Original Article

DOI: 10.5582/ddt.2025.01101

Verification of the protective effect of transfecting brain-derived neurotrophic factor and B-cell lymphoma 2 genes on retina ganglion cells in a rat model of optic nerve injury

Zijia Zhao[1], Yuan Fang[1], Gezhi Xu[1], Jiawei Xiong[1], Xiaofen Mo[1,2,3], Jiawen Fan[1,*]

[1] Eye Institute and Department of Ophthalmology, Eye & ENT Hospital, Fudan University, Shanghai, China;
[2] NHC Key Laboratory of Myopia (Fudan University); Key Laboratory of Myopia, Chinese Academy of Medical Sciences, Shanghai, China;
[3] Shanghai Key Laboratory of Visual Impairment and Restoration, Shanghai, China.

SUMMARY: This pilot study investigated the protective effect of transfecting brain-derived neurotrophic factor (*BDNF*) and B-cell lymphoma 2 (*bcl-2*) genes in retinal ganglion cells (RGCs) using *in vivo* electroporation in an adult rat optic nerve transection model. Sprague-Dawley rats were randomly divided into five groups: BDNF(+)/bcl-2(+), BDNF(+), bcl-2(+), empty plasmid (EP), and no surgery (NS). The plasmids were intravitreally injected and electroporated into the left eye. Seven days later, optic nerve transection was performed in all groups except the NS group. Protein expression was examined using Western blotting, RGC survival was quantified using 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) retrograde labeling, and apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) at multiple time points (7, 14, and 28 d after transfection). A significantly higher number of DiI (+) RGCs and lower number of apoptotic cells were observed in the BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups compared to those in the EP group at all time points. The number of DiI (+) RGCs in the three treatment groups was significantly lower than that in the NS group. However, there were no significant differences among the three treatment groups. The protective effects of gene transfection tended to be strongest in the BDNF(+)/bcl-2(+) group, followed by the BDNF(+) group and then the bcl-2(+) group. Thus, all gene transfection treatments had a protective effect against the loss of DiI(+) RGCs induced by optic nerve transection but did not result in full recovery. This study also confirmed the value of *in vivo* electroporation. The findings of this pilot study provide a working base for the development of gene therapy for blinding optic nerve disorders.

Keywords: electroporation, gene therapy, retinal ganglion cells, brain-derived neurotrophic factor, bcl-2

1. Introduction

Degeneration and death of retinal ganglion cells (RGCs) are observed in many blinding optic nerve disorders, with glaucoma being the most prevalent. Loss of RGCs contributes substantially to the development of irreversible vision loss (*1,2*). After optic nerve injury, axonal disruption triggers apoptotic RGC death, which is difficult to ameliorate with currently available treatments. Experimental studies have shown that if the optic nerve axon is compressed or severed, its connected ganglion cells will initiate apoptosis (*3*). Multiple mechanisms have been reported to be involved in the degeneration and death of RGCs, including intraocular pressure-independent oxidative stress, axonal transport disruption, neurotrophic factor deprivation, and activation of apoptotic and autophagic pathways (*4,5*).Currently, efficient treatments for RGC-related optic nerve injuries are insufficient. Accordingly, the development of novel treatments with satisfactory safety and efficacy has been an urgent task for ophthalmologists. Quigley proposed that the promotion of RGC growth, along with the prevention of apoptosis, is efficient in terms of protecting RGCs (*6*). Our previous work confirmed that transfection of the brain-derived neurotrophic factor (*BDNF*) gene into RGCs using an improved *in vivo* electroporation technique has a high transfection efficiency. Moreover, transfection of *BDNF* gene *per se* had protective effects on rat RGCs after optic nerve amputation (*7*).

Gene therapy has emerged as a promising strategy to protect RGCs by delivering neuroprotective or anti-apoptotic genes into RGCs; however, most existing studies have only focused on a single factor and have seldom observed multiple factors. For example, overexpression of BDNF *via* viral or other vectors results in significant RGC rescue; however, it only achieves

transient efficacy (*8*). Anti-apoptotic factors, such as members of the B-cell lymphoma (bcl) family (including bcl-2 and bcl-XL), oppose intrinsic apoptotic pathways. However, studies combining bcl-2 and neurotrophins in animal models of RGC injury are limited (*9*).

Based on the aforementioned information, we hypothesized that transfecting the *BDNF* (a neurotrophic factor) gene, the *bcl-2* gene (an anti-apoptotic gene), or co-transfecting both might result in yield protective effects in RGCs following optic nerve transection. Our main aim was to test whether transfection of *BDNF*, transfection of *bcl-2*, or co-transfection of both might result in better RGC survival and reduce cellular apoptosis. We believe that the findings of this pilot study will be helpful in developing a novel and effective gene therapy for the treatment of optic nerve injury.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley (SD) rats (9 weeks of age; weight, 200-250 g) were housed in a temperature-controlled room. The animals were kept on a 12-hour light-dark schedule and had free access to food and water. The treatment of all animals conformed to the National Institute of Health Principles of Laboratory Animal Care, the Association for Research in Vision and Ophthalmology (ARVO) Statement of the Use of Animals in Ophthalmic and Vision Research, and local institutional guidelines. All experiments were approved and supervised by the Ethics Committee for the Welfare of Laboratory Animals of the Eye and ENT Hospital of Fudan University (approval number: 2023DW003).

2.2. Plasmid construction and preparation

The plasmid vector, pbcl-2-green fluorescent protein (GFP), carrying the *bcl-2* and *GFP* genes, was obtained from the Institute of Neurobiology (Fudan University, Shanghai, China). The plasmid was transfected into *Escherichia coli* DH 5α competent cells, multiplied by culturing the *E. coli* cells, and then isolated and purified using an EndoFree Plasmid Maxi Kit (Qiagen, Santa Clarita, CA, USA). The plasmid was dissolved in Tris-HCl buffer with ethylenediaminetetraacetic acid (TE buffer) (Qiagen, Hilden, Germany) at a concentration of 2.5 μg/μL.

