• Basic Research •

Neuroprotective effects of gypenosides in experimental autoimmune optic neuritis

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Received: 2016-08-31 Accepted: 2016-12-05

Abstract

• AIM: To determine whether gypenosides have protective effects in experimental autoimmune optic neuritis (EAON).

• METHODS: Mice were randomly divided into seven groups: control group, model group, three different density gypenosides monotherapy, methylprednisolone monotherapy, combination of gypenosides and methylprednisolone group. The control group was subcutaneously injected with oil emulsion adjuvant and all other groups were subcutaneously immunized with an emulsified mixture of myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide to induce EAON. Mice in the gypenosides groups were administered injections daily with three concentrations (15 mg/kg, 30 mg/kg, 45 mg/kg) of gypenosides respectively. Mice in the methylprednisolone group and the combination treatment group were injected daily with methylprednisolone (20 mg/kg) or methylprednisolone (20 mg/kg) + gypenosides (30 mg/kg), respectively. After MOG immunization, visual evoked potential (VEP), optical coherence tomography (OCT), and histopathologic examination were performed at 14, 20, 30, and 40d post-inoculation (p.i.). All results were expressed as mean±SEM. The data were evaluated by one-way ANOVA followed by Tukey or Games-Howell test.

• RESULTS: Compared with the control group, p2 latency was prolonged in the model group (*P*=0.041). Combination treatment can alleviated the change in VEP at 20d p.i. (*P*=0.012). Average peripapillary retinal nerve fiber layer (RNFL) thickness was reduced in the model group (*P*= 0.000, 30d; *P*=0.000, 40d) and gypenosides treatment remarkably diminished the degree of RNFL degeneration at 30d and 40d p.i (*P*=0.000, 30d; *P*=0.000, 40d). The pathomorphological results showed a decrease in demyelination (*P*=0.020) and inflammatory reactions in the combination group compared with the model group (20d p.i.). Gypenosides treatment also alleviated the degree of axonal loss (40d p.i.) (*P*=0.003).

• CONCLUSION: Treatment with gypenosides exerts protective effects on retinal nerve fibers and axons in EAON. When combined with gypenosides, methylprednisolone reduces demyelination in the acute stage of EAON.

• **KEYWORDS:** gypenosides; experimental autoimmune optic neuritis; visual evoked potential; optical coherence tomography; histopathology

DOI:10.18240/ijo.2017.04.07

Zhang HK, Ye Y, Zhao ZN, Li KJ, Du Y, Hu QM, He JF. Neuroprotective effects of gypenosides in experimental autoimmune optic neuritis. *Int J Ophthalmol* 2017;10(4):541-549

INTRODUCTION

Optic neuritis (ON) is a common neuro-ophthalmologic inflammatory disease that results in persistent vision impairment in young adults. Idiopathic demyelinating optic neuritis (IDON), the most common clinical type, is strongly associated with multiple sclerosis (MS)[1]. Experimental autoimmune encephalomyelitis (EAE) is a commonly used model for studying of ON and MS[2]. Methylprednisolone pulse therapy has been the mainstay of treatment for the acute phase of ON. Corticosteroids accelerate the recovery of vision; however, they do not improve the visual prognosis[3]. Numerous undesirable side effects are associated with corticosteroids. One study even indicates that methylprednisolone accelerates neuron apoptosis in the central nervous system[4]. In addition to a wide range of demyelinating lesions in acute and chronic MS and ON, axonal loss and neuronal apoptosis occur and are closely related to irreversible loss of vision[5]. Therefore, alleviating demyelination and promoting neural protection during the acute inflammatory phase, should be a priority in the clinical treatment. With the serious side effects of corticosteroids and their lack of neuroprotective effect, patients with ON urgently need a novel medication

with anti-inflammatory properties, immune regulation, and neuroprotective effects. *Gynostemma pentaphyllum makino*, a popular herb used in traditional Chinese medicine, has been used in food and tea at length owing to its ability to prevent chronic diseases such as hepatitis, hypertension, and gastritis[6]. Gypenosides, saponin extracts from *Gynostemma pentaphyllum*, has been shown to provide various bioactivities such as antioxidation, hepatoprotection, antilipidemia and inflammation reduction. The neuroprotective effect of gypenosides has been proven and current research is mainly focused on ischemia-reperfusion injury, Alzheimer's Disease, Parkinson's Disease[7-9]. However, the protective effect of gypenosides in ON has not been established. In the present study, an *in-vivo* model was used to evaluate the potential therapeutic effects of gypenosides on functional and histological alterations of experimental autoimmune optic neuritis (EAON).

MATERIALS AND METHODS

Animals Four-week-old female C57BL/6 mice were purchased from the Slaccs-jingda (SLACCS, HUNAN). All animal experiments were performed in accordance with the approved guidelines of the Experimental Ethics Committee of Guangxi Medical University, Nanning, China. All animal procedures were done in strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement. The animals were housed in a standard animal room at 22℃-26℃ with a 12h light/dark cycle.

