Early Methylprednisolone Treatment Can Stabilize the Blood-Optic Nerve Barrier in a Rat Model of Anterior Ischemic Optic Neuropathy (rAION)

Tzu-Lun Huang,1-3 Yao-Tseng Wen,4 Chung-Hsing Chang,5,6 Shu-Wen Chang,1 Kuan-Hung Lin,7 and Rong-Kung Tsai3,4

1Department of Ophthalmology, Far Eastern Memorial Hospital, Banciao District, New Taipei City, Taiwan
2Department of Electrical Engineering, Yuan-Ze University, Chung-Li, Taoyuan, Taiwan
3Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan
4Institute of Eye Research, Buddhist Tzu Chi General Hospital, Hualien, Taiwan
5Department of Dermatology, China Medical University Hospital, Taichung, Taiwan
6Department of Dermatology, College of Medicine, China Medical University, Taichung, Taiwan
7Department of Neurology, Taiwan Adventist Hospital, Taipei, Taiwan

Correspondence: Rong-Kung Tsai, Institute of Eye Research, Buddhist Tzu Chi General Hospital, Tzu Chi University, 707 Sec. 3 Chung-Yung Road, Hualien 970, Taiwan; rktsai@tzu.chi.com.tw; tsai.rk@gmail.com.

TLH and YTW are joint first authors.

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PURPOSE. We investigated whether methylprednisolone (MP) treatment halting retinal ganglion cell (RGC) death and having anti-inflammatory effect over a narrow therapeutic window affects the integrity of the blood-optic nerve barrier (BOB) in a rat model of ischemic optic neuropathy (rAION).

METHODS. The optic nerve (ON) vascular permeability was determined by Evans blue extravasation. Changes in the levels of TNF-α and IL-1β cytokines were analyzed using quantitative RT-PCR (qRT-PCR) from day 1 to day 5 post-rAION. Rats were treated with MP starting on days 0, 1, 2, and 7 post-rAION. The survival and apoptosis of the RGCs were determined by fluorescein labeling and TUNEL assay, and the visual function was assessed with flash visual-evoked potentials (FVEPs) 4 weeks postinfarct. Inflammation of the ON was detected by immunohistochemical staining of ED1.

RESULTS. Macrophage recruitment in the ON was significantly reduced, which was compatible with the reduction in ON vascular permeability, after MP treatment starting on days 0 and 1 postinsult compared to PBS treatment (both, P < 0.05). There was significant reduction in TNF-α and IL-1β expression in MP-treated rats (all, P < 0.05). The survival number and antiapoptotic effect on RGCs, and the P1-N2 FVEP amplitude significantly improved with MP treatment starting on days 0 and 1 (all, P < 0.05).

CONCLUSIONS. Early treatment with MP halts RGC death and mitigates macrophage infiltration with decreased expression of proinflammatory cytokines in acute rAION. The very narrow therapeutic window is related to the quick stabilization of the disrupted BOB by early application of MP.

Keywords: methylprednisolone, rat model of ischemic optic neuropathy, blood-optic nerve barrier, retinal ganglion cell, optic nerve

Nonarteritic anterior ischemic optic neuropathy (NA-AION) is the most common acute optic neuropathy in people over the age of 50 years.1 There is currently no definite treatment for NA-AION that can halt the loss of retinal ganglion cells (RGCs) and rescue the visual impairment following ischemia. One retrospective study has reported positive effect of systemic steroid treatment in improvement of visual field and visual acuity in acute NA-AION.2 One prospective study, on the other hand, has reported conflicting conclusions regarding the neuroprotective effect of systemic steroid treatment on NA-AION.3 The therapeutic effects of any treatment are highly influenced by the fact that the treatment is administered too late to stop the inflammation cascade in acute NA-AION.

