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Erythropoietin Protects against Retinal Damage in A Rat Model of Optic Neuropathy via Glial Suppression

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Abstract

Objective: Traumatic optic neuropathy (TON) causes partial or complete blindness because death of irreplaceable retinal ganglion cells (RGCs). Neuroprotective functions of erythropoietin (EPO) in the nervous system have been considered by many studies investigating effectiveness of this cytokine in various retinal disease models. It has been found that changes in retinal neurons under conditions of glial cells are effective in vision loss, therefore, the present study hypothesized that EPO neuroprotective effect could be mediated through glial cells in TON model.

Materials and Methods: In this experiment study, 72 rats were assessed in the following groups: intact and optic nerve crush which received either the 4000 IU EPO or saline. Visual evoked potential and optomotor response and RGC number were assessed and regenerated axons evaluated by anterograde test. Cytokines gene expression changes were compared by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Density of astrocytes cells, assessed by fluorescence intensity, in addition, possible cytotoxic effect of EPO was measured on mouse astrocyte culture *in vitro*.

Results: *In vitro* data showed that EPO was not toxic for mouse astrocytes. Intravenous injection of EPO improved vision, in terms of visual behavioral tests. RGCs protection was more than two times in EPO, compared to the vehicle group. More regenerated axons were determined by anterograde tracing in the EPO group compared to the vehicle. Moreover, *GFAP* immunostaining showed while the intensity of reactive astrocytes was increased in injured retina, systemic EPO decreased it. In the treatment group, expression of *GFAP* was down-regulated, while *CNTF* was up-regulated as assessed by qRT-PCR in the 60th day post-crush.

Conclusion: Our study showed that systemic administration of EPO can protect degenerating RGCs. Indeed, exogenous EPO exerted neuroprotective and neurotrophic functions by reducing reactive astrocytic gliosis. Therefore, reduction of gliosis by EPO may be considered as therapeutic targets for TON.

Keywords: Erythropoietin, Retinal Ganglion Cells, Traumatic Optic Neuropathy

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Introduction

Traumatic optic neuropathy (TON), resulting from systemic diseases or any traumatic head injury, is the leading cause of partial or complete blindness in working adults. It has been recognized that changes in retinal neurons and glial cells precede development of vision loss; therefore, the present study focused on gliosis and retinal ganglion cell (RGC) protection. Erythropoietin (EPO), a glycoprotein cytokine which is well-known due to its hematopoietic effects, also plays a neuroprotective role in the retina, which has attracted much attention in recent years (1). Neurons and glial cells of the nervous system, particularly astrocytes, express both EPO and its receptor (EPO-R). EPO acts as a neurotrophic and neuroprotective agent in the cases of different brain injuries, such as hypoxia and ischemia, both *in vitro* and *in vivo* (2). EPO has shown significant effects on peripheral nervous system (PNS) injuries as well. It works as a neurotrophic and neuroprotective factor that improves functional recovery and nerve regeneration (3). An abundance of EPO and EPO receptors has been demonstrated in retinal cells (4). In disease models of the optic nerve, such as transection and optic nerve crush, EPO protected RGCs and promoted axon regeneration (5, 6). Based on several studies, administration of EPO in TON led to a significant increase in visual acuity, and a systematic literature review

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proposed systemic administration of EPO, as a safe and efficient treatment in TON; for review see. Highlighting neuroprotective, antioxidant, anti-inflammatory, angiogenic and antiapoptotic mechanisms of EPO were extensively studied (7). For example, after optic nerve injury, IL-6 was up-regulated, and its attachment to IL-6 receptors in the retina activated JAK/STAT3 transduction pathway, resulting in extended RGC survival and neurite outgrowth in cultures (8).

Retinal astrocytes, presented in RGCs and optic fiber layers, provide metabolic support to retinal neurons. Neuron survival in co-culture systems depends on the presence of astrocytes, and this interaction is now well-established (9). Interestingly, retinal glia cells provided cytokines such as ciliary neurotrophic factor (*CNTF*) and growth factors that may produce neuroprotective effects (10). Upon injury to the nervous system, astrocytes go through reactive astrogliosis marked by hypertrophy of soma and processes, increased expression of two intermediate filaments: glial fibrillary acidic protein (*GFAP*) and vimentin to try maintaining the microenvironment homeostasis (11). Eventually, activated glia lost their supportive role and gained toxic function in the progression of an environment, prohibiting axonal regeneration (12). Recently it has been shown that reactive gliosis was observed in glaucoma mouse models and degree of reactivity correlated with the inflammatory glaucoma process (13). Current solutions to attenuate retinal gliosis are limited. A pioneer study on cerebral ischemia as well as the study on *in vitro* model of spinal cord injury showed that EPO administration exerted significant decrease in inflammation and astrogliosis, while a strong trend was observed toward the promotion of neurite outgrowth (14). In this way, Hu et al. (15) study showed that exogenous EPO was able to reverse diabetes-induced reactive gliosis and acted indirectly neuroprotective effect.