Experimental Model of Optic Neuritis Mice in the model group and treatment groups were immunized subcutaneously with 150 μg of myelin oligodendrocyte glycoprotein (MOG) 35-55 (SBS Genetech, Beijing, China) emulsified in complete Freund's adjuvant (CFA) containing 8 mg/mL heat-killed *Mycobacterium tuberculosis* (BD, MD, USA). The mice were also injected intraperitoneally with 400 ng *Bordetella pertussis* toxin (Sigma, USA) the same day and 2d following MOG35-55 immunization. The control mice were injected with an equal volume of phosphate-buffered saline and CFA+ heat-killed *Mycobacterium tuberculosis*.

Experimental Design and Treatment The mice were randomly divided into 7 groups: control group, model group, three different density gypenosides monotherapy groups, methylprednisolone (Me)-monotherapy group, and a combination of gypenosides with methylprednisolone treatment group. Mice in the gypenosides groups received daily intraperitoneal injections of gypenosides (Jiatian biotechnology Co. LTD., Xi'an, Shaanxi province, China) at three different concentrations (15, 30 and 45 mg/kg) respectively, in 150 μL of 0.9% sodium chloride. Mice in the Me group received the intraperitoneal injections daily with methylprednisolone (20 mg/kg) in 150 μL of 0.9% sodium chloride. The combination treatment group was injected with gypenosides (30 mg/kg) in 150 μL of 0.9% sodium chloride and methylprednisolone (20 mg/kg) in 150 μL of 0.9% sodium chloride into the peritoneum. All treatment administrations were started on day 7 after immunization with MOG. Methylprednisolone was administered once daily for 3d, and gypenosides was administered once daily until mice were subjected to euthanasia (14, 20, 30, and 40d after immunization).

Visual Evoked Potential Recording Mice in all groups were anesthetized by intraperitoneal injection of 1% pentobarbital sodium in 0.1 mL. They were then placed in a quiet dark room and allowed to acclimate for 5min. Medical cotton pads and warm water bags were used to maintain body temperature of approximately 37℃. Mice were fixed to a platform, and a recording electrode was placed 5 mm behind the bregma. Meanwhile, a reference electrode was placed in the oral cavity in contact with oral mucosa. The ground electrode was placed on the tail of each animal. All recordings were performed monocularly with the opposite eye covered using a black patch. Stimulation was delivered 64 times at a frequency of 1.4 Hz. Visual evoked potential (VEP) signals were recorded by a Roland Electrophysiological Test Unit (Roland Consult, Germany) and P2 latency of VEP was analyzed.

Retinal Nerve Fiber Layer Thickness Measurements Average retinal nerve fiber layer (RNFL) thickness for 360° around the optic disc was measured by the optical coherence tomography (OCT) (Heidelberg, Germany). Animals were anesthetized by intraperitoneal injection of 1% pentobarbital sodium in 0.1 mL and the pupils were dilated with tropicamide (5 mg/mL). Mice were placed on a platform, and adjusted by another doctor to ensure that incident OCT beam entered through the pupil as well as perpendicular to the cornea. The region of measurement was defined as a circle, about 3.46 mm in diameter (system setting), using the optic disc as the center.

Histopathologic Evaluation of Optic Nerves After VEP and OCT measurement, mice were anesthetized with an injection of pentobarbiturate. Cardiac perfusion was then performed using 4% paraformaldehyde in phosphate buffer. The eyes and optic nerves (from globe to chiasm) were removed and fixed in 4% paraformaldehyde overnight. The samples were then dehydrated, cleared in butanol and embedded in paraffin. Five-micrometer-thick transverse sections were made and stained with hematoxylin and eosin (HE) (20d p.i.), Luxol fast blue (Lfb) (14-40d p.i.) and Bielschowsky's silver (14-40d p.i.) for evaluation of inflammation, demyelination, and axonal pathology.

Statistical Analysis Data are presented as mean±standard error (SE). Statistical analysis of results was performed by

