ORIGINAL ARTICLE

Protective effects of cerebrolysin in a rat model of optic nerve crush

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Abstract To investigate the effects of cerebrolysin (Cbl) on optic nerves (ON) and retinal ganglion cells (RGC) in a rat model of ON crush. Rats received intravitreal injection of Cbl (n = 20), intra-ON injection of Cbl (n = 20), intraperitoneal injection (IPI) of Cbl (n = 20), or phosphate buffered saline (PBS; n = 20) every day for 2 weeks after ON crush injury. At 3 weeks post-trauma, RGC density was counted by retrograde labeling with FluoroGold and visual function was assessed by flash visual-evoked potentials. Activities of microglia after insults were quantified by immunohistochemical analysis of the presence of ED1 in the optic nerve. At 3 weeks postcrush, the densities of RGCs in the Cbl-IVI group (1125 ± 166/mm²) and in the Cbl-IPI treatment group (1328 ± 119/mm²) were significantly higher than those in the PBS group (641 ± 214/mm²). The flash visual-evoked potential measurements showed that latency of the P1 wave was significantly shorter in the Cbl-IVI- and Cbl-IPI-treated groups (105 ± 4 ms and 118 ± 26 ms, respectively) than in the PBS-treated group (170 ± 20 ms). However, only Cbl IPI treatment resulted in a significant decrease in the number of ED1-positive cells at the lesion sites of the ON (5 ± 2 cells/vs. 30 ± 4 cells/high-power field in control eyes). Treatment with intra-ON injection of Cbl was harmful to the optic nerve in the crush model. Systemic administration of Cbl had neuroprotective effects on RGC survival and visual function in the optic nerve crush model.

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Introduction

Optic nerve (ON) injury leads to anterograde and retrograde degeneration, consequentially producing a scar at the site of injury and loss of retinal ganglion cells (RGCs). Although pulse steroid therapy is frequently used to treat acute traumatic optic neuropathy [1], there are no convincing data to indicate any effective treatment for traumatic optic neuropathy. In our previous studies we found that systemic human granulocyte colony-stimulating factor, but not corticosteroid treatment, had a neuro-protective effect in a rat model of optic nerve crush [2,3].

Neurotrophic factors that are upregulated by endogenous opioids are not sufficient to enable the rescue of damaged neurons [4]. Various neurotrophic factors are present in the central nervous system after injury, including brain-derived neurotrophic factor, nerve growth factor, ciliary neurotrophic factor, and glial cell line-derived neurotrophic factor [5]. Cerebrosylin (Cbl), the only drug available for clinical use that contains active fragments of some important neurotrophic factors [6,7], has been found effective in a number of clinical trials for the treatment of vascular dementia, stroke, and other neurodegenerative diseases [8–12]. Studies of the effectiveness of Cbl as a neuroregeneration treatment have been conducted in different injury models including traumatic brain injury, spinal cord injury, ischemic stroke, hyperthermia, drugs of abuse, and neuropathic pain [6,7,12–17]. In this pilot study that aimed to extend this research, we investigated whether Cbl has neuroprotective effects in an animal model of optic nerve crush.

Materials and methods

Cbl administration

Cbl; 215.2 mg/mL (EVER Neuro Pharma GmbH, Unterach, Austria) was used as the target drug in this crush model.

Animals

Adult male Wistar rats weighing 150–180 g (age 7–8 weeks) were obtained from the breeding colony of BioLASCO Co., Yu-Lan, Taiwan. The Institutional Animal Care and Use Committee at the Tzu Chi Medical Center, Hualien, Taiwan approved all animal experiments. All manipulations were performed with standard procedures as described in our prior paper [18].

Study design

A total of 80 rats underwent ON crush procedures in the right eyes and sham operations in the left eyes. Rats then received one intravitreal injection (IVI) of Cbl 2 μL (0.43 mg; n = 20), one intra-ON injection (IONI) of Cbl 2 μL (0.43 mg; n = 20), intraperitoneal injection (IPI) of Cbl (5 mL/kg; n = 20), or phosphate buffered saline (PBS; n = 20) every day for 2 weeks. Three weeks after surgery, RGC density was measured by retrograde labeling with FluoroGold (Fluorochrome LLC, Denver, CO, USA), and visual function was assessed by flash visual-evoked potentials (fVEP). Microglia activity after insult was quantified by immunohistochemical analysis of ED1 expression in the optic nerve.

Optic nerve crush injury experiments

ON crush injuries were induced as described in our previous reports [2,18]. Briefly, a standardized vascular clip (60-g microvascular clip; World Precision Instruments, Sarasota, FL, USA) was then applied to the ON at a distance of 2 mm posterior to the globe for 30 seconds. The left eyes received a sham operation that entailed optic nerve exposure without the crush procedure.