Plasmid pRc/cytomegalovirus (CMV) BDNF, containing mouse *BDNF* cDNA with a fragment length of 750 bp, was combined as previously described (*7*). Primers were designed and synthesized for amplification. The upstream primer sequence was 5'-ATG ACC ATC CTT TTC CTT ACT ATG-3', and the downstream primer sequence was 5'-CCA CTA TCT TCC CCT TTT AAT GG-3' (synthesized by Shanghai Sangon Biotech Co., Ltd., Shanghai, China). A polymerase chain reaction (PCR) reaction (consisting of 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s) was used to amplify the target genes. The resulting PCR product, approximately 750 bp in length, was electrophoresed on a 1% agarose gel, and the PCR product was purified.

The ligation reaction was performed in a final volume of 5 μL, containing 1 μL of purified PCR product, 1 μL of salt solution, 2 μL of sterile water, and 1 μL of TOPO vector provided in the GFP Fusion Protein Topoisomerase (TOPO) Expression Kit (NT-GFP Fusion TOPO TA Expression Kit; Invitrogen).

The reaction mixture was incubated at room temperature for 5 min and then transferred to ice. Then, 2 μL of the reaction mixture was added to competent *E. coli* cells and mixed gently. The mixture was incubated on ice for 30 min, subjected to heat shock for 30 s at 42°C, and then returned to ice. After adding 250 μL of Super Optimal broth with Catabolite repression (SOC) medium, the tube was capped, and the mixture was incubated at 37°C with shaking at 250 rpm for 1 h. After incubation, the cells were spread on pre-prepared Luria–Bertani Agar (LBA) plates, which were then placed in a 37°C incubator overnight. The next day, bacterial colonies on the plates were marked and single colonies were selected. Each colony was used as a template and subjected to PCR using the upstream primer for BDNF (as mentioned earlier) and the downstream primer BGH provided by the vector plasmid (5'-TAG AAG GCA CAG TCG AGG-3'). The PCR conditions were the same as those described previously (*7*). The PCR products were then electrophoresed, and colonies with bands of approximately 850 bp were considered positive. Positive colonies were streaked again to obtain single clones.

Single colonies from the streaked plates were transferred into LB medium containing ampicillin and incubated on a shaker at 37°C and 250 rpm overnight. A portion of the bacterial culture was stored, and the remaining portion was sent to Shanghai Sangon Biotech Co., Ltd. for sequencing. After sequencing, the stored bacterial culture was used to expand and extract plasmids using an Endofree Plasmid Purification Maxi kit (Qiagen). The purity of the plasmids was checked, and the concentration was adjusted to 2.5 μg/μL in 1 mM Tris-EDTA (TE) buffer (Qiagen).

2.3. Animal grouping, intravitreal injections, and electroporation

The entire grouping and experimental protocols are shown in Figure 1A. In rats, deep anesthesia was induced using an intraperitoneal injection of 500 mg of urethane, 11 mg of ketamine, and 14 mg of xylazine per kilogram of body weight (*7*). Subsequently, the left eye and left optic nerve (ON) were exposed (Figure 1B). The intraocular pressure was initially reduced through paracentesis before the surgery. A 31-gauge (G) needle was used to create a tiny hole in the rats' sclera,