However, as one of the unaddressed obstacles after optic nerve crush is the gliotic response, the possibility that EPO plays a neurotrophic role mediated through astrocytes in this phenomenon has not been explored yet. Based on these insights, we sought to determine the effect of EPO on optic nerve injury (ONI) and determine its effect on the visual behavioral and cellular levels. In the present study, we studied the effect of EPO on RGCs in rat models of optic nerve crush. We also investigated the impact of EPO on reactive and non-reactive astrocytes *in vitro*.

Materials and Methods

The optic nerve crush animal model and rh-EPO treatment

In this study, 72 adult male and female Lister Hooded rats (6-8 weeks old, Razi Institute, Iran) were recruited to randomly classify. Animals were fed with a standard diet ad libitum, kept on a standard light/dark cycle. All procedures, involving the animals concurred with the statement of The Association for Research in Vision and Ophthalmology (ARVO) for use of animals in ophthalmic and vision research and were approved by Royan Institute Committee for Ethics in Animal Research (EC/93/1139), Tehran, Iran). All types of manipulations were performed under general/local anesthesia: a mixture of intraperitoneally administered ketamine (50 mg/kg) and xylazine (5 mg/kg). The right eyes remained unaffected during the experiment and only their left optic nerve was submitted to the crush, performed as previously described (16). Briefly, under the guidance of an operating microscope (Olympus, Japan), each optic nerve was crushed for 30 seconds while carrying out the retinal blood supply. After the procedure, the mixed antibiotic eye ointments (Gentamicin and tetracycline, SinaDarou, Iran) was topically applied to prevent infection, and animals were kept under supervision until they were recovered from anesthesia. Two- and nine-days post-optic nerve crush, a group of rats received an intravenous injection of either 200 µl of phosphate-buffered saline (PBS) or 4000 IU/kg rh-EPO Alpha (PDpoetin 4000, Pooyesh Darou Biopharmaceutical, Iran).

Visual evoked potential recording

To assess the functional integrity of the rat optic apparatus, we recorded visual evoked potential prior to the crush and at 7-, 14-, 28-, and 56-days post-treatment. Visual evoked potential (VEP) recordings were performed as previously described (16). Briefly, after anesthetizing, head of the animals were fixed into a stereotaxic apparatus (Narishige, Japan). The reference electrode was implanted at the anterior part of the skull and a monopolar electrode was implanted on the surface of the occipital cortex (AP: 27.0 and L: +3.0 referred to Bregma) as the recording electrode. Three hundred times Flashlight stimulation at a frequency of 0.5 Hz was delivered by a general stimulator (ScienceBeam Co., Iran). For each VEP record, we measured amplitude of the N1P2 wave and reported it as amplitude recovery.

Optomotor response

To assess spatial visual acuity of the animals at 60th days post-injury, an OptoDrum system (Striatech technologies, Germany) was used. The OptoDrum is comprised of an elevated platform in the center, a camera on top of the device, and four computer monitors arranged in a square, displaying black-and-white stripes that rotate clockwise or counterclockwise around the animal. The clockwise stripes only trigger the optomotor reflex of the left eye, while counterclockwise stripes only trigger the response of the right eye (17). At first, stripes were rotated at a low spatial frequency, and if the moving stripes could be seen, the animal responded with reflexive head movements. Then spatial frequency of the stripes gradually increased until it reached the threshold of the animal's vision, and the optomotor reflex no longer was evoked. In this way, visual acuity of the animals was determined and reported on cycle/degree.

Retinal flat mount imaging for retinal ganglion cell survival analysis

To reliably quantify RGCs in different groups, whole mount immunostaining was performed. After 60 days of

injection, the eyes were enucleated, and the retina was dissected out after fixation in 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) overnight. The isolated retinas were kept in 0.5% Triton™-X100 (Sigma-Aldrich, USA) for 15 minutes at -80°C. Then retinae were thawed, washed, and incubated with related primary antibodies anti-Brn3a (1:250, Santa Cruz Biotechnology, USA) or *GFAP* (1:1000, Sigma-Aldrich, USA) produced in goat or rabbit, while they were diluted in 0.2% Triton™-X100 and 5% normal sheep or goat serum and incubated overnight at 4°C. After washing three times in PBS, retinae were incubated with the secondary antibodies Alexa fluor Sheep anti-goat IgG (1:500, Invitrogen Inc., USA) or Alexa fluor goat anti-rabbit IgG (1:500, Invitrogen Inc., USA) 2 hours at room temperature. The stained retinas were mounted and photographed with a fluorescent microscope (BX71, Olympus, Japan). Total number of Brn3a positive (n=16 image from each retina; n=4 eye for each group) were manually counted by a blinded observer and expressed as a percentage at each retina normalized to the control group.