Evidence from rat models of anterior ischemic optic neuropathy (rAION) suggests that reducing inflammation and optic nerve (ON) edema in the acute phase may be effective in protecting neurons from degeneration.4-8 From the histological viewpoint, the modulation of inflammation at an early stage of rAION may be a useful approach in the treatment of NA-AION.9-12 After the induction of ON ischemia, the breakdown of the blood-optic nerve barrier (BOB) occurs within hours,8,13 and the recruitment of extrinsic macrophages and the activation of resident microglia at the ischemic core are identified as early as 3 days after the insult.14-17 Functional changes such as in the amplitude of visual-evoked potentials (VEPs) were also mitigated early before the permanent degradation following rAION induction.18 There is evidence suggesting that the administration of corticosteroids can effectively decrease tissue edema by increasing the expression levels of the occludin and claudin-5 genes and stabilizing the blood-brain barrier (BBB) in models of brain osmotic insult, as well as models of retinal diseases.19-21 The optic nerve is a part
of the white matter in the brain; ischemia results in the increase in proinflammatory signals and cytokines and the disruption of the BBB. In our previous study, we proved that the granulocyte colony-stimulating factor (G-CSF) could rescue visual function and RGCs survival within a narrow therapeutic window.14 Systemic MP treatment showed both the antiapoptosis of RGCs and the anti-inflammatory effect on the ON.24,25 However, the therapeutic window for MP in the rAION model has not yet been elucidated. We hypothesized that the therapeutic window for MP is dependent on the stabilization of the BOB, as well as the infiltration level of extrinsic macrophages in the ischemic core in the ON. We sought to examine if the different timing of the initiation of MP treatment causes significant differences in the neuroprotection it offers in the rAION model.

METHODS

Animals
A total of 126 adult male Wistar rats weighing 150 to 180 g (7-8 weeks old) were used in this study. The rats were obtained from the breeding colony of BioLASCO Co., Taiwan. The 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 the Tzu Chi Medical Center approved all the animal experiments.

Study Design
In the present study, we investigated the therapeutic window of methylprednisolone (MP) sodium succinate (Medason, Nang Kuang Pharmaceutical Co., Taiwan) and the role of MP in regulating ON inflammation in a rAION model. The summary of the animal numbers used in our study is illustrated in the Figure 1. After rAION had been induced, the rats were randomly divided into five groups (n = 12 in each group). The four groups were administered the MP treatment at different time points after the ischemic insult. In the MP-treated group, the MP injections were administered within 30 minutes (MP-0d), 24 hours (MP-1d), 48 hours (MP-2d), and 7 days (MP-7d) after the rAION. The treatment control group received phosphate-buffered saline (PBS) alone via intraperitoneal injection after the ischemic insult for 14 consecutive days (n = 12). The sham group received laser treatment without the use of photosensitizing agents (n = 12). In the treated group, each rat was injected intraperitoneally with MP (Kirin, Japan) for 14 consecutive days. The animals receiving MP were given a first bolus dose of 30 mg/kg via intraperitoneal administration, followed by tapering doses for 2 weeks, as described in our previous report.25,26 All the animals tolerated this treatment without any complications, and all of them survived until the end of the treatment. The rats were euthanized using CO2 insufflation 4 weeks postinfarct.

In order to follow the 3Rs (replacement, reduction, and refinement) principle for humane animal research, we determined the n = 6 for every test.

rAION Induction
The rAION induction protocol was the same as that used in our previous report. Briefly, after general anesthesia, Rose Bengal (RB; Sigma-Aldrich Corp., St. Louis, MO, USA) was administered intravenously through the tail vein using a 28-gauge needle (2.5 mM RB in PBS/1 mL/kg animal weight). The sham laser treatment consisted of illuminating the ON region with an argon laser without RB. After the administration of RB and pupil dilatation, the right optic discs of the rats were directly treated with an argon green 532 nm/500 μm/80 mW spot laser (MC-500 Multicolor laser, Nidek Co., Ltd., Tokyo, Japan) with 12 pulses of 1 second duration each.

FIGURE 1. Summary of the study design. Total number of rats is 126. There were two main studies: one is the neuroprotective study, which we gave MP or PBS of the same dose at 0, 24, 48 hours and 7 days after rAION injury then did RGC counting, FVEP, TUNEL, and ED1 analysis at day 28. The other one is the permeability study, which we started treatment of MP or PBS of the same dose at 0, 24, 48, 72, and 96 hours after injury and did EB and cytotoxic studies following 1 day (at the day of 1, 2, 3, 4, and 5).

Flash Visual-Evoked Potentials (FVEP)
The detailed procedure of recording FVEPs has been described in our previous reports. Briefly, we used a visual electrodiagnostic system (UTAS-E3000, LKC Technologies, Gaithersburg, MD, USA) to measure the FVEP. To exclude the possibility that the contralateral fellow eye contaminated the FVEP test, we covered the fellow eye while performing the stimulation. We compared the amplitude of the P1/N2 wave in each group to evaluate visual function (n = 6 rats per group).

