RGC Neuroprotection Following Optic Nerve Trauma Mediated By Intranasal Delivery of Amnion Cell Secretome

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PURPOSE. Intranasally delivered ST266, the biological, proteinaceous secretome of amnion-derived multipotent progenitor cells, reduces retinal ganglion cell (RGC) loss, optic nerve inflammation, and demyelination in experimental optic neuritis. This unique therapy and novel administration route delivers numerous cytokines and growth factors to the eye and optic nerve, suggesting a potential to also treat other optic neuropathies. Thus, ST266-mediated neuroprotection was examined following traumatic optic nerve injury.

METHODS. Optic nerve crush injury was surgically induced in C57BL/6J mice. Mice were treated daily with intranasal PBS or ST266. RGC function was assessed by optokinetic responses (OKRs), RGCs were counted, and optic nerve sections were stained with luxol fast blue and anti-neurofilament antibodies to assess myelin and RGC axon damage.

RESULTS. Intranasal ST266 administered daily for 5 days, beginning at the time that a 1-second optic nerve crush was performed, significantly attenuated OKR decreases. Furthermore, ST266 treatment reduced damage to RGC axons and myelin within optic nerves, and blocked RGC loss. Following a 4-second optic nerve crush, intranasal ST266 increased RGC survival and showed a trend toward reduced RGC axon and myelin damage. Ten days following optic nerve crush, ST266 prevented myelin damage, while also inducing a trend toward increased RGC survival and visual function.

CONCLUSIONS. ST266 significantly attenuates traumatic optic neuropathy. Neuroprotective effects of this unique combination of biologic molecules observed here and previously in optic neuritis suggest potential broad application for preventing neuronal damage in multiple optic nerve disorders. Furthermore, results support intranasal delivery as a novel, noninvasive therapeutic modality for eyes and optic nerves.

Keywords: traumatic optic neuropathy, retinal ganglion cells, neuroprotection, ST266, amnion cell–derived therapy, intranasal drug delivery

Traumatic optic neuropathy1 can result from direct optic nerve injury in penetrating orbital trauma, but more commonly occurs indirectly due to optic nerve stretching from blunt head trauma. Traumatic optic neuropathy is one of the leading types of ocular injury sustained in military combat,2,3 as well as noncombat traumatic brain injury, in which 60% of traumatic head injuries result in neuro-ophthalmic abnormalities,4 with 50% of those involving the optic nerves or visual pathways. Traumatic optic nerve injury can result in retinal ganglion cell (RGC) axonal damage and irreversible RGC loss with permanent visual deficits. Medical and surgical treatments have all failed to improve outcomes, and some treatments carry significant risks.1,5 Thus, novel neuroprotective therapies are needed to prevent neuronal damage and loss of vision following optic nerve trauma.

ST266 (formerly amnion-derived cellular cytokine solution; ACCS) is the biological proteinaceous, secretome of a novel population of cells called amnion-derived multipotent progenitor (AMP) cells that contains physiologic levels of multiple growth factors and cytokines that can stimulate a variety of anti-inflammatory and neuroprotective responses in human cells.6–7 Intranasal delivery results in rapid accumulation of high levels of proteins from ST266 in the eye and optic nerve, and prevents visual dysfunction, RGC loss, and inflammation in an animal model of optic neuritis, an inflammatory disorder of the optic nerve.8 Detection of ST266 proteins in the eye and optic nerve within 30 minutes after intranasal administration suggests that proteins gain direct access into the central nervous system and are transported locally,9 representing a novel delivery method to target the eye that does not depend on systemic vascular absorption through the nasal mucosa. The combination of cytokines and growth factors present in ST266 suggests that it exerts effects by multiple mechanisms, but it remains unknown whether effects of ST266 in optic neuritis are due mainly to suppression of inflammation, direct neuroprotection of RGCs, or perhaps both. Interestingly, one pathway upregulated by ST266 in experimental optic neuritis involves the SIRT1 deacetylase.10 When activated, SIRT1 deacetylase has previously been shown to prevent RGC loss and preserve vision in both optic neuritis9–12 and traumatic optic nerve injury.13 Common
ST266 Neuroprotection in Optic Nerve Crush