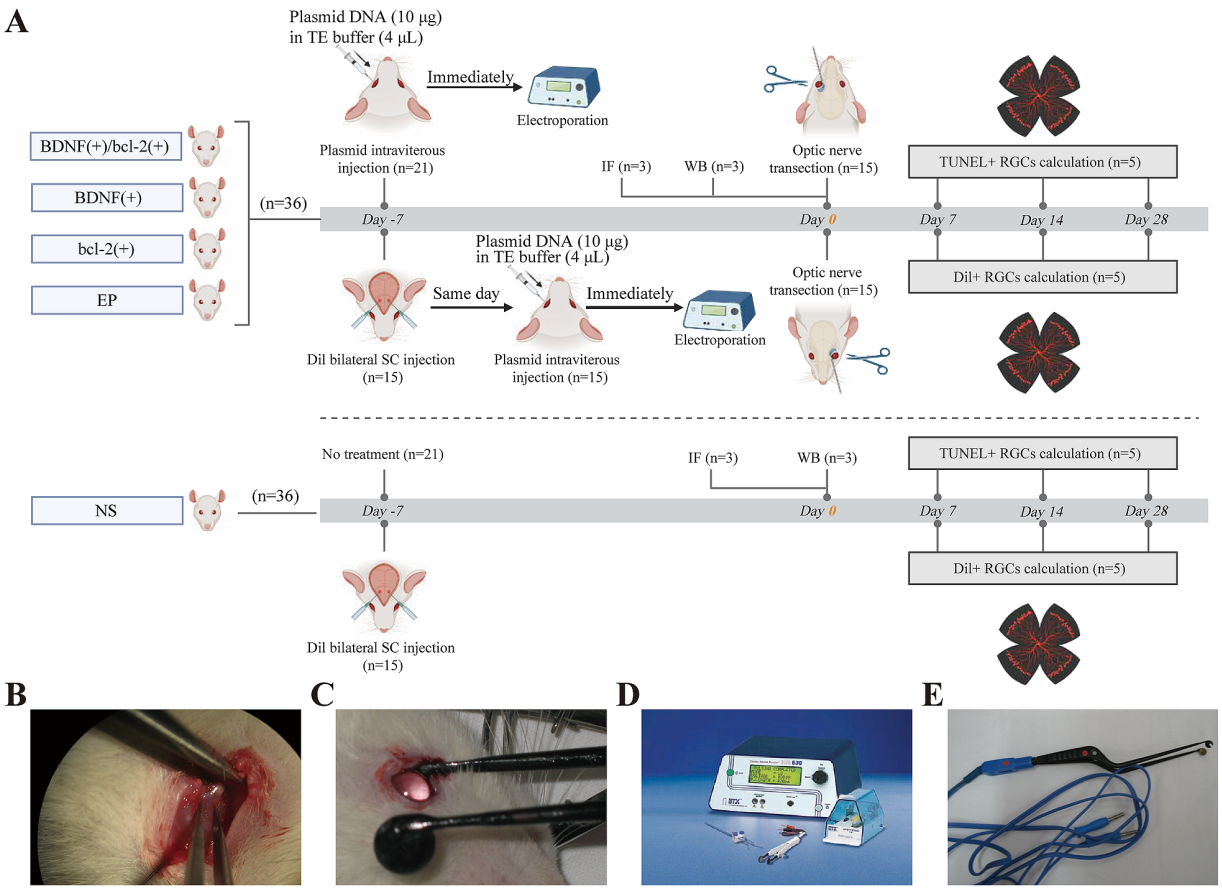

Figure 1. Diagram of the experimental protocol. (**A**) Entire grouping and experimental protocol; (**B**) Optic nerve exposure during surgery; (**C**) Self-developed electrodes, with the cathode connected to the corneal electrode and the anode connected to the scleral electrode; (**D**) ECM830 electroporator from BTX; (**E**) Self-developed electrode for rat experiments. bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; EP, empty plasmid; IF, immunofluorescence; NS, no surgery; RGC, retinal ganglion cell; SC, superior colliculi; TE buffer, Tris-HCl buffer with ethylenediaminetetraacetic acid; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WB, Western blotting.

positioned 0.5 to 1.0 mm posterior to the limbus. The tip of the 31G needle was observed after entering the vitreous at a 40 to 50-degree angle through the dilated pupil. Subsequently, 10 μg of plasmid DNA in 4 μL of TE buffer was gently injected into the vitreous body (*10,11*).

Subsequently, the eye was immediately held and grasped using forceps-type electrodes, with the cathode connected to the corneal electrode and anode connected to the scleral electrode (Figure 1C), as previously described (*11,12*). Square-wave pulses lasting 99 ms were then applied, with a field strength of 12 V/cm, and five pulses were delivered. This process was repeated twice with a 5-min interval between applications using an Electro Square Porter T820 device (BTX, Holliston, Massachusetts, USA) (Figures 1D and E). Rats that received 4 μL of the *BDNF* gene before electroporation were referred to as the BDNF(+) group. Rats receiving 4 μL of the *bcl-2* gene solution before electroporation were referred to as the bcl-2(+) group. Rats receiving 2 μL of the *BDNF* gene and 2 μL of the *bcl-2* gene along with electroporation were referred to as the BDNF(+)/bcl-2(+) group, and those rats injected with 4 μL of plasmid pRc/CMV with electroporation but containing no *BDNF* cDNA or *bcl-2* cDNA were referred to as the empty plasmid (EP) group. Rats that did not receive an intravitreal injection were referred to as the no surgery (NS) group.

2.4. Fluorescence observation in retinal whole mounts

To confirm the transfection of *BDNF* and *bcl-2* into RGCs, connected GFP fluorescence was observed in the rat retina in the BDNF(+) (n = 3), bcl-2(+) (n = 3), BDNF(+)/bcl-2(+) (n = 3), EP (n = 3), and NS (n = 3) groups. One week after *in vivo* electroporation, all rats were killed by intracardiac perfusion of 4% paraformaldehyde in 0.1 M PBS under deep ether anesthesia. The left eyeball was then immediately removed, post-fixed in 4% paraformaldehyde for one hour, and then placed in phosphate-buffered saline (PBS) at 4°C. For the observation of fluorescence in retinal wholemounts, retinas were isolated and cut into a four-leaf clover structure within 24 h after eyeball isolation.

Retinas were then mounted using glycerol buffer and observed under a fluorescence microscope (Leica, Wetzlar, Hesse, Germany) to visualize the expression of green fluorescent protein.

2.5. Western blotting (WB)

WB was performed to study the changes in *BDNF* and *bcl-2* gene expression. Treated retinal tissues were lysed using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA.) with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein concentrations were measured using bicinchoninic acid assays (QuantiPro BCA Assay kit, Sigma-Aldrich). Lysate proteins were separated on NuPAGE Novex 4–12% Bis-Tris 1 mm-thick mini-gels (Life Technologies, Carlsbad, CA, USA) by electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for blotting. The membranes were incubated overnight at 4°C with affinity-purified rabbit polyclonal anti-human BDNF (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-human bcl-2 (bcl-2-100, Invitrogen) primary antibodies. After three washes in tris-buffered saline (TBS)-T (0.1% Tween 20 in TBS), the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (Cell Signaling, Danvers, MA, USA) diluted 1:1,000 in TBS-T for 2 h at room temperature and washed again in TBS-T. Detection was performed using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden). β-actin (Cell Signaling#4967, 1:2,000) served as the loading control. Protein bands were observed using an IMAGE READER LAS-3000 imaging system (Fujifilm, Tokyo, Japan) and analyzed using the software IMAGE READER LAS-3000 (V2.2, Fujifilm).