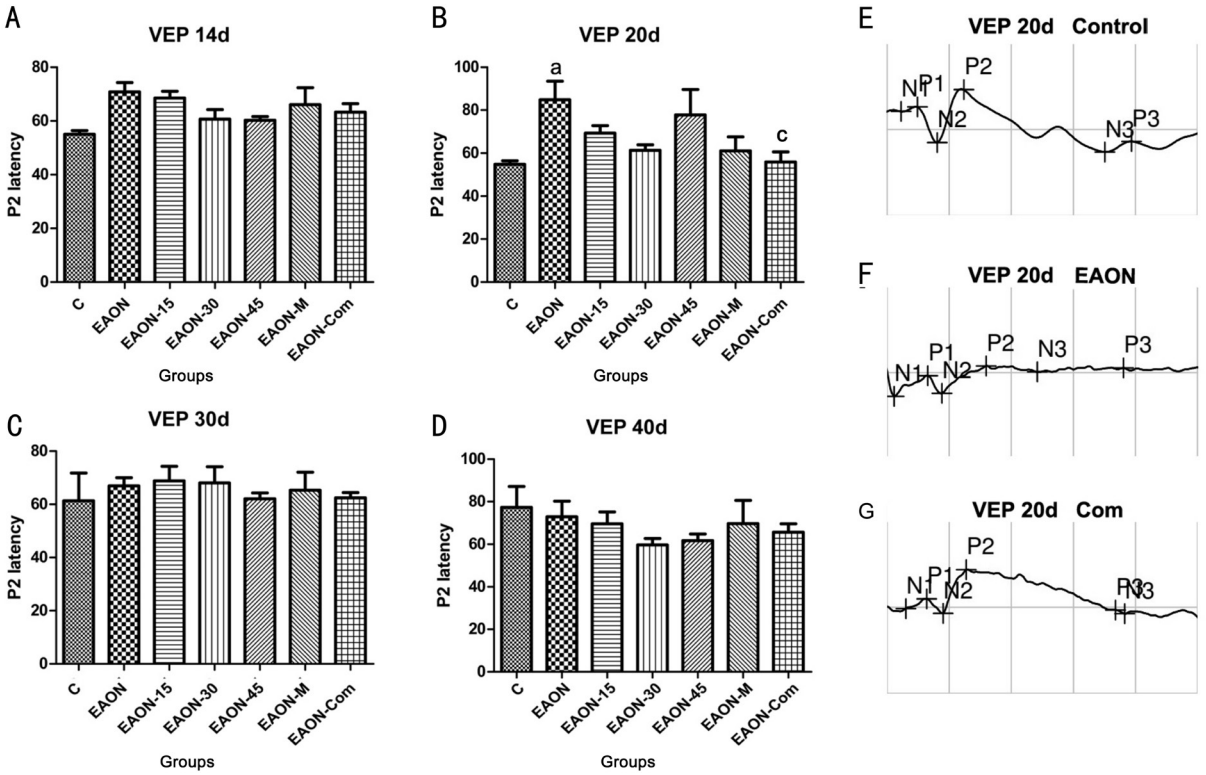

Figure 1 Effect of gypenosides and methylprednisolone on VEPs in EAON A-D: The average P2 latency of VEP at 14, 20, 30 and 40d p.i. A statistical significance is observed at 20d p.i. where the P2 latency of EAON mice is significantly prolonged. Combination treatment can alleviate the deterioration of VEP latency ($^aP<0.05$ when compared with the control group; $^cP<0.05$ compared with the EAON group). E-G: Representative VEP traces from control, model, and combined treatment groups at 20d p.i.

one-way analysis of variance (ANOVA), followed by Tukey or Games-Howell test. Values of $P<0.05$ were considered statistically significant. Statistics were processed using SPSS statistics 16.0 (SPSS Inc., USA).

RESULTS

Latency Delay in Visual Evoked Potentials P2 latency variations on day 14 to day 40 p.i. are shown in Figure 1. At day 14 p.i., there was no significant difference for each group ($P=0.134$), although there did appear to be a slight prolongation of latency in the model and treatment groups. At day 20 p.i., the model group VEP appeared to have a marked prolongation of latency ($P=0.041$) compared with the control group, but no significant increase in latency was observed in any of other treatment groups when compared with the control group. It is important to note the significant differences in latency between the combination group and EAON group ($P=0.012$), suggesting combination therapy can improve VEP outcome in the pathogenic period of disease (Figure 1B). By contrast, no improvement was observed in the other treatment groups when compared with the EAON model group. Although methylprednisolone therapy presented similar results to the combination treatment, the statistical analysis indicated no improvement ($P=0.094$) (Figure 1B). Representative VEP traces in individual mice at 20d p.i. are shown (Figure 1E-1G). Each group presented no statistical difference at days 30 and 40 p.i. ($P=0.932$, 30d; $P=0.738$, 40d) (Figure 1C, 1D).

Changes in Retinal Nerve Fiber Layer Thickness OCT was performed to evaluate RNFL thickness in each group. As showed in Figure 2, there was no significant difference in each group at 14d p.i. and 20d p.i ($P=0.100$, 14d; $P=0.087$, 20d) (Figure 2A, 2B). Pronounced RNFL reductions were confirmed in the model group at 30d p.i. when compared with the control ($P=0.000$) and treatment groups ($P=0.000$) (Figure 2C). Obvious differences were revealed in the last period, 40d p.i., the RNFL was significantly decreased in the model ($P=0.000$) and the methylprednisolone group ($P=0.000$) versus control. The RNFL thickness in the model group relatively thin compared with the other groups apart from the gypenosides (30 mg/kg) group ($P=0.155$) and the methylprednisolone treatment group ($P=0.194$). The RNFL was relatively thick in gypenosides groups (15 mg/kg, 45 mg/kg) and the combination group when compared with the model ($P=0.00$). Our findings demonstrate that EAON has a reduction of RNFL attenuation by 78% after combination treatment (gypenosides+methylprednisolone) compared to methylprednisolone alone (Figure 2D).