Retrograde and anterograde tracing

Retrograde and anterograde tracings were performed to investigate the rat eye-brain connectivity in each group, 60 days after injection. Retrograde tracer DiIC18 (DiI, Molecular Probes, UK) was injected into the superior colliculus of the rats to label retina-projecting neurons. Cholera Toxin B, conjugated to Alexa488 (CTB-488, Invitrogen Inc., USA), was injected as anterograde tracer into the vitreous body for tracing visual cortex-originating nerves. In 5-7 days, the samples were collected and processed as described in the previous publications (18). The left eyes from the intact, vehicle, and EPO groups received 2 µl of DiI in the superior colliculi or 3 µl of toxin in the vitreous, while the right eyes were intact (n=4 for each group). The 8 µm longitudinal slices of optic nerves were imaged using a fluorescent microscope (IX71, Olympus, Japan).

Quantitative reverse transcript polymerase chain reaction

Total RNA fraction was extracted from the four independent replicates by the RNeasy micro kit (Qiagen, Germany) and reverse transcribed by the Quantitate Reverse Transcription synthesis system (Qiagen, Germany). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using QuantiTect SYBR Green PCR Kit (Qiagen, Germany) according to the manufacturer's instructions. Expression levels of the genes were normalized to *GAPDH* using the standard curve method. Primers used in this study was as following:

GAPDH-

F: 5′-TGCTGAGTATGTCGTGGAGT-3′
R: 5′-CGGAGATGATGACCCTTTTG-3′

Neurotrophin 3 (*NT3*)-

F: 5′-CCTCTCCTTACCCAGCATCTC-3′
R: 5′-GGCATCCGTATGACTATTTCCA-3′

brain derived neurotrophic factor (*BDNF*)-

F: 5′-TGAAAGAGACAAGAACACAGGA-3′
R: 5′-CAAGAGGTAAAGTGTAGAAGGGAC-3′

CNTF-

F: 5′-TTCCATCAGGCAATACATACTC-3′
R: 5′-TGAGAAGAAATGACACGAAGG-3′

growth associated protein 43 (*GAP43*)-

F: 5′-AGGAGGAGGGCAGCAAAG-3′
R: 5′-CGGCGAGTTATCAGTGGAAG-3′ (19)

GFAP-

F: 5′-CGAGCCATTGAACTTACC-3′
R: 5′- ATTTCCTTCCATTACAGACCC-3′.

Fluorescence intensity quantification

Fluorescence intensity quantification was performed seven days after the optic nerve crush on the whole retina, which stained as described above. To reach this, retinal cryo-sections from rat retinas were immunoassayed with *GFAP*, while 49, 69-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) was used to stain nuclei. Mean intensity levels of four retinas from the untreated group and four retinas from the vehicle and EPO treated group were analyzed. For each retina, 16 images, randomly acquired from the whole retina, were analyzed using Image J software (https://imagej. nih.gov; National Institutes of Health, USA), by calculating the area in the ganglion cell layer outlined in each section of images. Additionally, the whole signal was measured in flat-mounted samples expressed as pixel gray-scale values. Average means fluorescent value per image, normalized by the control group, was used for statistical analysis.

Axon density quantification in optic nerves crossed sections

To assess glial covering optic nerve area, optic nerves were harvested and after immersion in 4% PFA/5% glutaraldehyde (TAAB, G002, USA) for seven days, they were post-fixed in 1% osmium tetroxide (TAAB, O002, USA) for 3 hours. They were next dehydrated and embedded in Araldite resin (TAAB, E009, USA). The semi-thin cross-sectional at a distance 3 mm distal to the globe was stained with toluidine blue. The mean axon density was randomly counted manually on 7-8 non-overlapping frames for each nerve group.