2.6. Transection of the optic nerves

Optic nerve transection was performed to induce RGC apoptosis 7 d after intravitreal injection combined with electroporation, as described earlier (*13*). The protocol was improved by our group and implemented in all groups except the NS group. Briefly, the rats were anesthetized with 1 mL/kg of ketamine and 0.0625 g/kg of xylazine *via* intramuscular injection. After anesthesia, the orbital tissues were re-isolated and the optic nerve was exposed. A sharp blade was used to open the optic nerve sheath to expose the optic nerve and avoid blood vessels. Micro-scissors were used to cut the optic nerve at a position approximately 1.0 mm and 1.5 mm behind the eye, and a segment of approximately 0.5 mm was removed to ensure complete transection of the optic nerve. The orbital tissues were carefully restored, and the conjunctiva was sutured. The fundus was observed using indirect ophthalmoscopy through dilated pupils to confirm normal retinal circulation.

2.7. Retrograde tracing of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) to count living RGCs after optic nerve transection

Retrograde tracing of DiI refers to a method used to retrogradely trace the projections of neurons using DiI fluorescent labeling. In this technique, DiI dye is injected into the target area, and the labeled neurons are subsequently observed in a retrograde manner based on their inherent fluorescence properties, both near the injection site and distant from the injection site. This approach is widely used in neuroscience research, enabling researchers to gain insights into the connections and neural circuits of neurons (*14,15*). We used DiI (Molecular Probes, Invitrogen) dissolved in *N*,*N*-dimethylformamide (DMF). Fifteen randomly selected SD rats from each group were anesthetized with an intramuscular injection of 1 mL/kg ketamine and 0.0625 g/kg xylazine and then fixed in a stereotaxic apparatus. The skin on the top of the head was incised to expose the skull, and bilateral holes were drilled at the corresponding positions of the dorsal lateral geniculate nucleus using an electric drill. According to Paxinos' rat brain stereotaxic atlas (*16*), the superior colliculi (SC) was localized 20 mm anterior to the bregma, 12 mm lateral to the midline, and at a depth of 32 mm. A total of 5 μL of DiI at a concentration of 150 μg/μL was injected into each side using a 10-μL microsyringe. After the injection, the scalp was sutured layer by layer. On the same day as the DiI injection, a combination of intravitreal injection of the plasmid and *in vivo* electroporation was performed.

Seven days after retrograde tracing with DiI, the optic nerves were transected as described above. Five rats from each group at each time point were euthanized 7, 14, and 28 d after optic nerve transection. Retinal wholemounts were prepared as described in the 'Fluorescence observation' section above. Wholemounts were sealed with glycerol buffer and observed under a fluorescence microscope (DRM model; Leica) to visualize DiI-labeled positive RGCs. Images were collected and analyzed using the software QWin (V3, Leica) to calculate the area and number of RGCs. Calculations were performed in four quadrants, with the optic disc at the center. In each quadrant, DiI-labeled positive cells were counted at 1.5, 2.5, and 3.5 mm from the center, with a total of 12 regions for observation and counting in each sample (*7*). The results were calculated and converted to the number of surviving RGCs per square millimeter of the area.

2.8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

To analyze RGC apoptosis 7, 14, and 28 d after optic nerve transection, five rats from each group at each time point were euthanized, and retinal tissue paraffin sections (six sections per eye) were prepared. After pretreatment,

an *In Situ* Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA) was used for proteinase K digestion and terminal deoxynucleotidyl transferase (TdT) labeling, followed by biotin-labeled fluorescence conjugation. The sections were then sealed with 20% glycerol buffer and observed under a fluorescence microscope to identify the apoptotic RGCs. The number of apoptotic RGCs per millimeter of length was counted and calculated for each group.

2.9. Statistical analysis

In this study, statistical analyses were performed using the software SPSS (V 29.0.1.0, IBM, Chicago, IL, USA) and GraphPad Prism 10 (V10.2.3, GraphPad Software, MA, US). Each experiment was performed in triplicate. The normal distribution of the data was confirmed using the Shapiro-Wilk test. Data are expressed as the mean ± standard deviation (SD). Comparisons between the two groups were done using paired or unpaired *t*-tests. Statistical evaluation among multiple groups was done using one-way analysis of variance (ANOVA) followed by Bonferroni's test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Confirmation of *BDNF* and *bcl-2* gene expression in RGCs

As shown in Figure 2, no green fluorescence of GFP was observed in the NS and EP groups (Figure 2A). In contrast, distinct green fluorescence signals were detected in the BDNF(+), bcl-2(+), and BDNF(+)/bcl-2(+) groups 7 d after electroporation and plasmid transfection (Figure 2B). Accordingly, expression of the *BDNF* and *bcl-2* genes in RGCs was confirmed

3.2. Verification of gene expression after transfection of the *BDNF* and *bcl-2* genes

Figure 3 shows the results of gene transfection 7 d after plasmid transfection, as indicated by WB. The BDNF(+) group exhibited the highest expression of BDNF protein compared to the other groups, and the BDNF(+)/bcl-2(+) group also had a higher level of BDNF protein expressed than the bcl-2(+), EP, and NS groups (Figures 3A and 3C), indicating successful transfection of the *BDNF* gene. The bcl-2(+) group exhibited the highest level of bcl-2 protein expression compared to the other groups, and the BDNF(+)/bcl-2(+) group also exhibited a higher level of bcl-2 protein expression than the BDNF(+), EP, and NS groups (Figures 3B and 3D), indicating successful transfection of the *bcl-2* gene. Thus, the BDNF(+), bcl-2(+), and BDNF(+)/bcl-2(+) groups were successfully established and could be used for subsequent experiments (Figure 3).