Retrograde labeling of RGCs with FluoroGold and morphometry of the RGCs

The detailed procedures have been described in our previous reports [2,3,18]. We performed retrograde labeling of the RGCs 1 week prior to when the rats were euthanized. In brief, the rats were anesthetized and placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). The 1.5 μL of 5% FluoroGold was injected into the superior colliculus on each side through a Hamilton syringe. One week after the labeling, the eyeballs were harvested after the animals had been euthanized. The retinas, examined with a 400× epi-fluorescence microscope (Axioskop; Carl Zeiss Meditec Inc., Thornwood, NY, USA), were examined for RGCs at a distance of 1 mm from the center to provide the central RGC densities. We counted at least five randomly chosen areas of 62,500 μm² each in the central regions of each retina, and their averages were taken as the mean density of RGCs per retina (n > 6 in each group).

fVEP

An isolated silver plate electrode was placed extradurally through a 2-mm diameter craniotomy over the visual cortex using stereotactic coordinates (bregma –8 mm, lateral 3 mm) [18,19]. We used a visual electrodiagnostic system (UTAS-E3000, LKC Technologies, Gaithersburg, MD, USA) to measure the fVEP [18]. When the wave was nonrecordable, the latency of P1 was set at 200 ms for comparison.

Immunohistochemistry (IHC) of ED-1 (CD68) in the ONs

The experiment was performed as described previously [3,20]. In short, the primary antibody (1:50; AbD Serotec, Oxford, UK) was applied and incubated overnight at 4°C. The secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was applied at room temperature for 1 hour. Counterstaining was performed using 4’,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma, St Louis, MO, USA). For comparison, the ED1-positive cells
were counted in six high-power fields (HPFs) at the ON lesion site.

**Statistical analysis**

Data are presented as mean values with standard deviations. One-way analyses of variance (ANOVA) was used to evaluate the differences between treated groups. A $p$ value of $<0.05$ was considered to represent statistical significance.

**Results**

**RGC densities after ON crush**

The mean density of RGCs in the center of normal eyes was 2535 ± 123/mm². Three weeks after ON crush, the mean central RGC density was 641 ± 214/mm². The densities of the RGCs in the center of the retina were significantly higher in the Cbl-IVI group (1125 ± 166/mm²) and the Cbl-IPI group (1328 ± 119/mm²) than in the PBS group (641 ± 214/mm²; both $p < 0.001$). However, there were no significant differences in RGC count between the Cbl-IONI group and the PBS group (Fig. 1).

**fVEP**

The fVEP changes were measured 3 weeks after ON crush induction. The latencies of the $P_1$ wavelet were 119 ± 1 ms in the sham group, 170 ± 20 ms in the PBS group, 105 ± 4 ms in the Cbl-IVI group, 161 ± 35 ms in the Cbl-IONI group, and 118 ± 26 ms in the Cbl-IPI group. Latency of the $P_1$ wavelet was more significantly preserved in the Cbl-IVI and Cbl-IPI groups than in the PBS group (Fig. 2, $n = 6$ in each group, both $p < 0.001$). Cbl-IONI had no effect on shortening the latency of the $P_1$ wavelet.

**ED1 expression in optic nerves**

Very few ED1-positive cells were noted in the sham group (1 ± 1 cells/HPF); however, a prominent number of ED1-positive cells were noted in the Cbl-IVI and Cbl-IPI groups.

![Figure 1. Improvement in retinal ganglion cell (RGC) density in central retinas after cerebrolysin (Cbl) treatment via intravitreal (IVI) and intraperitoneal (IPI) injection was noted at the 3rd week postcrush. (A) In the sham group, the density of RGCs was 2535 ± 123/mm². (B) RGC densities were significantly decreased in the phosphate-buffered saline (PBS)-treated group (641 ± 214/mm²). (C) The density of RGCs was significantly higher in the Cbl-IVI treatment group (1125 ± 166/mm²) than in the PBS-treated group ($p < 0.001$). (D) After Cbl intraoptic nerve injection, the density of RGCs increased to 924 ± 112/mm²; however, no significant difference was noted between that group and the PBS group. (E) After Cbl-IPI treatment, the density of RGCs significantly increased to 1328 ± 119/mm². **$p < 0.001$. $n > 6$ in each group. Bar = 50 μm. C = crush.](image-url)
positive cells in the lesion areas was noted in the crush-PBS group (30 ± 4 cells/HPF). ED1 in the ON was detected in 33 ± 5 cells/HPF in the IVI-Cbl group, in 39 ± 9 cells/HPF in the IONI-Cbl group, and in 5 ± 2 cells/HPF in the IPI-Cbl group. The number of ED1-positive cells was significantly lower in the IPI-Cbl group than those of the other treatment groups (Fig. 3, n = 6 in each group, p < 0.001). There was no significant difference in the number of ED1-positive cells between the Cbl-IVI group or the Cbl-IONI group and the PBS-treated group.