Isolation of mouse astrocytes

NMRI neonate mice aged 5-7 days were used to extract astrocytes. The brains were extracted, the meninges of the cortex were eliminated. The tissues were then sectioned into small pieces, enzymatically dissociated, and incubated for 7-10 minutes at 37°C in 0.5% trypsin (Invitrogen, USA). The suspension was centrifuged at 1500 g for 5 minutes after filtering by a 100-µM cell strainer (Corning, USA). The cells were cultured in the astrocyte cell culture

media (DMEM/high glucose; Sigma-Aldrich, USA), included 15% fetal bovine serum (FBS, Gibco, USA). To remove other cell types, the cells were agitated for 3 hours at 240 rpm on day 7 and overnight at 180 rpm on day 14. The cells were purified with passage and ready to be examined after 2-3 passages.

Scratch-wound model

The medium was first changed every 2-3 days to remove any debris and dead cells, and the cells were permitted to grow to 99% confluence in DMEM/high glucose+15% FBS. The media was then changed to DMEM/high glucose+7.5% FBS, and the cells were kept for at least another two weeks. During this time, the medium was changed every 3-4 days. After that, the cells were moved to DMEM/high glucose+1% FBS for 2-3 days before wounding. Two parallel scratches were made using a p1000 sterile pipette tip, followed by two further scratches at a right angle to the previous two. To generate a 37-40% damage rate, the cells were scratched in a simple grid. The dishes were washed once with DMEM-high glucose+15% FBS shortly after damaging to eliminate debris and floating cells.

Immunocyto fluorescence

The cells were fixed for 15-20 minutes with 4% PFA, then at 30 minutes, permeabilized with 0.5% Triton™ X-100 in PBS. The cells were then maintained overnight at 4°C with primary antibody (1:200, *GFAP*, Sigma-Aldrich, USA) in blocking solution and it was subsequently incubated with secondary antibody (1:1000, Invitrogen, USA) for one hour in room temperature (RT). Ultimately, the cells were stained for 5 minutes with DAPI and observed using a microscope (Olympus IX71, Olympus, Japan).

Astrocyte viability using MTS assay

Amount of soluble formazan, produced by cellular reduction of MTS (Promega, G5421, Italy) was used to determine cell viability. Astrocytes were cultured at a density of 1×10^4 cells per well in a 96-well culture plate. The cells were cultured for 24 hours. Then, they were treated with different concentrations of EPO (6 IU/ml and 60 IU/ml) (20-22) for 1 and 7 days. Twenty microliters of MTS solution were added to each well at the end of treatment, and the plates were incubated at 37°C for 3 hours. Absorbance reading of each well was then determined at 490 nm using a microplate reader.

Live and dead staining by acridine orange and propidium iodide

Cell viability was evaluated by acridine orange-propidium iodide (AO/PI) (23) staining after 24 hours of *in vitro* culture. In a nutshell, Dulbecco's solution (Gibco, USA) was used to prepare stock solution (AO: 670 mmol/l, PI: 750 mmol/l), which was then stored at 4°C in dark. One milliliter PI and 0.01 ml AO were diluted 10 times with Dulbecco's solution. They were next mixed before using and a 0.22 mm filter membrane was used to filter the mixture. The cells were examined under a fluorescence microscope after a 10-minute incubation with the AO/PI mixture. Dead cells were red in color (PI), while live cells were green (AO).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 9.2.0.332, USA). Data are expressed as the mean ± standard deviation (SD) and analyzed by unpaired two-tailed student t test or one-way ANOVA, followed by the student-Newman-Keuls test. A mixed model of ANOVA and LSD post-hoc was used for quantitative data of the N1P2 waves in different groups. $P<0.05$ were considered statistically significant.

Results

Erythropoietin recovered rat visual functions against crushed retinal ganglion cells axons

Schematic Figure 1A shows the overall experimental design. Animals were divided into three groups (Intact: the same aged, Vehicle: 10 µl/kg saline, and experimental: 4000 IU/kg EPO injected at 2- and 9-days post-crush). Visual evoked potential test, reflected the visual pathways' functional integrity, was done 56 days after treatment. Protective effect of EPO and its effect on the repair process of the left injured optic nerve were evaluated by comparing N1P2 wave amplitude before and after the crush. As shown in Figure 1B, the lowest amplitude in both groups was observed on day 9 (equal to 7 days after the first injected EPO). The amplitude continued to be reduced in subsequent days in the vehicle (n=10); there was no significant difference between the tested days. However, in the EPO treatment animals (n=11), we observed a highly significant increasing trend of the N1P2 wave. Interestingly, this continuous increasing amplitude in EPO treatment group was more than two-fold higher than the amplitude on days 0, 14, 28, and 56 (*P=0.02) compared to the same days in the control group. We considered another visual behavioral assessment, as complementary data, according to the functional recovery of the injured optic nerve detected by VEP recording.