Retrograde Labeling of RGCs With FluoroGold and Morphometry of the RGCs
The detailed protocol of FluoroGold labeling has been described in our previous reports. In brief, the retinas were examined for RGCs at a distance of 1 and 3 mm from the center to provide the central and midperipheral RGC densities, respectively. We counted at least eight randomly chosen areas in the central (approximately 40% of the central area) and midperipheral (approximately 30% of the midperiphery) regions of each retina, and their averages were used as the mean density of RGCs per retina (n = 6 rats per group). The retinal ganglion cell survival percentage 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.

ON and Retinal Sample Preparation

ON Preparation. A segment of the ON approximately 5 to 7 mm in length between the optic chiasm and the eyeball was
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Vascular Permeability of the BOB Gradually Recovered Before Day 2 Postischemia

Methylprednisolone treatment starting on day 0 and day 1 postischemia significantly reduced the ON vascular permeability by 2.2- and 2.1-fold, respectively, compared to the PBS-treated groups (both \( P < 0.05, n = 6 \) per group; Fig. 3). After

In Situ Nick End-Labeling (TUNEL) Assay
To ensure the use of equivalent fields for comparison, all the paraffin or frozen sections of the retina were prepared with retinas at 1 to 2 mm distance from the ON head. We detected apoptotic cells using the TUNEL reaction (DeadEnd Fluorometric TUNEL System, Promega). The TdT-DUTP terminal nick-end labeling-positive cells in the RGC layer of each sample were counted in 10 high-powered fields (HPF; 400X), and an average from three sections per retina was used for further analysis (\( n = 6 \) rats per group).

Immunohistochemistry (IHC) of ED1+ Cell in the ON
The detailed procedure of ED1 labeling has been described in our previous reports. Briefly, longitudinal sections of the ONs were stained with hematoxylin-cosin for morphologic evaluation. Since ED1 antibodies react against extrinsic macrophages, IHC of ED1 (CD68) using a monoclonal antibody (1:50, AbD Serotec, Oxford, UK) was performed. For comparison, the ED1+ cells were counted in six HPFs (400X magnification) at the ON lesion site (\( n = 6 \) rats per group).

Statistical Analysis
All the measurements were performed in a blinded fashion including histology interpretation. Statistical analysis was performed using a commercial software (IBM SPSS Statistics 19, International Business Machine Corp., Armonk, NY, USA). We used the Kruskal-Wallis and Mann-Whitney U tests to evaluate differences in the number of cells between groups. Results with \( P \) values less than 0.05 were considered statistically significant.

RESULTS
Extrinsic Vascular-Borne Macrophages (ED1+ Cells) Significantly Reduce in Number With MP Treatment on Day 0 and Day 1 Postischemia
There was no ED1+ cell infiltration in the sham group (0 cells/HPF). After rAION induction, the number of ED1+ cells infiltrated in the injured area of the ON was observed in the PBS-treated group (118 ± 13 cells/HPF). The numbers of ED1+ cells infiltration in the ON were 14 ± 2, 72 ± 16, 139 ± 34, and 126 ± 26 cells/HPF in the day 0-MP, day 1-MP, day 2-MP, and day 7-MP-treated rats postinfarct, respectively (Fig. 2). The differences in the decreasing number of ED1+ cells were statistically significant in both day 0-MP and day 1-MP groups compared with that in the PBS-treated group (both \( P < 0.05, n = 6 \)). There was no significant difference in the ED1 infiltration between MP groups starting on the second day and the seventh day postischemia compared to the PBS group (all \( P > 0.05, n = 6 \)).

Vascular Permeability of the BOB Gradually Recovered Before Day 2 Postischemia
Methylprednisolone treatment starting on day 0 and day 1 postischemia significantly reduced the ON vascular permeability by 2.2- and 2.1-fold, respectively, compared to the PBS-treated groups (both \( P < 0.05, n = 6 \) per group; Fig. 3). After

Evans Blue (EB) Dye Extravasation of the ONs
Optic nerve vascular leakage was quantified using modifications of the published techniques. The details of the vascular permeability test have been described in our previous report. The summary of the animal numbers used in the vascular permeability study is illustrated in Figure 1. After the rats were anesthetized, 2% EB (Sigma-Aldrich Corp., St. Louis, MO) was injected into the tail vein (final dosage: 4 mL/kg). The optic nerves were cut and weighed when dry. The samples were collected 1 day after EB injection in groups with MP or PBS treatment starting on day 0 to day 3 postinsult. We killed animals 1 day following the treatment. Therefore, we got the vascular permeability in earlier 5 consecutive days after injury. Briefly, the EB dye conjugated to serum albumin in the ON was extracted using formamide (0.2 mL per retina) at 78°C overnight and kept at −20°C until further processing. The details have been described in our previous reports.