FIGURE 1. ST266 preserves visual function following ONC. The right optic nerves of C57BL/6j mice were surgically exposed and crushed for 1 second with fine-tip forceps. Left eyes served as controls. Mice (n = 6 mice/treatment group) were treated daily with one intranasal drop of ST266 or sham-treated with PBS. OKR responses were measured before ONC injury (day 0) and repeated through 5 days post-ONC. ONC induced significant decreases in OKR responses as compared with normal OKR responses in control eyes (**P < 0.001). Treatment with daily ST266 led to significantly higher OKR responses in ONC eyes as compared with ONC eyes from PBS-treated mice (**P < 0.05). Data from one of two representative experiments shown.

METHODS

Mice

Seven- to 8-week-old C57BL/6j wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were housed at an animal facility at the University of Pennsylvania. Care and treatment of the animals, as well as all performed procedures, were conducted in accordance with the guidelines for the Institutional Animal Care and Use Committee at the University of Pennsylvania, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optic Nerve Crush

Optic nerve crush (ONC) was performed on 8- to 12-week-old C57BL/6j wild-type mice as in our prior studies, with minor modifications as described. Mice were anesthetized systemically with xylazine and ketamine and topically with 0.5% proparacaine eye drops. Under a dissecting microscope, the conjunctiva was lifted with fine forceps and cut using scissors, exposing the sclera. Forceps were used to manipulate and retract orbital fat and muscles, allowing for exposure of the optic nerve. Injury to the optic nerve was induced using curved fine-tip forceps to induce a focal crush injury to the optic nerve approximately 1 to 2 mm behind the globe. Maximal pressure was used to close the forceps for either 1 second or 4 seconds, as indicated in each experiment. Use of fine-tip forceps facilitated avoidance of orbital vessels, in an attempt to avoid ocular ischemia. Bleeding during the procedure was categorized as none, minimal, moderate, and large. Mice were excluded for more than moderate bleeding. In each experiment, the ONC was performed in one eye only, allowing the contralateral uninjured eye to serve as a control.

ST266 Treatment

ST266 was provided by Noveome Biotherapeutics, Inc. (Pittsburgh, PA, USA) and administered intranasally similar to prior studies. ST266 was shipped in aliquots on dry ice and stored at −20°C before use. Fresh aliquots were thawed and refrigerated, and were warmed to room temperature for treatment each day. Mice were randomized to treatment groups and treated with a single 20-µL drop of either undiluted ST266 or PBS delivered intranasally once daily; 20-µL drops were used, as this was the maximum volume that could be consistently administered into the nares without excess fluid draining outside the nose. Treatment began immediately after ONC and mice were treated once daily until they were killed.

RGC Labeling and Quantification

RGC immunolabeling and counting was performed as previously described. Summary; eyes were removed immediately after mice were killed and fixed with 4% paraformaldehyde (PFA). Retinas were carefully dissected and whole-mounted flat on prelabeled glass slides. They were then washed with PBS and permeabilized with 0.5% Triton X-100 in PBS at −70°C. The retinas were then thawed and rewashed with the 0.5% Triton X-100 solution, and incubated overnight with goat anti-Brown3a (RGC marker) antibody from Santa Cruz Biotechnology (Dallas, TX, USA), which was diluted 1:100 with blocking buffer solution for 1 hour, then washed with PBS four to five times. The retinas were then mounted with vectashield mounting medium for fluorescence. RGCs were photographed at a magnification of ×40 in 12 standardized fields at one-sixth, three-sixths, and five-sixths of the retinal radius from the center of the retina in four quadrants. A blinded investigator counted RGCs in each field using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA) software.

Optokinetic Responses (OKRs)

OKR was used as a measure of visual function in the mice before and after ONC injuries. OKR was assessed using OptoMotry software and equipment (Cerebral Mechanics, Inc., Medicine Hat, AB, Canada) as described in previous studies. OKR function is measured by determining the contrast grid, projected at different spatial frequencies. Data are reported in units of cycles/degree.