Gypenosides Treatment Reduces Optic Nerve Demyelination Optic nerve sections from each groups were stained with Lfb (Figure 3A) to examine whether gypenosides can protect against demyelination during EAON. We did not find a

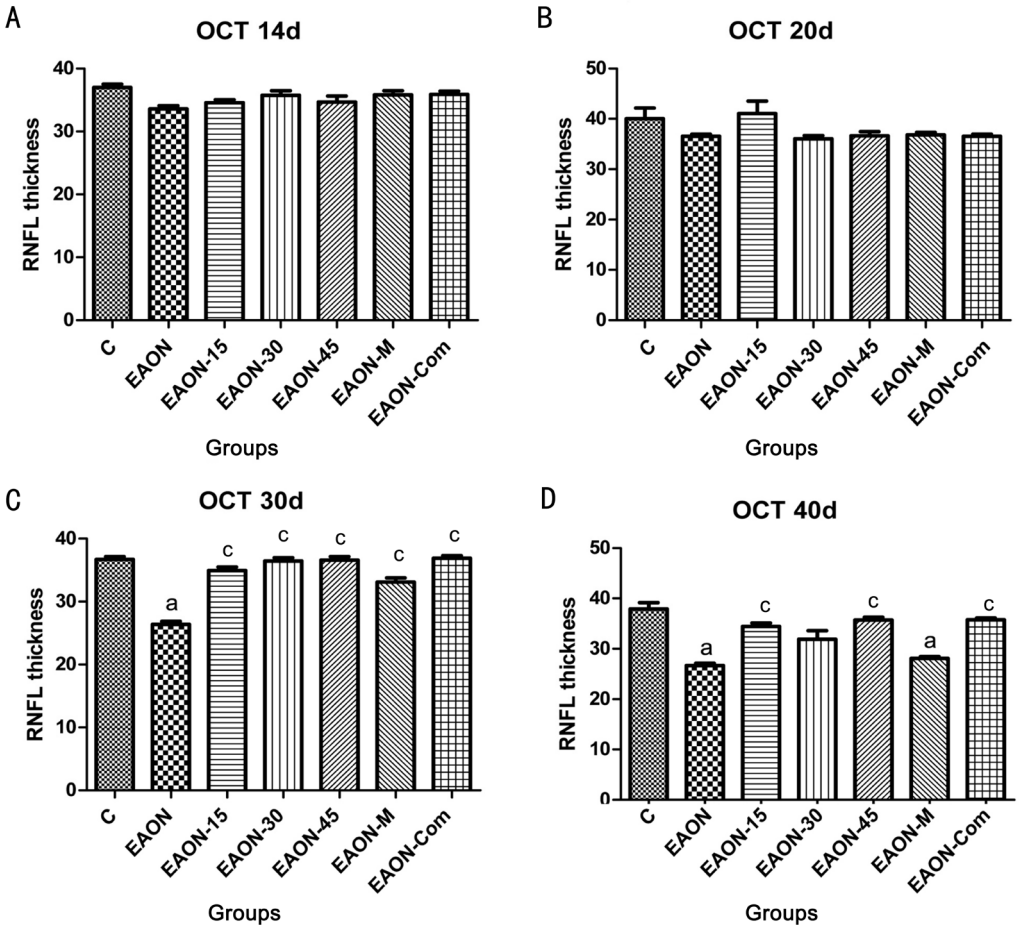

Figure 2 Effect of gypenosides and methylprednisolone on OCT in EAON A-D: Averaged RNFL thickness at 14, 20, 30, 40d p.i in each groups. Both gypenosides and methylprednisolone alleviated the degeneration of RNFL at 30d p.i. In the late phase of disease (day 40 p.i.), only gypenosides treatment opposed the degeneration of RNFL. $^{a}P<0.05$ when compared with the control group; $^{c}P<0.05$ compared with the EAON group.

qualitative change in myelin staining until 20d p.i. (Figure 3C1, 3C2). Demyelination occurred in optic nerves from EAON mice, with a significant density decrease compared with the control (P=0.000) (Figure 3B, 3C2). There were no significants difference among the model group and treatment groups (Figure 3C2), except for the combination group (P=0.020). At day 30 p.i., optic nerves density in the methylprednisolone (P=0.027) and the combination group (P=0.025) showned significant increase in Lfb staining compared with optic nerves of EAON mice (Figure 3C3). Quantification of blue density demonstrated variation in each group, but did not reach significance at 40d p.i., although the model group did show a relatively low density (P=0.491) (Figure 3C4).

Gypenosides Treatment Reduces Axonal Loss in the Optic Nerve To determine whether gypenosides treatment regulates axonal loss, Bielschowsky silver staining was performed and the averaged hole ratio in each group was analyzed (Figure 4A, 4B). No significant difference was found in any group at 14d p.i and 20d p.i. (Figure 4B, 4B2). Hole ratio in the model group increased significantly at 30d p.i. (P=0.007), indicating a significant axon damage (Figure 4B3). At 40d p.i., we also saw a significant axonal loss in optic nerves from the model group (P=0.021), but mice in the treatment groups showed no significant difference compared with control mice (Figure 4A, 4B4). The hole ratio in the gypenosides (30 mg/kg) group (P=0.003) and the combination group (P=0.030) showed significant improvement compared to the model group, but methylprednisolone monotherapy group showed no improvement (Figure 4B4).