Detection of IL-1β and TNF-α mRNA by quantitative RT-PCR (qRT-PCR)
Total RNA was isolated from ON samples with TRIzol (Invitrogen, Waltham, MA, USA). The samples were collected every day from day 1 to post-rAION induction with MP or PBS treatment starting on day 0 to day 3 postinsult. We killed animals 1 day following the treatment. Finally, we got the change of cytokines in earlier 4 consecutive days after injury. First-strand cDNA was synthesized with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) in 10 μL RT reaction mixture using random hexamers. Polymerase chain reaction amplification of the cDNA in the presence of primers specific for IL-1β, TNF-α, and gyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used: IL-1β, forward: 5′-AGCCTTCAGGAAGGAGTTGTC-3′; reverse: 5′-TCAGACAGCACGAGGCATTT-3′; TNF-α, forward: 5′-TACTGAACCTCGGGTGATGGTGC-3′; reverse: 5′-CAGCCTGTCCGTTCGAAGAAGCC-3′; GypA, forward: 5′-CCAGTGGCTACGAGACAGAAAG-3′; reverse: 5′- CACCGTGCTCAGAGCACGAAAG-3′. The polymerase chain reaction amplification conditions were maintained according to the manufacturer’s protocol. The qRT-PCR was performed with the Power SYBR Green PCR Master Mix on the StepOnePlus System (Thermo Scientific, Waltham, MA, USA) according to standard procedures. GypA was used as an internal control to account for loading differences.
the infarct, the disruption of BOB occurred immediately with PBS treatment, and this disruption gradually stabilized at 2 days postinfarct. There was no significant difference in the vascular permeability between the MP and PBS treatment groups after the second day post-rAION. The window time for the EB extravasation in the ON within day 0 and day 1 after the insult represented that early treatment with MP could reduce the BOB disruption in the acute rAION model, which was compatible with the time course of macrophage recruitment to the injured area in the ON (Fig. 2).

**Proinflammatory Cytokine (IL-1β and TNF-α) Production Reduced With MP Treatment on Day 0 and Day 1 Postischemia**

The mRNA levels of proinflammatory cytokines, IL-1β and TNF-α, in the ON were highest 1 day after the insult and then gradually decreased till day 3 postinfarct in the PBS groups (Fig. 4). There was a significant decrease in the IL-1β/GAPDH (0.278 ± 0.026 to 0.021 ± 0.007) and TNF-α/GAPDH (0.159 ± 0.031 to 0.057 ± 0.009) ratios in the MP groups (both P < 0.01; n = 6 per group). In the day 1-MP treatment group, there was a significant decrease in the IL-1β/GAPDH and TNF-α/GAPDH ratios from 0.159 ± 0.018 to 0.028 ± 0.013 and 0.056 ± 0.022 to 0.019 ± 0.01, respectively (P < 0.05 compared with the PBS-treated group; n = 6 per group). However, no significant differences in the IL-1β/GAPDH and TNF-α/GAPDH ratios were seen between the MP and PBS treatment groups on day 2 postischemia.