Myelin Staining

Demyelination of the optic nerve was assessed by methods outlined in a previous publication, through staining with luxol fast blue (LFB). After mice were killed, optic nerves were isolated and fixed in 4% PFA, then embedded in paraffin and cut longitudinally into 5-μm sections. The optic nerve sections were mounted, deparaffinized using a heating block, and stained with fast blue.
rehydrated. Sections were then stained with LFB, and the relative amount of staining was quantified for relative demyelination using a 0- to 3-point scale by a blinded investigator, where 0 = no demyelination, 1 = scattered small focal areas of demyelination, 2 = prominent areas of demyelination, and 3 = large confluent areas of demyelination.

Neurofilament Staining

Neurofilament (NF) staining was performed and quantified in accordance with previously published methods. Deparaffinized, rehydrated optic nerve sections were incubated with blocking solution from Vectastain Elite ABCkit (Vector Laboratories, Burlingame, CA, USA) in PBS, then incubated overnight at 4°C using a primary rabbit anti-neurofilament antibody (AbCam, Cambridge, UK) diluted 1:100 with PBS. The sections were then washed four times in PBS and incubated with goat biotinylated anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 hour at 37°C. The sections were then washed with PBS four times and incubated with ABC reagent using the Vectastain Elite ABC kit. The sections were then developed with DAB (diaminobenzidine) substrate from the DAB substrate kit (Vector Laboratories). Sections were placed in a running water bath to stop the DAB reaction, then dehydrated and mounted using Refrax mounting medium. The NF-stained sections were then photographed by a blinded investigator using a standard exposure at three distinct fields within each nerve, at the distal, central, and proximal regions.

Figure 2. ST266 reduces RGC axon loss. Mice (n = 6 mice/treatment group) were treated daily with one intranasal drop of ST266 or sham-treated with PBS beginning immediately after 1-second ONC was performed on the right eye only. Retinas and optic nerves were removed 5 days post-ONC. (A) ONC induced significant RGC loss, measured by Brn3a labeling, as compared with control eyes in retinas of PBS-treated mice (*P < 0.05). In contrast, RGC numbers were not significantly decreased compared with control eyes in ST266-treated mice, but showed only a trend toward increased RGC numbers compared with ONC eyes from PBS-treated mice. (B) Representative images (original magnification ×40) of RGCs from each group are shown. (C) ONC induced a significant decrease in RGC axon immunostaining with neurofilament antibodies as compared with control eyes in optic nerves of PBS-treated mice (***P < 0.001), and RGC axon staining was significantly higher in ONC eyes of ST266-treated mice compared with ONC eyes from PBS-treated mice (##P < 0.001). (D) A collage of images (original magnification ×40) of optic nerves from each group show variable neurofilament staining in ONC eyes reflective of focal areas of axon loss. Data from one of two experiments shown.
myelin loss in the ONC eye of an ST266-treated mouse (original magnification ×20). Data from one of two experiments shown.

Staining was quantified by measurement of optical density of each photograph, using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistics

Differences in OKR responses measured over multiple days were compared by ANOVA of repeated measures. Differences in RGC numbers, NF staining intensity of RGC axons, and LFB staining scores of demyelination were measured at single time points and compared using 1-way ANOVA. All ANOVA analyses were followed by Tukey’s Multiple Comparison post hoc testing to determine differences between individual groups. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SEM. Differences were considered statistically significant at \( P < 0.05 \).

Results

Intranasal ST266 Improves Visual Function and Reduces Optic Nerve Damage Induced by ONC

The right optic nerves of adult C57BL/6j mice were surgically exposed and briefly crushed with fine-tipped forceps for 1 second to induce a focal but significant axonal injury. Left eyes served as controls. Beginning immediately after ONC, mice were treated with an intranasal drop (20 μL) of either ST266 or PBS. ONC induced a severe decrease in OKR responses, measured daily over the next 5 days, that was significantly attenuated in mice treated with ST266 as compared with those treated with PBS (Fig. 1).