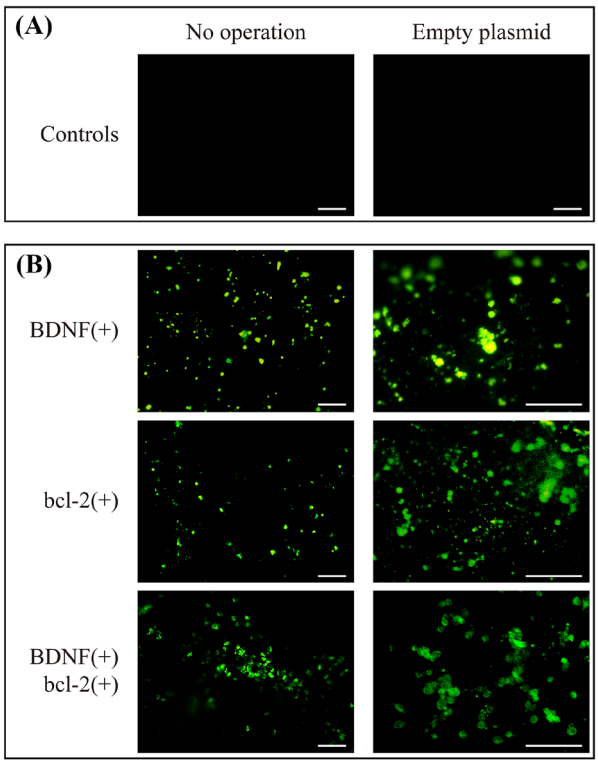

Figure 2. Analysis of gene expression 7 d after electroporation and plasmid transfection. (**A**) Images from controls. The column on the left shows an image of no surgery (NS), and the column on the right shows an image of the empty plasmid (EP). (**B**) Images from the BDNF (+), bcl-2 (+), and BDNF (+)/bcl-2 (+) groups. The column on the left is at a magnification of 1×, and the column on the right is at a magnification of 2×. Scale bar = 100 μm. bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor.

3.3. Verification of the morphological manifestation of Dil-labeled (Dil(+)) RGCs in the NS group

Figures 4A–4C depict the morphological manifestation of DiI(+) RGCs at different sites in the retina, namely the central retina (Figure 4A), parafoveal retina (Figure 4B), and peripheral retina (Figure 4C). The DiI(+) RGCs observed in the central retina had a diameter of 7–10 μm (Figure 4A), which increased from the central to the peripheral region (Figures 4B and 4C). In the peripheral retina, the diameter of the cells was 9–13 μm with a plump shape (Figure 4C). Figure 4D shows the density of DiI(+) RGCs in the central retina at 7, 14, and 28 d. There were significant differences in cellular density at 7, 14, and 28 d. The stability of DiI(+) RGCs was confirmed in the NS group, indicating that it was suitable for use as a blank control.

3.4. Verification of the protective effects of transfecting the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2* on the loss of DiI(+) RGCs induced by optic nerve transection

Figure 5 shows the effects of transfecting the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2* 7, 14, and 28 d later. As shown in Figure 1A, the number of DiI(+) RGCs in

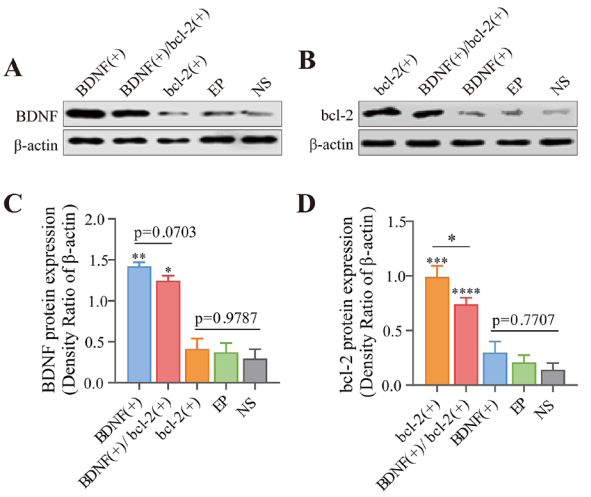

Figure 3. Expression of BDNF and bcl-2 7 d after gene transfection. (**A**) BDNF expression. The BDNF(+) and BDNF(+)/bcl-2(+) groups had higher levels of BDNF expression than the bcl-2(+), EP, and NS groups. (**B**) bcl-2 expression. The bcl-2(+) and BDNF(+)/bcl-2(+) groups had higher levels of bcl-2 expression than the BDNF(+), EP, and NS groups. (**C**) Quantitative data on BDNF expression. BDNF protein expression in the BDNF(+) and BDNF(+)/bcl-2(+) groups was significantly upregulated compared to that in the control group but did not differ significantly in the bcl2(+), EP, and NS control groups. (**D**) Quantitative data on bcl-2 expression. bcl-2 protein expression in the bcl-2(+) and BDNF(+)/bcl-2(+) groups was significantly higher than that in the control group but did not differ significantly in the BDNF(+), EP, and NS control groups. *means $P < 0.05$, **means $P < 0.01$ and ***means $P < 0.001$, ****means $P < 0.0001$, (vs NS control group). n = 3/group. Independent repeats = 2 samples. bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; EP, empty plasmid; NS, no surgery.