Discussion

In vivo and in vitro data show that Cbl substantially augments neurogenesis and neuroplasticity [13,17,21–23]. Our study demonstrated that the neuroprotection induced by Cbl-IVI and Cbl-IPI may contribute to the improved visual functional outcomes after optic nerve crush injury.

In consideration of prior clinical reports indicating the good efficacy of subtenon injection as well as intravitreal injection in the treatment of ocular disease, we designed direct injection of cerebrolysin into the ON sheath (IONI) [24–26]. Low-molecular-weight peptides of cerebrolysin are able to cross the blood–retinal barrier [27] and, with its crossing, it is possible that systemic cerebrolysin can pass through the blood retinal barrier and induce the positive effect in neuroprotection in the optic crush model. Although local administration of the drug may yield better results and fewer side effects than systemic applications in ON insults, IONI may cause additional trauma to the optic nerve, and for this reason our data show no neuroprotection effect in the IONI group.

Applications of Cbl in different animal models have been reported [4,14,16,17,28]. Sharma et al. [29] studied Cbl treatment in a rat model of spinal cord injury and found that a high dose of Cbl (5 mL/kg) was very effective in reducing the degree of axonal changes in morphology after injury. They also found that Cbl markedly reduced the degree of neurotoxicity induced by hyperthermia [15]. In a neurodevelopmental rat model of schizophrenia, Vázquez-Roque et al. [28] found that chronic administration of Cbl ameliorates the behavioral and morphological changes induced by lesions of the neonatal ventral hippocampus. In addition, in a rat model of embolic artery occlusion, Zhang et al. [17] found that Cbl treatment administered <48 hours after stroke enhances neurogenesis and improves functional outcome, also, using a transgenic model of Alzheimer’s disease, Rockenstein et al. [16] found that Cbl reduced amyloid burden and improved synaptic plasticity.

Results of the double-blind, placebo-controlled randomized clinical trial of Cbl (30 mL/day for 10 days) in acute ischemic stroke in Asia has shown favorable outcomes in patients with National Institutes of Health Stroke Scale scores >12 who were treated with Cbl [30]. Two large clinical trials of Cbl as treatment for Alzheimer’s disease have also been conducted [9,11]. One of the trials studied the effect of two doses of Cbl (10 mL and 30 mL, 5 days/week for 4 weeks) on patients with mild to moderately severe vascular dementia and found that patients who received Cbl showed better cognitive performance than patients who received placebo [11]. The other trial studied the effect of three doses of Cbl (10 mL, 30 mL, and 60 mL for 12 weeks) on patients with moderate to moderately severe Alzheimer’s disease and found that patients in all three Cbl treatment groups had significantly better global clinical function than patients who received placebo [9].

Zhang et al. [17] reported that Cbl activated Akt in neural progenitor cells and that blockage of the PI3K/Akt pathway with a PI3K/Akt inhibitor (LY294002) abolished Cbl-induced cell proliferation. In our previous study, we found that granulocyte colony-stimulating factor also upregulated the PI3K/Akt signaling to protect RGCs from apoptosis in an animal model of optic nerve crush [2]. Other studies have shown that Cbl protects against nerve damage by downregulating microglial activation as well as by controlling IL-1 beta expression [31,32]. Our results demonstrate that only Cbl administration by systemic application

Figure 2. Improvement in latency of P1 in flash visual-evoked potential after cerebrolysin (Cbl) treatment via intravitreal (IVI) and intraperitoneal (IPI) injection was noted at the 3rd week postcrush. Both Cbl-IVI- and Cbl-IPI-treated groups had shorter P1 latency (105 ± 4 ms and 118 ± 26 ms, respectively) than the phosphate buffered saline (PBS)-treated group (170 ± 20 ms; ** all p < 0.001). No significant shortening of P1 wave latency was noted in the Cbl-IONI treatment compared with the PBS-treated group (161 ± 35 ms vs. 170 ± 20 ms, respectively). n = 6 in each group. C = crush.
can reduce the infiltrations of microglia/macrophage cells at the lesion site of optic nerve from the evidence of ED1 stain, which was not decreased in the IVI group. A possible explanation for this is that there was only one shot in this IVI group and/or the concentration of drug (0.43 mg/2 μL) may not have reached the therapeutic level in this optic nerve crush model. We need further evaluation for optimal dosage of Cbl in the IVI treatment because of dose-specific effects [31]. Further in-depth studies for signal pathway with inhibitors such as PI3K/Akt or ERK pathway with a PI3K/Akt inhibitor (LY294002) are needed.

In conclusion, systemic administration of Cbl seems to afford neuroprotection in the rat model of optic nerve crush, as evidenced by RGCs surviving and visual function assessment. These findings support the possibility that Cbl can have positive effects in the management of traumatic optic neuropathy.

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References