Mice were euthanized 5 days post-ONC, and retinal RGC numbers as well as axonal integrity of RGC axons in the optic nerve were quantified. ONC eyes from PBS-treated mice had significantly decreased numbers of surviving RGCs compared with control, non-ONC eyes (Figs. 2A, 2B). In contrast, RGC numbers were not significantly decreased in ONC eyes from mice treated with ST266, compared with control eyes, although ST266 did not increase RGC survival significantly compared with PBS-treated ONC eyes. Loss of RGC axon staining in ONC crush eyes of PBS-treated mice was significantly attenuated by ST266 treatment (Figs. 2C, 2D).

Examination of myelin staining along optic nerves showed significant loss of myelin induced by ONC in PBS-treated mice as compared with control, noncrush eyes (Fig. 3). Although ONC also induced significant demyelination in ST266-treated mice, the level of demyelination was significantly lower than that induced in PBS-treated mice with ONC injury. ST266 itself had no effect on healthy optic nerves, as there was no significant difference between control (non-ONC) eyes from PBS-treated versus ST266-treated mice in the number of RGCs (Figs. 2A, 2B), axon staining (data not shown), or demyelination score (data not shown).

Intranasal ST266 Exerts Partial Neuroprotective Effects Induced by More Severe ONC Injury

Initial results shown above indicate intranasally delivered ST266 can preserve visual function and reduce histopathologic damage induced by brief yet harsh optic nerve trauma. To determine whether these neuroprotective effects can be mediated in response to a more severe optic nerve injury, the right optic nerves of adult C57BL/6j mice were surgically exposed and crushed with fine-tipped forceps for 4 seconds to induce more severe RGC loss than found after 1-second ONC. Beginning immediately after ONC, mice were treated with an intranasal drop (20 μL) of either ST266 or PBS. ST266 was unable to attenuate a severe decrease in OKR responses induced by ONC, measured daily over the next 5 days (Fig. 4A); however, ST266 treatment did lead to a small yet statistically significant increase in RGC survival following ONC, with a nonsignificant trend toward increased RGC axon staining (Figs. 4B, 4C). ONC eyes from PBS-treated mice had significant demyelination compared with control, non-ONC eyes (Fig. 4D). In contrast, demyelination was not significantly increased in ONC eyes from mice treated with ST266, compared with control eyes, although ST266 did not decrease demyelination significantly compared with PBS-treated ONC eyes.

FIGURE 3. ST266 reduces demyelination. Optic nerve sections from mice \(( n = 6 \) mice/treatment group) treated daily with one intranasal drop of ST266 or sham-treated with PBS following 1-second ONC performed on the right eye only were stained with LFB to assess myelin. Optic nerves were removed 5 days post-ONC. (A) ONC induced significant demyelination as compared with control eyes in PBS-treated mice \(( *** P < 0.001 \), and ST266 treatment significantly reduced the level of demyelination induced by ONC \(( ^{\circ}P < 0.05 \) versus PBS-treated ONC eyes). (B) Representative images of myelin staining from each group show normal myelin in control eyes, focal myelin loss in an ONC eye from a PBS-treated mouse, and less myelin loss in the ONC eye of an ST266-treated mouse (original magnification ×20). Data from one of two experiments shown.
Intranasal ST266 Maintains Partial Neuroprotective Effects Up to 10 Days Following ONC Injury