For functional outcome assessment, we injected EPO compared to saline in 12 rats in the same condition described in schematic Figure 1A. We investigated whether the EPO exhibited functions typically observed in intact rats. To assess this visual behavioral function, optomotor response analysis was done in animals which had left optic nerve crush (ONC) in their eyes. The control group included the same-aged rats with saline injection (vehicle). Optomotor response was based on head movement and following the visual grating stimuli when exposed to 4 monitors around the rats. We observed that EPO-treated animals dramatically showed increased visual acuity compared to vehicles. At a 2-month post-crush, visual acuity (cycle/degree) in rat eyes with

EPO treatment was significantly preserved compared to vehicle-operated eyes (**P=0.001, Fig.1C). These results demonstrated a significant improvement in visual behavioral tests following EPO injections.

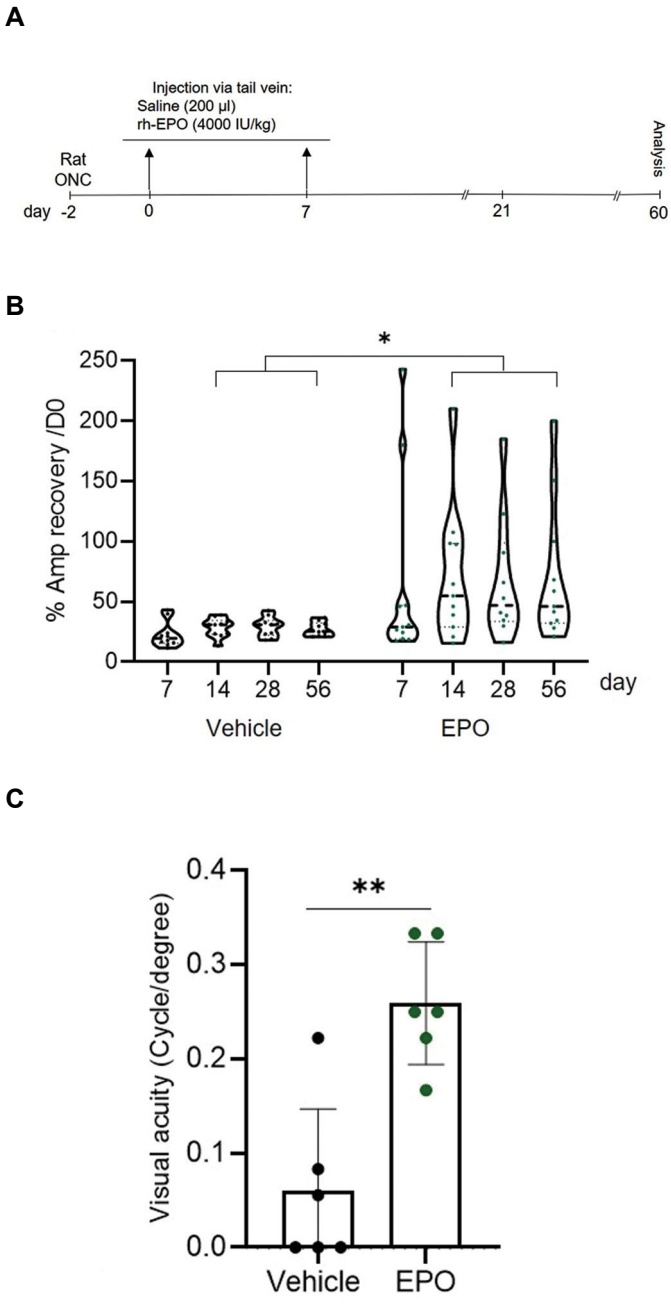

Fig.1: Experimental design and visual behavioral assessment. **A.** Rat optic nerve crushing was done on day -2, and then animals randomly divided into the vehicle and EPO-treated groups. 4000 IU/kg EPO intravenous injections were administrated to experimental rats 2- and 7-days post-crush; the control group received 200 µl saline at the mentioned time-points. At the end of eight weeks, animals were sacrificed and retina, in addition to the optic nerve, were collected for analyzing retinal ganglion cell survival rate, retro and anterograde tracing, as well as glial and gene expression measurements. **B.** Changes in amplitude of N1P2 wave were evaluated during eight weeks post-optic nerve crush in vehicle and EPO treated groups. Although reducing the amplitude was seen on the first-week post-crush, increasing significant constant recovery was observed in the experimental animals until the 60th day; P=0.02, data represent mean ± SD, n=10-11 per group. C. The graph, showing EPO administration in the injured retina, significantly preserves visual acuity 2 months after injections, as assessed using optomotor test. P=0.001, data represent mean ± SD, n=6 per group. rh-EPO; Recombinant human erythropoietin, ONC; Optic nerve crush, *; P<0.05, and **; P<0.01.