Gypenosides Treatment Reduces Optic Nerve Inflammation Sections of the optic nerves and retina were stained by HE at 20d p.i. to determine the anti-inflammatory effect of gypenosides. In the retinal section, inflammatory cells were detected in the ganglion cell layer in the model and the gypenosides (15 and 30 mg/kg) group (the arrow points, Figure 5A). In the EAON model group, a remarkable increased thickness of the inner plexiform layer (IPL) was revealed (the arrowheads points the IPL, Figure 5A). Significant inflammatory cell infiltration appeared in optic nerves in the model group (the arrow points in Figure 5B). In the treatment groups, there was less infiltration of inflammatory cells in

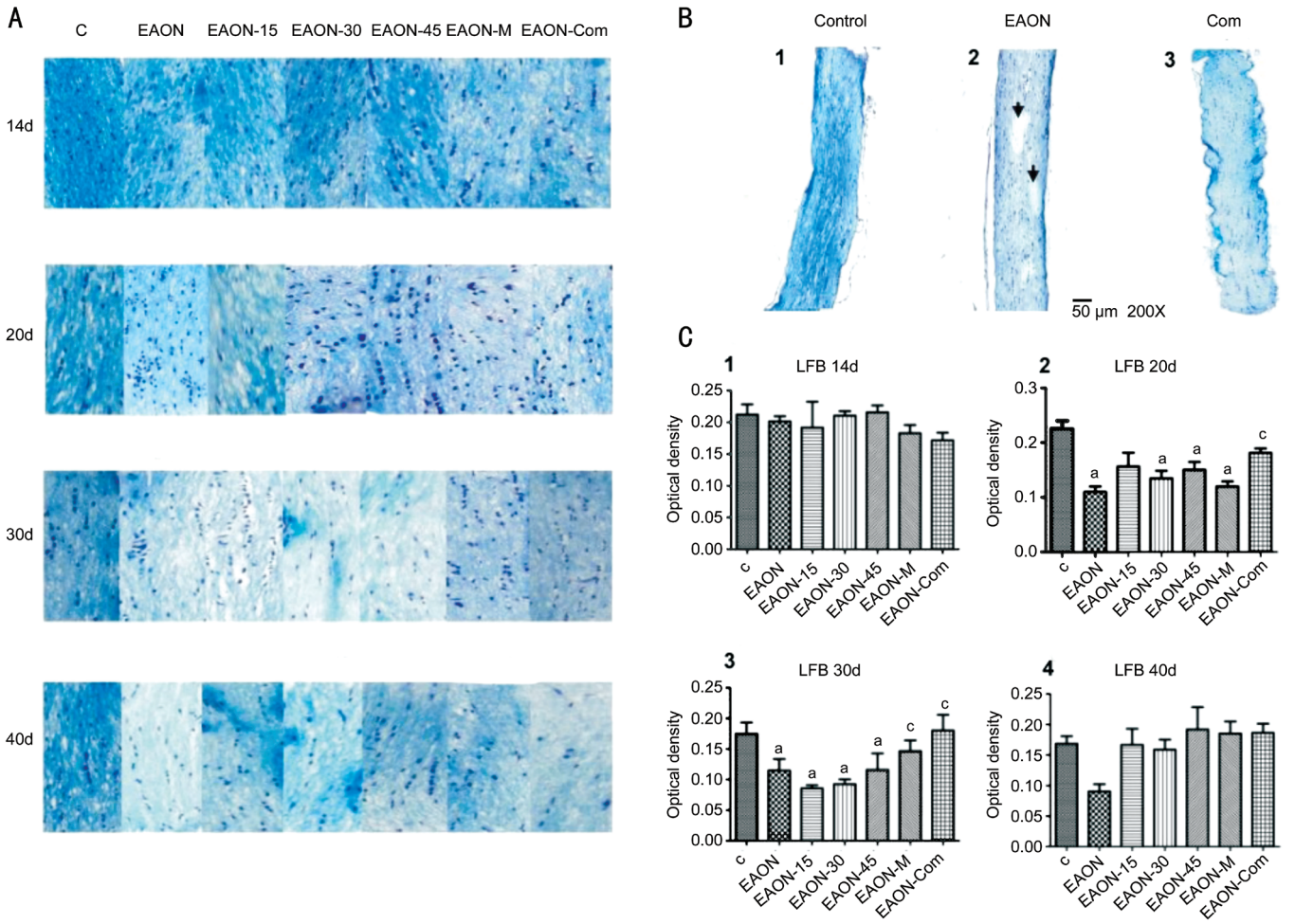

Figure 3 Gypenosides and methylprednisolone combination treatment attenuated demyelination in the optic nerve during EAON, stained with luxol fast blue A: Representative sections from optic nerves at 14-40d p.i. in each group; B: An optic nerve from control, model and combination treatment groups at 20d p.i. (1-3). Arrows point to regions of demyelination of the optic nerve in mice with EAON. C: Average optical density of Lfb staining at 14, 20, 30 and 40d p.i in each group (1-4). [a]P<0.05 when compared with the control group; [c]P<0.05 compared with the EAON group.