Although initial experiments (Figs. 1–3) show intranasally delivered ST266 can preserve visual function and reduce histopathologic damage up to 5 days after brief, yet harsh, optic nerve trauma, it is possible ongoing damage may continue to accumulate over time with or without treatment. To determine whether these neuroprotective effects can be maintained longer, the right optic nerves of adult C57BL/6j mice were surgically exposed and crushed with fine-tipped forceps for 1 second, and, beginning immediately after ONC, mice were treated with an intranasal drop (20 μL) of either ST266 or PBS for the next 10 days. ST266 led to a notable, though nonsignificant trend toward improved OKR responses over the course of 10 days as compared with ONC eyes from PBS-treated mice (Fig. 5A); ST266 treatment prevented a statistically significant decrease in RGC axon staining compared with control eyes, as was induced in ONC eyes of PBS-treated mice (Fig. 5B). However, ST266 only led to a nonsignificant trend toward increased axon staining compared with ONC eyes of PBS-treated mice. ST266 treatment induced a trend toward increased RGC survival compared with control eyes (**P < 0.01), and ST266 treatment significantly increased RGC survival (***P < 0.001) after ONC compared with ONC eyes of PBS-treated mice. (D) OnC induced significant demyelination as compared with control eyes in PBS-treated mice (**P < 0.01), but not in ST266-treated mice. Data from one of two representative experiments shown.

DISCUSSION

Results show that intranasal delivery of ST266 significantly preserves visual function and reduces RGC damage and loss of myelin in the optic nerve following traumatic ONC injury. PBS-treated mice demonstrate an almost 80% decrease in visual function 1 day post-ONC, and 90% by day 2 (Fig. 1), suggesting...
that most RGC axons are injured during the crush. However, fewer than 50% of RGCs and their axons are lost in ONC eyes from PBS-treated mice (Fig. 2), suggesting that surviving crushed axons normally do not continue to convey visual signals. Thus, in addition to increasing the number of surviving RGCs, ST266 also preserves or restores visual function within those RGC axons that do survive. These effects are similar to neuroprotective effects of daily intranasal ST266 on vision and RGC survival in experimental optic neuritis, suggesting that ST266 can modulate conserved pathways leading to RGC survival in multiple optic neuropathies. Alternatively, the array of growth factors and cytokines present in ST266 may use different mechanisms to prevent optic nerve damage following trauma than it used to prevent damage in optic neuritis, as the complex nature of this biologic therapy is likely to stimulate multiple different signaling pathways.

Indeed, it was unknown whether ST266 would be able to exert similar neuroprotective effects in traumatic optic neuropathy as found in optic neuritis. Optic neuritis is an inflammatory disorder of the optic nerve associated with multiple sclerosis, and is frequently studied in autoimmune animal models of multiple sclerosis. ST266 itself, as well as the AMP cells that produce ST266, have been shown to exert significant anti-inflammatory effects, often studied in models of wound healing. Thus, prior effects observed in autoimmune optic neuritis may have been largely mediated by anti-inflammatory mechanisms that might not have been effective in a direct traumatic nerve injury model. Interestingly, some prior evidence suggested a potential direct neuroprotective effect of ST266, as local ST266 administration into the central nervous system reduced neurologic dysfunction and loss of neurons in a rodent model of traumatic brain injury. The current results suggest similar effects can be mediated by ST266 in optic nerve trauma using a noninvasive delivery method.

Figure 5. ST266 effects 10 days post-ONC. The right optic nerves of C57BL/6J mice were surgically exposed and crushed for 1 second with fine-tip forceps. Left eyes served as controls. Mice (n = 5 mice/group; ONC eye from one mouse in the PBS-treated cohort was not used in analyses, as the optic nerve was not well exposed and surgical crush could not be confirmed) were treated daily with one intranasal drop of ST266 or sham-treated with PBS for 10 days. (A) ONC induced significant decreases in OKR responses as compared with normal OKR responses in control eyes (***P < 0.001), with or without ST266 treatment, and ST266 treatment led to a trend toward increased OKR responses following ONC. (B) ONC induced a significant decrease in neurofilament staining of RGC axons 10 days post-ONC (*P < 0.05) that was not present with ST266 treatment. (C) ONC induced significant RGC loss compared with control eyes, with (***P < 0.01) or without (**P < 0.01) ST266 treatment, with ST266 inducing a trend toward increased RGC survival. (D) ONC induced significant demyelination as compared with control eyes in PBS-treated mice (**P < 0.01), and ST266 treatment significantly reduced the level of demyelination induced by ONC (P < 0.05 versus PBS-treated ONC eyes).
The combination of biologic molecules in ST266, including important growth factors and cytokines\textsuperscript{6,7} that can stimulate a variety of responses, makes it unlikely that all mechanisms underlying its neuroprotective effects can be fully determined, but contributions of specific pathways may be examined in the future. One candidate is the SIRT1 deacetylase pathway, which was shown previously to be upregulated in retina and optic nerve following intranasal ST266 treatment.\textsuperscript{8} This is particularly intriguing because treatment with genetic overexpression and pharmacologic activators of SIRT1 have previously been shown to exert similar RGC neuroprotective effects in both experimental optic neuritis as well as ONC injury models.\textsuperscript{9–13} As found in the current studies with ST266, together, these findings suggest it may be useful in future studies to examine the ability of ST266 to prevent RGC loss in the presence of SIRT1 inhibitors or genetic deletion of SIRT1 from specific retinal and inflammatory cells.