Systemic erythropoietin injection protects retinal ganglion cells

To study neurotrophic or neuroprotective effects of the treatments following ONC, we assessed effects of EPO injections on RGC survival. We used the RGCs transcription factor, *Brn3a*, to evaluate the effect of the treatments on degenerating RGCs in the crushed rat retina (Fig.2A). Number of the labeled RGCs within the retina was quantified and compared between the groups when normalized to animals that had an intact visual system. Eight weeks after the injury in the vehicle group, the mean number of the labeled RGCs was reduced to 7.22 ± 1.07 % in comparison with the healthy retina. In contrast, the EPO injections significantly protected the RGCs from neurodegeneration, as the mean number of the labeled RGCs were up to 20.41 ± 1.08 % (***P=0.00) compared to the control retina. Next, we assessed effects of the EPO injections on RGC projections to the brain. A retrograde tracing approach that used microinjections of DiI into the superior colliculi and its detection within the RGCs layer of the retina was used. Our findings showed that the EPO group had more axonal preservation than the vehicle group. To study the ganglion axonal integrity, we employed the anterograde tracing test using conjugated Cholera toxin, which passes through axonal terminals. We assessed around 500 µm distal to crush area and found significantly more CTB+ axons in this region of the EPO group than the vehicle (Fig.2B). Overall, these results showed that EPO injection increased axonal integrity of RGCs following the crush model.

Erythropoietin down-regulated expression of *GFAP* in the injured retina

To determine whether recovery effects of EPO on the injured retina may be persuaded by the cytokines, we performed molecular analysis of some key known neuroprotective and neuroregenerative markers within the retina and optic nerves. Differential expressions of cytokines and growth factors, such as *NT3*, *BDNF*, *CNTF*, *GAP43* as a regenerative marker and *GFAP* area representative of glial cells, were assessed by qRT-PCR in EPO treated and control rats Figure 3A. Retinal *CNTF* gene expression at presence of EPO was significantly high, up to 3.5-fold, relative to control (*P=0.01). No significant difference was found for *NT3*, and *GAP43* in the EPO treatment group in comparison with the control group, while the expression of *BDNF* and *GFAP* mRNA was significantly decreased following EPO injections (*P=0.00). To validate decreased expression of *GFAP*, we performed *GFAP* immunostaining (the micrographs on the bottom panel of Fig.3B), Immunostaining confirmed that at one week after ONC, most glial cells in the whole retina acquired reactive phenotype and expressed more *GFAP* protein in an untreated group than the control. However, the images and its related quantitative graph showed that EPO-treatment effectively reduced retinal gliosis. In this way, dramatic up-regulation of glia was evident, with low axon density on vehicle optic nerve cross-sectioned, while it was not significant

in EPO treated nerve sections. A rat optic nerve was unaffected by diseases in the middle panel of Figure 3B, demonstrating an axon packing density which was controversy with glial expansion. In optic nerves, axons are bundled in well-defined fascicles separated by a thin astrocyte glial process (15). We found that with ONC, nerves became disorganized with smaller and more irregular fascicles farmed by hypertrophic astrocyte processes. Interestingly, they were notably containing larger axons, as shown in the vehicle group with low axon density ($1.07\times10^5 \pm 0.07$ axons/mm^2). Retinal glial suppression, following EPO injections, was confirmed with no significant difference in mean axon density between the normal retina and EPO (respectively, $1.93\times10^5 \pm 0.07$ and $1.70\times10^5 \pm 0.08$ axons/mm^2). Interestingly, two months after ONC, a little, green-immunoassayed related to *GFAP*-positive astrocytes were detected in the layers of retinal neurons of the normal control group. However, more green-stained cells were seen in the vehicle compared to the EPO injected. These results were in line with the changes of *GFAP* in the retina on the 7th injured day.