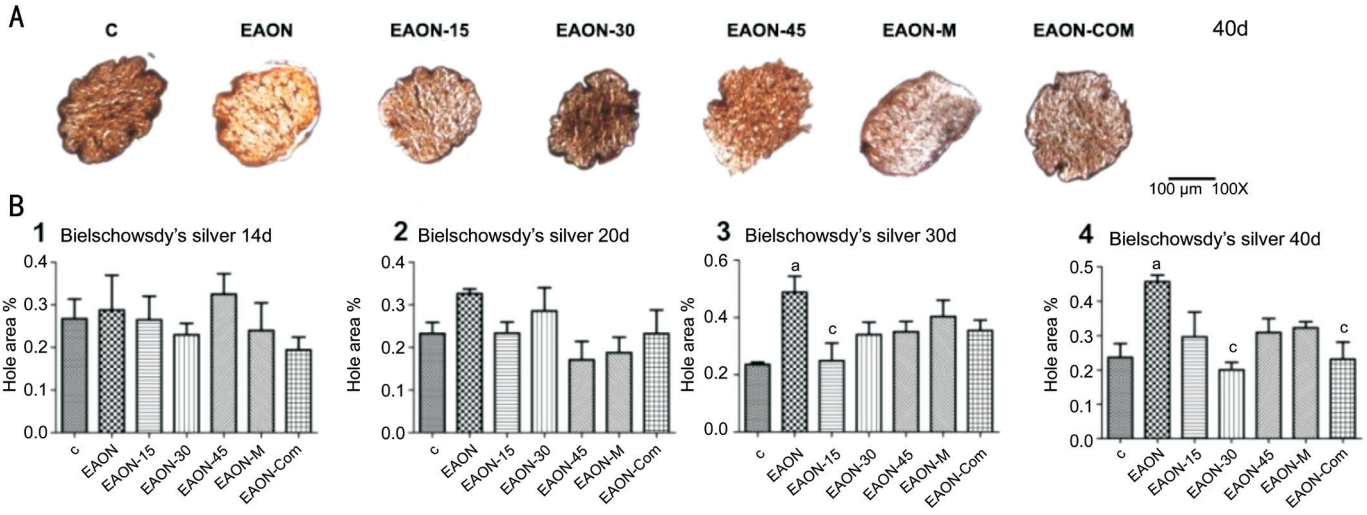

Figure 4 Gypenosides treatment reduces axonal loss in optic nerve during EAON A: Representative cross sections of optic nerve stained with Bielschowsky's silver at 40d p.i. Optic nerves from gypenosides-treated mice show high-density axonal staining compared to those from EAON mice; B: Averaged hole ratio of Bielschowsky's silver staining in each groups were analyzed at 14-40d p.i. (1-4). At 40d p.i., hole ratio in EAON optic nerves show significant increase compared to control optic nerves. Treatment with gypenosides (30 mg/kg) and combination therapy (gypenosides 30 mg/kg and methylprednisolone 20 mg/kg) could significantly reduce optic nerve hole ratio when compared with EAON, but methylprednisolone monotherapy showed no improvement. [a]P<0.05 when compared with the control group; [c]P<0.05 compared with the EAON group.