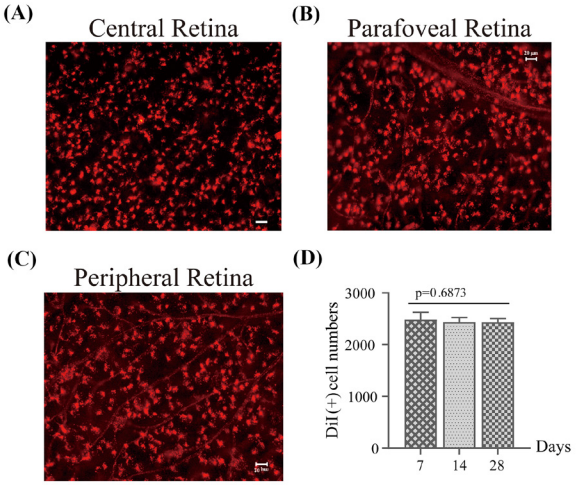

Figure 4. Morphology of DiI(+) RGCs in the retina of the NS group. (**A**) RGCs in the central retina (around the optic nerve) were small, with a diameter of 7-10 μm, and densely distributed. (**B**) RGCs in the parafoveal retina were slightly larger, with a diameter of 9-11 μm, and distributed more sparsely than those in the central retina. (**C**) RGCs in the peripheral retina were larger, with a diameter of 9-13 μm, and sparsely distributed. (**D**) There were no significant differences in the NS group at the three experimental time points (one-way ANOVA, $P = 0.6873$). Scale bar = 20 μm. DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; NS, no surgery; RGC, retinal ganglion cell.

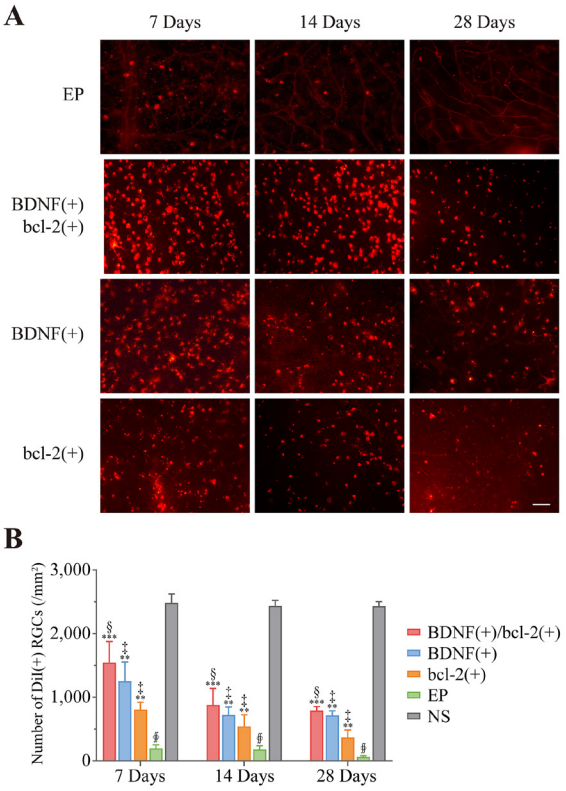

Figure 5. Transfecting the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2* enhanced survival of DiI(+) RGCs. (A) Images of DiI(+) RGCs at 7, 14, and 28 d after gene transfection. The EP group had the lowest number of DiI(+) RGCs (bright-red cells). The BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups exhibited higher numbers of DiI(+) RGCs. Scale bar = 50 μm. (**B**) Quantitative data on the number of DiI(+) RGCs 7, 14, and 28 d after gene transfection. **$P <$ 0.01 and ***$P < 0.001$, *vs.* EP group. $^{‡}P < 0.01$, $^{§}P < 0.001$ and $^{¶}P <$ 0.0001, *vs.* NS group. bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; EP, empty plasmid; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; NS, no surgery; RGC, retinal ganglion cell.

the BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups gradually decreased over time. At the same timepoint, the BDNF(+)/bcl-2(+) group had the highest number of cells. The number of cells was greatest in the BDNF(+)/bcl-2(+) group, followed by the BDNF(+) group and then the bcl-2(+) group. The EP group had the lowest number of cells. Apoptotic cells (reduced cell volume, condensed nuclei, and loose cytoplasm) were observed, and their number increased with time (Figure 5A). Figures 5B shows the quantitative data on cell counts. Trends were similar at the three time points. The number of DiI(+) RGCs in the BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups was significantly greater than that in the EP group and lower than that in the NS group. This result indicates that all gene transfection treatments resulted in protective effects against the loss of DiI(+)-labeled RGCs induced by optic nerve transection but did not result in full recovery. The number of DiI(+) RGCs tended to be greatest in the BDNF(+)/bcl-2(+) group, followed by the BDNF(+) group and then the bcl-2(+) group, there were no significant differences among these three groups