ST266 was administered once daily in the current study, as in prior studies of ST266 treatment for experimental optic neuritis.\textsuperscript{8} A higher volume (20 vs 6 μL/drop) was used in the current ONC experiments, although due to limitations of how much of that volume covers the area of absorption, likely through the cribriform plate, it is unclear whether increasing the volume of a single application leads to a significant increase in delivery of ST266 proteins to the eye and optic nerve. To better examine dose-effect responses to increasing ST266 therapy in ONC, future studies should entailing administration of ST266 multiple times daily instead of the once-daily treatment studied here, and direct delivery to the cribriform plate of the nasal passages using a targeted intranasal delivery device. Alternatively, concentrating the proteins present in ST266 in smaller volumes may also lead to increased efficacy. Interestingly, although once-daily ST266 for 5 days resulted in significant effects on visual function, RGC axons, and myelin, following a 1-second ONC, only partial neuroprotective effects were observed 10 days after similar injury as well as 5 days following a more severe injury induced by a 4-second ONC. Thus, increased dosing may indeed be necessary and should be considered for prolonged or severe injury.

Irrespective of the dosage used, the current findings further support intranasal drug delivery as a potential novel route of delivery for retinal and optic nerve disease. Although intranasal drug delivery has been used experimentally to bypass the blood-brain barrier to target the brain,\textsuperscript{20–31} as well as in clinical trials for Alzheimer’s disease,\textsuperscript{22,25,26} the use of intranasal delivery to target optic nerve degeneration has not been pursued as extensively reported before the recent ST266 studies in experimental optic neuritis.\textsuperscript{8} It has been shown that proteins targeted specifically to the nasal mucosa adjacent to the cribriform plate are able to travel along the olfactory nerve into the central nervous system,\textsuperscript{34} and this appears to be an important part of the mechanism by which ST266 proteins reach the eye. The mechanism by which proteins delivered intranasally subsequently reach the eye and optic nerves after gaining access to the olfactory nerve remains unclear, and warrants further investigation. The ability of intranasal proteins to reach the eye within 30 minutes after administration found in prior studies\textsuperscript{8} suggests perhaps some type of localized diffusion through lymphatic channels or along neuronal pathways may occur, and importantly indicates that the mechanism does not involve systemic distribution through the blood. Additional studies are needed to examine whether targeted intranasal delivery is feasible in humans and leads to similar accumulation in the eye, to establish clinical relevance. Intravitreal injection has become a common delivery method for treatment of retinal disease\textsuperscript{35}; however, intranasal administration of ST266 might allow for more frequent dosing, reduced patient discomfort, and reduced risk of complications compared with intravitreal medication delivery.

Overall, results suggest that ST266 significantly attenuates traumatic optic neuropathy. Neuroprotective effects of this unique combination of biologic molecules observed here and previously in optic neuritis suggest potential broad application for preventing neuronal damage in multiple optic nerve disorders. In addition, results support targeted intranasal delivery as a potential novel, noninvasive therapeutic modality for eyes and optic nerves that should be investigated further.

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