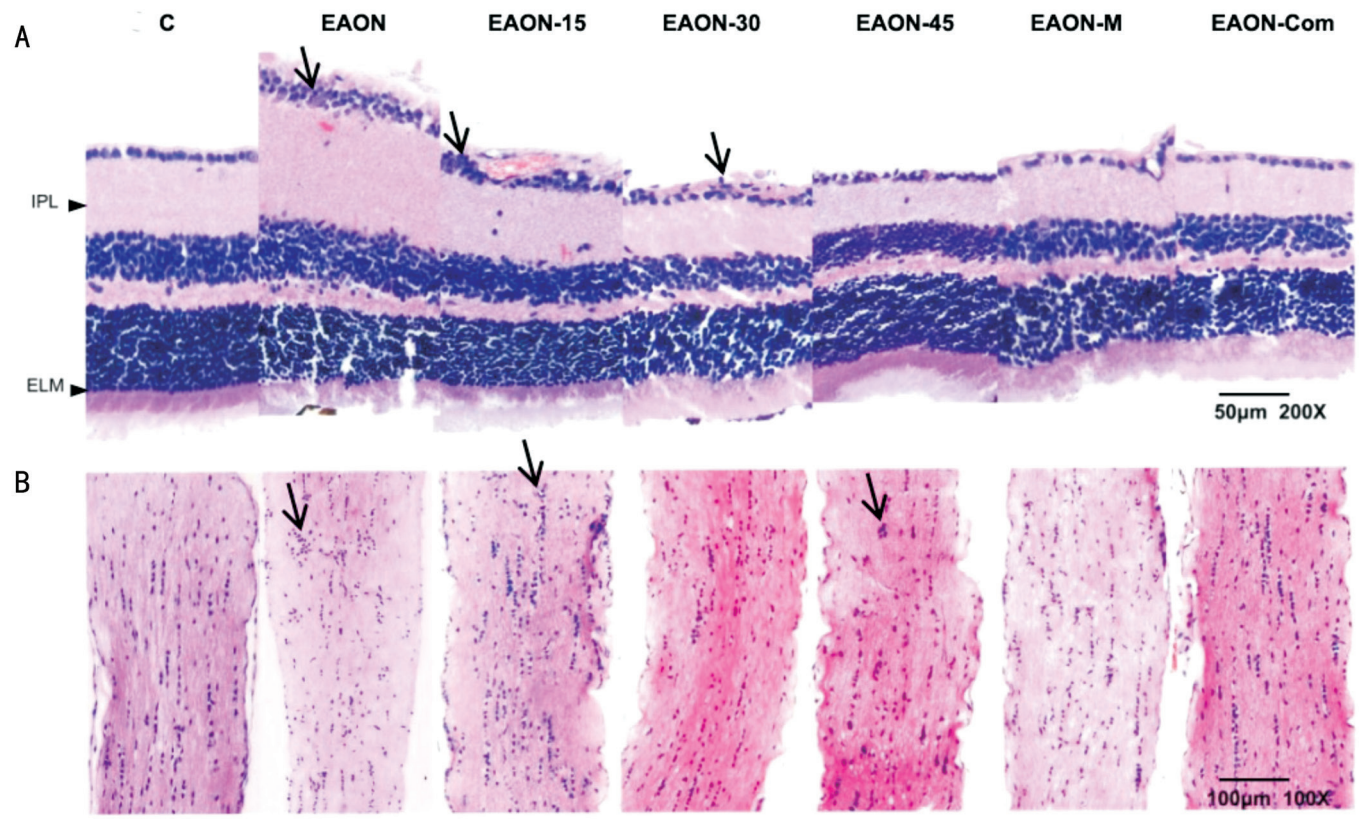

Figure 5 Gypenosides and combination treatment reduces inflammation in the retina and optic nerve during EAON at 20d p.i. A: A series of photographs of HE staining of the retina. Inflammatory cell infiltration is found in the model group and the gypenosides (15 and 30 mg/kg) group. We observe that the inflammation on the retina is reduced significantly in the gypenosides group (45 mg/kg), methylprednisolone group and combination group (arrows). Arrowheads indicate the IPL and ELM. IPL thickness increase in EAON mice indicate swelling might occur in the retina. B: A series of photographs of HE staining in the optic nerve. Abundant inflammatory cells are show in EAON optic nerves 20d p.i. Decreased inflammation is present in optic nerves from EAON mice treated with gypenosides and almost no inflammation is present in the combination treatment group (EAON-Com). Arrows point to regions of inflammatory cell infiltration.

the gypenosides (15 and 45 mg/kg) groups and almost no inflammation was present in the combination treatment group (Figure 5B).

DISCUSSION

Corticosteroids are commonly used in the treatment of ON, although their pharmacological effects are based on decreasing inflammation, not neuroprotection. Therefore, they have limited effects on improving the prognosis of visual acuity. Thus, a novel medicine that targets the neurodegeneration of MS and ON may be particularly beneficial. Gypenosides has been proven to have neuroprotective and anti-inflammatory effects in other diseases. In the present study, we tested the therapeutical effects of gypenosides and combination therapies (gypenosides + methylprednisolone) in EAON.

VEP is a noninvasive tool for investigating the function of the visual system and has been used to study the visual pathway function in models of EAE[10]. In general, VEP latency prolongation in ON may be related to the conduction velocity decreasing in demyelinating lesions. One study has observed that demyelination coincided with VEP latency prolongation[11]. In the present study, we utilized VEP to measure demyelination in ON. At day 20 p.i. (acute stage), VEP P2 latency of the model group was delayed compared with the control group. Compared with the model group, combination therapy significantly improved the outcome of VEP, suggesting a protective effect against demyelination. Meanwhile, we evaluated the optic nerve myelin by Lfb staining and obtained a similar result. We observed significant demyelination in the model group at 20d p.i., with the combination treatment group displaying decreased demyelination. Both VEP and myelin staining confirmed a protective effect of combination therapy but did not demonstrate such effect in gypenosides groups or Me group. These results suggested that gypenosides and methylprednisolone may have collective effects on demyelination in the acute stage. At 30d p.i. (chronic stage), VEP showed no significant differences between each group including the model group. However, Lfb staining showed methylprednisolone and combined treatment reduced demyelination. This may suggest a remyelination in the chronic stage. Although we found demyelination microscopically, there

were no differences in myelin conduction function. For further study, ultramicroscopic evaluation of several transections using transmission electron microscopy may further assess the demyelination.

By HE staining we also observed the protective effect. In the model group, many inflammatory cell infiltrates were observed in the retinal ganglion cell layer and optic nerve at 20d p.i. Further more, retinal thickness was increased, especially in the inner plexiform layer (IPL), indicated swelling may occur in the retina. Inflammation in the gypenosides groups was relatively decreased compared to that of the model group. In the methylprednisolone and the combination group, we discovered almost no inflammatory reaction. It is generally believed that methylprednisolone plays a strong role in the control of inflammation although the anti-inflammatory effect of gypenosides need to be further confirmed. At present, we have no direct evidence linking gypenosides to decreased inflammation. Further experimentation to assess the effects of gypenosides include evaluating T-helper (Th) 1 cell and Th 17 cell infiltration , characteristic in EAE, cytokines relating to these cells such as IFN-γ and IL-17, and inflammatory factors (chemokine, IL-6).

