs and an anti-inflammatory effect on ONs.

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