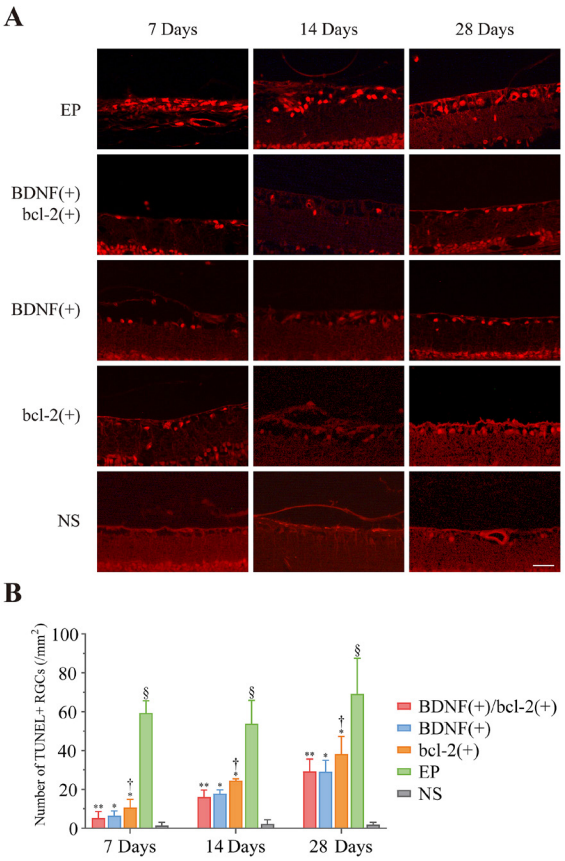

Figure 6. Transfecting the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2* reduced apoptosis of RGCs (TUNEL(+) cells). (A) Images of TUNEL(+) cells 7, 14, and 28 d after gene transfection. The EP group had the highest number of apoptotic cells (TUNEL staining, bright-red cells). The BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups had fewer apoptotic cells, and no apoptotic cells were found in the NS group. Scale bar = 20μm. **(B)** Quantitative data on TUNEL(+) cells at 7, 14, and 28 d after gene transfection. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, *vs.* EP group. $^{\dagger}P < 0.05$ and $^{\S}P < 0.001$ *vs.* NS group. bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; EP, empty plasmid; NS, no surgery; RGC, retinal ganglion cell; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

(Figure 5B).

TUNEL-positive apoptotic RGCs were also observed in the same experimental system. Figure 6A shows the changes in apoptotic RGCs over the period of transfection. Figure 6B shows the cell counts of TUNEL+ cells in each group (Figure 6). Trends were similar at the three time points, namely the number of TUNEL+ cells in the BDNF(+)/bcl-2(+), BDNF(+), and bcl-2(+) groups was significantly lower than that in the EP group and higher than that in the NS group. There were no significant differences among the three treatment groups. However, cellular apoptosis tended to be least prevalent in the BDNF(+)/bcl-2(+) but greater in the BDNF(+) group and even greater in the bcl-2(+) group; the number of DiI(+) RGCs followed the reverse of that trend.

The productive effects of gene transfection were verified based on the observation of DiI (+) and TUNEL(+) cells.

4. Discussion

This study revealed that transfection of the *BDNF* and *bcl-2* genes and co-transfection of both genes *via in vivo* electroporation significantly improved the survival of RGCs and reduced cellular apoptosis. The protective effects on DiI(+) RGCs were significant in these three groups but full recovery was not achieved. Thus, the protective effects of transfection with the genes *BDNF* and *bcl-2* and co-transfection with both genes on the loss of RGCs induced by optic nerve transection were verified. There were no significant differences among the groups with transfection of the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2*, but protective effects tended to be strongest in the BDNF(+)/bcl-2(+) group, followed by the BDNF(+) group and then the bcl-2(+) group. To extent known, this is the first study to verify the effects of gene transfection with a neurotrophic support-related gene, an anti-apoptosis-related gene, and co-transfection of both genes in animals that underwent optic nerve transection. We believe that the findings of the present study are pivotal to the development of a novel gene treatment for RGC-related optic nerve injury.

After electroporation and plasmid transfection for 7 d, the expression of BDNF(+), bcl-2(+), and BDNF(+)/bcl-2(+) was clearly observed (Figure 2), indicating that *in vivo* electroporation can result in the successful transfection and co-transfection of the *BDNF* and *bcl-2* genes into RGCs. Co-transfection of the *BDNF* and *bcl-2* genes *via in vivo* electroporation was developed in our previous study (*7*). We found that BDNF expression was significantly upregulated by transfection with the *BDNF* gene, but not by transfection with the *bcl-2* gene. Likewise, the expression of bcl-2 was significantly upregulated by transfection with *bcl-2*, but not by transfection with the *BDNF* gene. However, transfection of *BDNF*(+)/*bcl-2*(+) upregulated both (Figure 3). Transfection of the genes *BDNF*, *bcl-2*, and *BDNF* plus *bcl-2* resulted in significant protective effects (less reduction in/apoptosis of Dil(+) cells). The number of RGCs in the three treatment groups was significantly higher than that in the EP group but still lower than that in the NS group, indicating that the treatments were effective but did not result in full recovery. There were no significant differences among the BDNF (+), bcl-2 (+), and BDNF (+)/bcl-2 (+) groups, but efficiency tended to be highest in the BDNF(+)/bcl-2(+) group (Figure 5). Analysis of apoptosis yielded coincident results, namely there were significantly fewer apoptotic cells (TUNEL+) in the BDNF(+), bcl-2(+), and BDNF(+)/bcl-2(+) groups than in the EP group, and there were no significant differences among the three treatment groups. However, the protective effects of transfection tended to be strongest in the BDNF(+)/bcl-2(+) group (Figure 6). These results partly agree with the findings of previous studies, in which the administration of BDNF and anti-apoptotic Z-DEVD_fmk (*17*) and the combination

of bcl-2 and growth-related protein-43 promoted the regeneration of vertebrate peripheral nerve axons (*18*). We demonstrated that co-transfection of the *BDNF* and *bcl-2* genes did not result in better efficiency than transfection of *BDNF* or *bcl-2* alone. Thus, this study did not observe any synergistic effects of neurotrophic support genes and anti-apoptotic therapy. However, the survival of DiI(+) RGCs tended to be greater in the BDNF(+)/bcl-2(+) group while it tended to have fewer apoptotic cells (*vs.* transfection of *BDNF* or *bcl-2* alone). Indeed, the quantity of transfected plasmids of each gene in the BDNF(+)/bcl-2(+) group was only half that of the groups with transfection of a single gene. These trends imply that co-transfection has a better efficacy than that of transfection alone. This warrants further verification. In addition, we observed that the protective effects diminished over time. As indices, the number of RGCs and apoptotic cells exhibited similar trends. Confirmation of this phenomenon requires a longer follow-up period in future studies.

The main limitation of this study was the small sample size in each experimental group. This may have led to biased conclusions. In addition, the follow-up period was only 28 d, and the long-term effects were unclear. These issues should be addressed in future studies.