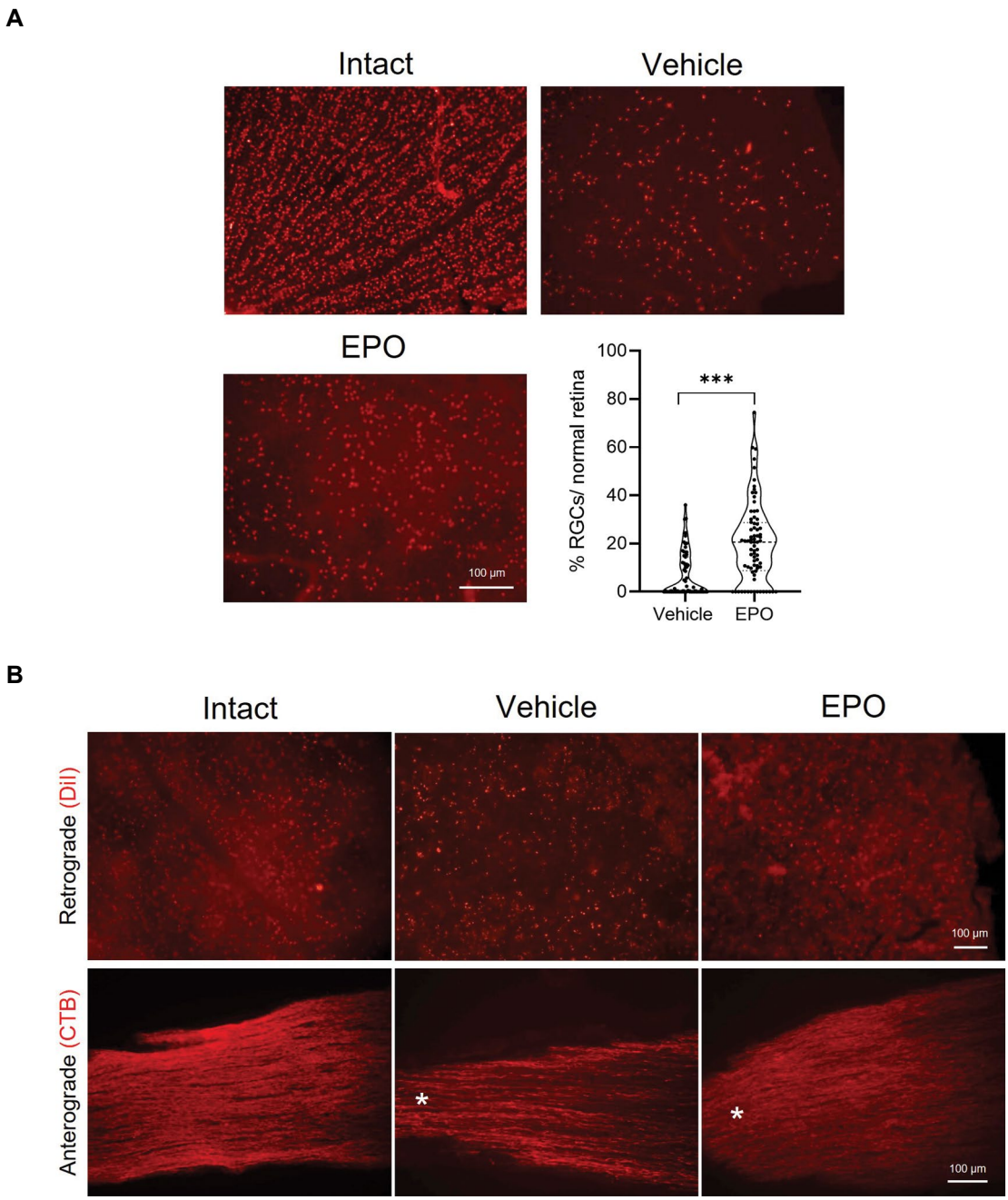

Fig.2: Neuroprotective effects of EPO administration on retinal ganglion cells of the crushed optic nerve. **A.** Quantitative analysis of RGCs survival as number per retina of different groups on day 60th post-crush. n=4, P=0.00 as determined by student t test. **B.** Representative micrographs of retro and anterograde labeled retinal ganglion cells and axons in the different retina and optic nerves, including intact, vehicle, and EPO-treated groups. Both protected cells and labeled axonal in EPO injected groups are higher than vehicle and they are the same as intact group (scale bar: 100 µm). *; Optic nerve crush site, EPO; Erythropoietin, and RGCs; Retinal ganglion cells.