**The RGC Survival Significantly Improved With MP Treatment on Day 0 and Day 1 Postischemia**

The retinal ganglion cell densities in the central and midperipheral retinas of the laser-stimulated eyes (sham) were 1576 ± 174/mm² and 1294 ± 99/mm², respectively. In the fourth week postinfarct, the central and midperipheral RGC densities in the PBS-treated group decreased to 518 ± 165/mm² and 371 ± 87/mm², respectively. In the day 0-MP group, the RGC densities lesser declined to 1168 ± 139/mm² and 875 ± 127/mm² in the central and midperipheral retinas, respectively (P = 0.021 and P = 0.021 compared to the PBS-treated group, n = 6 per group). In the day 1-MP group, the RGC densities also lesser declined to 924 ± 226/mm² and 610 ± 190/mm² in the central and midperipheral retinas, respectively (P = 0.02 and P = 0.01 compared to the PBS-treated group, n = 6 per group). However, the RGC densities did not significantly change in the day 2-MP group, and remained at 661 ± 187/mm² and 430 ± 86/mm² in central area and midperipheral retinas, respectively (P = 0.47 and P = 0.39 compared to the PBS-treated group, n = 6 per group; Fig. 5). The survival rates of RGCs in the day 0, day 1, day 2, and...
FIGURE 4. The expression levels of TNF-α and IL-1β in the ON tissues. (A) Analysis of TNF-α and IL-1β expression using qRT-PCR. Methylprednisolone treatment starting on day 0 to day 4 post-rAION reduced the level of proinflammatory cytokines (IL-1β and TNF-α). There was a significant decrease in the IL-1β/GAPDH (0.278 ± 0.026 to 0.021 ± 0.007) and TNF-α/GAPDH (0.159 ± 0.031 to 0.057 ± 0.009) ratios with MP treatment (day 0 postinsult) compared to those observed with PBS-treated groups (both, *P < 0.01; n = 6 per group, Mann-Whitney U test). However, no significant differences were observed in the IL-1β/GAPDH and TNF-α/GAPDH ratios between MP (day 2 after injury) and PBS treatment groups.

FIGURE 5. Morphometry of RGCs in rAION-induced rats with PBS and MP treatment on day 0, 1, 2, and 7 post-rAION induction. Representatives RGC numbers in the central retinas (A) and the midperipheral retinas (B) in each group. In the immediate MP (day 0)-treated group, the RGC densities lesser declined to 1168 ± 139/mm² and 875 ± 127/mm² in the central and midperipheral retinas, respectively (*P = 0.021 and P = 0.021 compared to the PBS-treated group, n = 6 per group). In the day 1-MP group, the RGC densities lesser declined in the central and peripheral retinas, respectively (924 ± 226/mm² and 610 ± 190/mm²; both, *P < 0.05 compared to the PBS-treated group; n = 6 per group; scale bars: = 50 μm). The asterisk indicates P < 0.05 (Mann-Whitney U test).
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The changes in FVEPs after rAION were measured in the fourth week after the infarct. The amplitude of the P1-N2 wavelet in the sham was 52 ± 6 μV. The amplitudes of the P1-N2 waves in the PBS-group and the day 0, day 1, day 2, and day 7 MP and PBS groups showed 68%, 47%, 20%, and 29%, respectively. Treatment with MP in the day 0 and day 1 groups led to higher preservation of RGCs compared to that seen in the other groups.

Visual Function Significantly Improved With MP Treatment on Day 0 and Day 1 Postischemia

The changes in FVEPs after rAION were measured in the fourth week after the infarct. The amplitude of the P1-N2 wavelet in the sham was 52 ± 6 μV. The amplitudes of the P1-N2 waves in the PBS-group and the day 0, day 1, day 2, and day 7 MP-treated groups were 11 ± 4 μV, 50 ± 4 μV, 29 ± 11 μV, 14 ± 9 μV, and 13 ± 7 μV, respectively (Fig. 7). There was a significant preservation of the P1-N2 amplitude in the FVEPs in the day 0 and day 1 MP-treated groups compared to that in the PBS treatment (both, \( P = 0.02; n = 6 \) per group).

DISCUSSION

Our observations demonstrate that early treatment with MP 0 and 1 day postinfarct leads to the better rescue of RGC death and visual function following the induction of rAION compared to that seen with deferring the treatment. The narrow therapeutic window of rAION probably can be explained with the observation of faster recovery of the disrupted BOB on day 2 postinjury. On the other hand, early treatment with MP 0 and 1 day after infarct can quickly stabilize the disrupted BOB and simultaneously affect the recruitment of vascular-borne macrophages (ED1+) and their infiltration in the ON, thus further decreasing the expression of TNF-α and IL-1β in the ON in a time-dependent manner (Fig. 8). We suggested that the ischemic injury in rAION is largely through the proinflammatory mechanism and macrophage activation and migration through a disrupted BOB.