Our experiments showed that gypenosides and methylprednisolone combination therapy reduced demyelination in the acute phase of ON. The mechanism may be related to antioxidant effects of gypenosides Oxidative stress plays an important role in demyelination caused by ON. Specific CD4 + T cells trigger the inflammatory cascade, and the generation of oxygen free radicals causes the damage of oligodendrocytes. This oligodendrocyte-myelin-axonal unit injury induces nerve signal conduction disorders, and causes secondary symptoms of neurological deficit[12]. In addition, the production of a massive reactive oxygen species (ROS) attacks lipids and proteins and exceeds the antioxidant capacity of the body. The central nervous system is prone to damage by lipid peroxidation due to its high oxygen consumption and rich presence of lipid. Experiments show that the body's endogenous active oxygen scavenger is insufficient to protect the central nervous system against ROS-induced damage. Administration of an exogenous ROS scavenger can reduce blood-brain barrier injury and demyelination in EAON[13]. Recently, many experiments have confirmed the anti-oxidative effect of gypenosides, including as an anti-inflammatory. As an activator of peroxisome proliferator-activated receptor α (PPAR-α), gypenosides can inhibit the tissue factor activation and over-expression that occur in inflammatory processes mediated by lipopolysaccharides[14]. Gypenosides also reduce lipid peroxidation and DNA damage in low perfusion models in the cerebral cortex and hippocampus of rats[15]. At present, we have no direct evidence to prove gypenosides antioxidant effects in tissue, such as decreasing ROS or malondialdehyde (MDA), the final product of lipid peroxidation. This subject deserves further research.

Furthermore, axonal damage and loss of neurons are widely found in ON and MS. In the early stage of ON, axon loss and neuron injury may be a main cause of irreversible vision dysfunction[16]. The subsequent retrograde axonal degeneration causes thinning of the RNFL[17]. Our OCT measurement proved this point. At 40d p.i. (late stage), RNFL thickness in the model group and the methylprednisolone treatment group decreased significantly; however, no significant change was found in the gypenosides treatment groups compared with the control group. RNFL thickness in gypenosides (15 and 45 mg/kg) groups and the combination treatment group were relatively increased from that of the model group and methylprednisolone treatment group. This result suggests that gypenosides treatment, rather than methylprednisolone, provides the neuroprotective effect in the ON model. Furthermore, RNFL in the methylprednisolone treatment group deteriorated as much as it did in the EAON group, suggesting an adverse effect in neuroprotection, gypenosides combined therapy improves degeneration in late stage. Similarly, we observed axonal damage in the ON experimental group by Bielschowsky silver staining until 40d p.i. A significant axonal injury was found in the model group. The gypenosides group (30 mg/kg) and the combination therapy group (gypenosides 30 mg+Me 20 mg) alleviated axonal damage when compared with the EAON model. However, methylprednisolone alone did not alleviate the damage. In the late stage of EAON, gypenosides alleviates neurodegeneration and provides a protective effect in both OCT and axon staining .

Activated immune cells and glial cells release a variety of substances, such as inducible nitric oxide synthase (iNOS), matrix metalloproteinases (MMP), tumor necrosis factor, cytokines and oxygen free radicals[18]. Meanwhile, the accumulation of amyloid protein or metabotropic glutamate in axonal demyelination lesions can also lead to axonal damage[19]. Amyloid precursor protein, a characteristic sign in axonal damage, have been observed at demyelinating lesions[20]. A recent study has found gypenosides attenuates β amyloid-induced inflammation by reducing microglial activation, increasing the levels of iNOS expression, and increasing neurotrophic factor and IL-10 releases[21]. It is possible that in our study gypenosides plays a similar role in EAON.

Gypenosides neuroprotective effects in other diseases have been confirmed. The current studies are mainly focused on

cerebral ischemia-reperfusion injury, Parkinson's disease, and Alzheimer's disease. Studies have found that gypenosides protects cortical cells by multiple antioxidative actions, including improving intracellular reduced glutathione, suppressing cytosolic Ca^{2+} elevation and blocking apoptosis induced by glutamate[22]. In other experiments, gypenosides shows a dose-dependent effect in attenuated MMP (+) -induced oxidative damage. The neuroprotective effect of gypenosides may be attributed to increased glutathione content and enhanced activity of glutathione peroxidase and superoxide dismutase in nigral culture[23-24]. Gypenosides-17, a novel phytoestrogen isolated from *Gynostemma pentaphyllum*, conferred protection in a cell model of Alzheimer disease through activation of PI3K/Akt and Nrf2/ARE/HO-1pathways and inactivation of GSK-3β[25]. This experiment provided a novel perspective into the mechanism for the neuroprotective effects of gypenosides.

It is well known that corticosteroids are the main therapy for ON but will not improve the visual outcome. Moreover, they have adverse effects on neuron survival[4]. Therefore, corticosteroids together with an anti-inflammatory and neuroprotective medication, such as gypenosides, may bring a new hope to those patient suffering from ON.

In conclusion, this is the first report to demonstrate the ability of gypenosides to preserve visual functions in experimental optic neuritis, by providing neuroprotection and reducing demyelination and axon loss. These neuroprotective effects may be attributed to antioxidant or anti-inflammatory effects, or other mechanism such as alleviating β Amyloid, MMP, or glutamate-induced damage. Therefore, it is worth further investigation. Gypenosides may have a chance to be an effective adjuvant drug when combined with methylprednisolone in the future treatment of ON. With its neuroprotective effects presented in our study, gypenosides along with corticosteroids overcomes the deficiencies traditional therapy and may improve the vision prognosis in ON.

ACKNOWLEDGEMENTS

Foundations: Supported by the National Natural Science Foundation of China (No.81260149; No.81360152; No.81560162).

Conflicts of Interest: Zhang HK, None; **Ye Y**, None; **Zhao ZN**, None; **Li KJ**, None; **Du Y**, None; **Hu QM**, None; **He JF**, None.

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