5. Conclusion

In conclusion, this study demonstrated that transfection of the *BDNF* and *bcl-2* genes and co-transfection of both genes significantly enhanced retinal ganglion cell survival and reduced apoptosis in animals *via in vivo* electroporation. Accordingly, results confirmed the protective effects of transfection of these genes on the loss of RGCs. These findings imply that gene therapy is a promising strategy for treating traumatic and degenerative optic neuropathies. Approaches to translate this strategy into clinical applications are highly anticipated. Moreover, results confirmed the value of *in vivo* electroporation. This study provides a foundation for further use of gene therapy in blinding optic nerve disorders. Future studies should focus on exploring the underlying signaling networks, observing long-term functional outcomes, and exploring potential clinical applications.

Funding: This study was supported by the Shanghai Pujiang Talent Program Project - Class A (Research and Development Category) (22PJ1401500) in China.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Quigley HA. Glaucoma. Lancet. 2011; 377:1367-1377.
2. Calkins DJ. Critical pathogenic events underlying progression of neurodegeneration in glaucoma. Prog Retin Eye Res. 2012; 31:702-719.
3. Levin LA, Gordon LK. Retinal ganglion cell disorders: Types and treatments. Prog Retin Eye Res. 2002; 21:465-484.
4. Si Z, Fan Y, Wang M, Zhao J, Zhang Y, Liu D, Zheng Y. The role of RGC degeneration in the pathogenesis of glaucoma. Int J Biol Sci. 2025; 21:211-232.
5. Cui N, Jia J, He Y. Glaucomatous retinal ganglion cells: Death and protection. Int J Ophthalmol. 2025; 18:160-167.
6. Quigley HA. Ganglion cell death in glaucoma: Pathology recapitulates ontogeny. Aust N Z J Ophthalmol. 1995; 23:85-91.
7. Mo X, Yokoyama A, Oshitari T, Negishi H, Dezawa M, Mizota A, Adachi-Usami E. Rescue of axotomized retinal ganglion cells by BDNF gene electroporation in adult rats. Invest Ophthalmol Vis Sci. 2002; 43:2401-2405.
8. Osborne A, Khatib TZ, Songra L, Barber AC, Hall K, Kong GYX, Widdowson PS, Martin KR. Neuroprotection of retinal ganglion cells by a novel gene therapy construct that achieves sustained enhancement of brain-derived neurotrophic factor/tropomyosin-related kinase receptor-B signaling. Cell Death Dis. 2018; 9:1007.
9. Donahue RJ, Fehrman RL, Gustafson JR, Nickells RW. BCLXL gene therapy moderates neuropathology in the DBA/2J mouse model of inherited glaucoma. Cell Death Dis. 2021; 12:781.
10. Li Y, Schlamp CL, Nickells RW. Experimental induction of retinal ganglion cell death in adult mice. Invest Ophthalmol Vis Sci. 1999; 40:1004-1008.
11. Yokoyama A, Oshitari T, Negishi H, Dezawa M, Mizota A, Adachi-Usami E. Protection of retinal ganglion cells from ischemia-reperfusion injury by electrically applied Hsp27. Invest Ophthalmol Vis Sci. 2001; 42:3283-3286.
12. Dezawa M, Takano M, Negishi H, Mo X, Oshitari T, Sawada H. Gene transfer into retinal ganglion cells by *in vivo* electroporation: A new approach. Micron. 2002; 33:1-6.
13. Mey J, Thanos S. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats *in vivo*. Brain Res. 1993; 602:304-317.
14. Yu YL, Li HY, Zhang PX, Yin XF, Han N, Kou YH, Jiang BG. Comparison of commonly used retrograde tracers in rat spinal motor neurons. Neural Regen Res. 2015; 10:1700-1705.
15. Makarenko IG. DiI tracing of the hypothalamic projection systems during perinatal development. Front Neuroanat. 2014; 8:144.
16. Paxinos G, Franklin KBJ. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates. Academic Press, 2019.
17. Magharious MM, D'Onofrio PM, Koeberle PD. Optic nerve transection: A model of adult neuron apoptosis in the central nervous system. J Vis Exp. 2011; 51:2241.
18. Cristino L, Pica A, Della Corte F, Bentivoglio M. Co-induction of nitric oxide synthase, bcl-2 and growth-associated protein-43 in spinal motoneurons during axon regeneration in the lizard tail. Neuroscience. 2000; 101:451-458.
19. Korsching S. The neurotrophic factor concept: A reexamination. J Neurosci. 1993; 13:2739-2748.
20. Daadi MM, Saporta S, Willing AE, Zigova T, McGrogan MP, Sanberg PR. *In vitro* induction and *in vivo* expression of bcl-2 in the hNT neurons. Brain Res Bull. 2001; 56:147-

152.
21. Holm KH, Cicchetti F, Bjorklund L, Boonman Z, Tandon P, Costantini LC, Deacon TW, Huang X, Chen DF, Isacson O. Enhanced axonal growth from fetal human *bcl-2* transgenic mouse dopamine neurons transplanted to the adult rat striatum. Neuroscience. 2001; 104:397-405.
22. Goldberg JL, Espinosa JS, Xu Y, Davidson N, Kovacs GTA, Barres BA. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron. 2002; 33:689-702.
23. Lambricht L, Lopes A, Kos S, Sersa G, Préat V, Vandermeulen G. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opin Drug Deliv. 2016; 13:295-310.

Received September 20, 2025; Revised October 26, 2025; Accepted November 1, 2025.

**Address correspondence to:*
Jiawen Fan, Eye Institute and Department of Ophthalmology, Eye & ENT Hospital, Fudan University, Shanghai, China, 200031.
E-mail: jiawen.fan@fdeent.org

Released online in J-STAGE as advance publication December 13, 2025.