Proinflammatory cytokines like the TNF-α and interferon-γ (IFN-γ) trigger the conversion of macrophages and microglia into classically activated proinflammatory phenotypes in a nontraumatic spinal cord neuroinflammation rat model. This may exacerbate the area of the initial injury in the nerve, resulting in cytotoxic environments. As a quick response within 24 hours after central nervous system (CNS) injury, theoligodendrocyte precursor cells exhibiting a reactive morphology would cause negative effects due to the influx of solutes and macromolecules from the disrupted BBB after microinjection of lipopolysaccharide for inducing CNS injury in the rat model. In the experiment involving compression injuries to the spinal cord, the inhibition of the persistent activation of macrophages resulted in neuroprotective effect on neuronal death associated with local microenvironmental changes through the production of TNF-α, IL-1, and IL-4. In an animal model of ischemic optic neuropathy, the TUNEL+ cells were identified in the RGC layer between 7 and 15 days, and the apoptotic signal is largely complete 31 days after induction. These previous reports concluded the relatively longer window period of RGC death secondary to ischemic optic neuropathy implies that the available window of treatment after an ON stroke may be longer than was previously believed. However, our current results showed that the therapeutic window is limited to 1 day postinfarct, with the evidence of quick stabilization of disrupted BOB postinfarct; the same observations were also described in another G-CSF report.

The therapeutic window for human NA-AION is unclear, as it may be complicated by the multifactorial nature of NA-AION pathogenesis, with factors such as aging, systemic vascular comorbidities, small cupping, and severity of infarct influencing the prognosis. When we administered anti-inflammatory drugs like MP within the therapeutic window of rAION, MP hastened the recovery of BOB breakdown to decrease the recruitment of
FIGURE 7. Evaluation of visual functional assessments through FVEP recordings in the fourth week after infarct. (A) Representative FVEP wavelet after rAION in each group. (B) Bar charts showing the P1-N2 amplitudes. There was a significant improvement in the VEP in the day 0-MP and day 1-MP groups compared to that in the PBS treatment group (29.6 ± 4.2 μV and 28.8 ± 10.8 μV, respectively; both, *P = 0.02; n = 6 per group). Data are expressed as mean ± SD. The asterisk indicates *P < 0.05 (Mann-Whitney U test).

FIGURE 8. Mechanisms of neuroprotection in MP treatment. In normal rats, the BOB is a tight junction. After rAION induction, the disruption of BOB occurs quickly as massive numbers of vascular-borne macrophages (ED1+ cells) infiltrate the core of the ON. After early treatment with MP, the disrupted BOB can be quickly stabilized, thereby decreasing the recruitment and infiltration of ED1+ cells, as well as the expression levels of inflammatory cytokines such as TNF-α and IL-1β in the ON.
blood-borne macrophages into the ON, thus further improving this negative impact on the ON (within 1 day of the ischemic injury). Proinflammatory cytokines (TNF-α and IL-1β) induce the upregulation of the expression of metalloproteinases (especially MMP-9) in the vascular endothelium cell and discontinuities in the ZO-1 junctional expression related to vascular permeability. A previous report of rAION demonstrated that prostaglandin J2 improved vascular perfusion with the evidence of reduced fluid leakage from the disrupted BBB when they injected the drug within 1 day of the rAION. The fact that early treatment with G-CSF stabilized the BOB to reduce macrophage infiltration also supported our hypothesis that early application of MP can stabilize the BOB, decreasing macrophage infiltration also supported our hypothesis that early application of MP can stabilize the BOB, decreasing the inflammatory cell (ED1+ cells) recruitments and further decreasing the proinflammatory cytokine production. These anti-inflammatory events will decrease the secondary degeneration of RGCs after rAION induction, as demonstrated by the better protection of RGCs from death, as well as the preservation of visual function (FVEPs) at 4 weeks postinfarct. The stop of BOB disrupt seems to be more critical than the preservation of visual function (FVEPs) at 4 weeks postinfarct. The time of treatment will influence the efficacy of barrier remodeling. Based on our results, we support that treatment with steroid or other BOB-stabilizing agents is not necessary after 2 days postinjury.

In conclusion, early treatment (day 0 and day 1) with MP within the therapeutic window provides significant antiapoptotic effects on the RGCs and anti-inflammatory effects on the ON with evidence for the prevention of RGC death and anti-inflammatory events will decrease the secondary degeneration of RGCs after rAION induction, as demonstrated by the better protection of RGCs from death, as well as the preservation of visual function (FVEPs) at 4 weeks postinfarct. The stop of BOB disrupt seems to be more critical than the rescue of secondary death of RGC in acute rAION. The time of treatment will influence the efficacy of barrier remodeling. Based on our results, we support that treatment with steroid or other BOB-stabilizing agents is not necessary after 2 days postinjury.

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