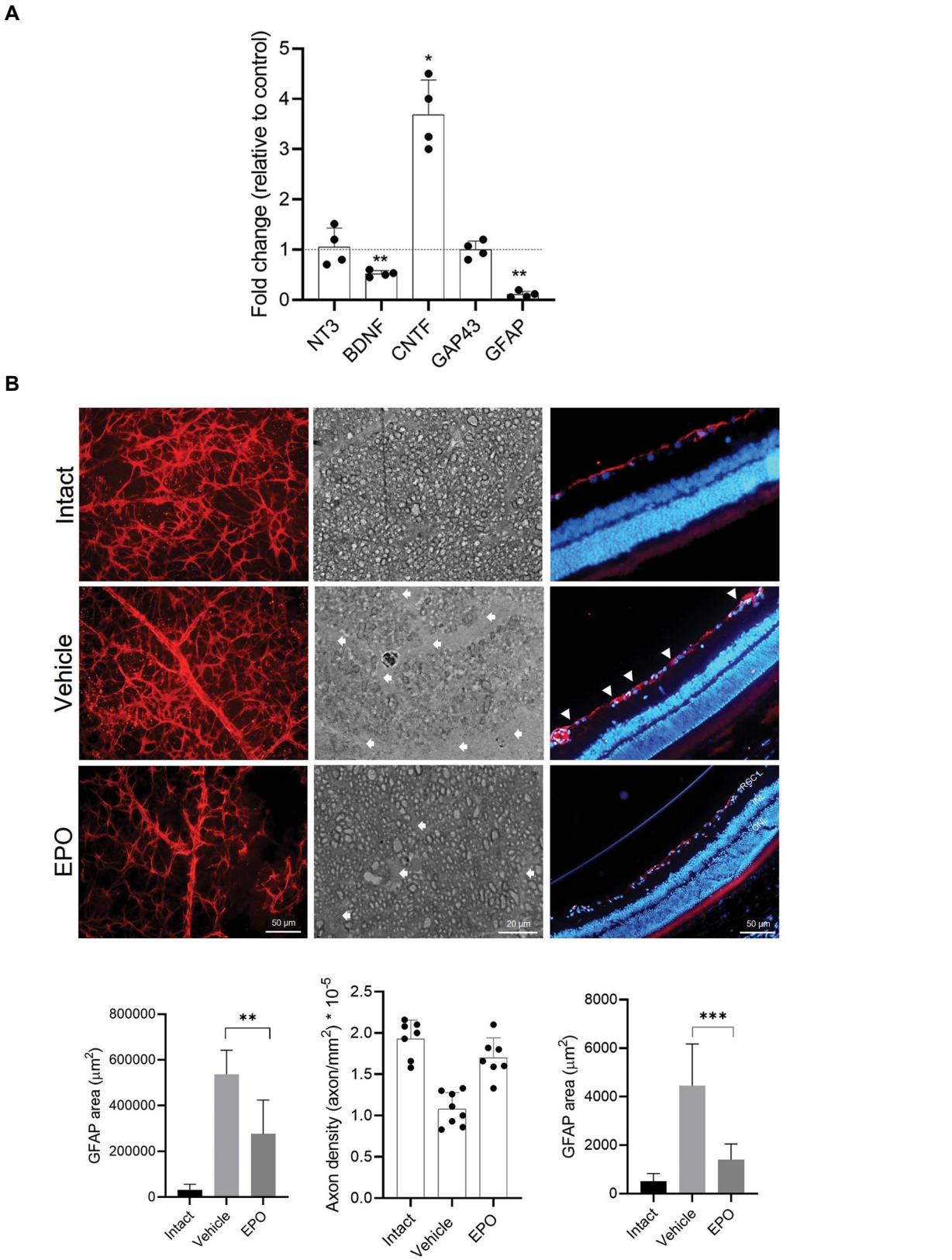

Fig.3: Assessment of EPO treatment effect on gene expression of cytokines and glial markers. **A.** qRT-PCR analysis of the effect of EPO injection on expression of different cytokines, regenerative and astrocytic markers; the qRT-PCR test was performed on the retina and optic nerves. Expression of both *BDNF* and *GFAP* markers was significantly lower in the EPO recipient group, than the vehicles (P=0.00), whereas *CNFT* had significantly higher ratio in the EPO group (*; P=0.01). Data showed that by treating with EPO, mRNA levels of *NT3* and *GAP43* were not significantly up regulated in the control group. **B.** To further investigate the *GFAP* expression, we did an immunofluorescence staining for the *GFAP* marker on the 7th and 60th days post-injury. Analysis of this protein revealed that GFAP expression was significantly reduced in the EPO group compared to the vehicle and the intact group (P=0.00). The middle panel of Figure section B is related to the high-magnification light micrograph on cross-section, demonstrating tight packing of axons interpreted with thin astrocyte process in the intact and EPO treated group. The same conditioned nerve from the vehicle group with diminished axon packing and increased astrocyte hypertrophy (arrowheads) was seen in the middle. RGCL; Retinal ganglion cell layer, INL; Inner nuclear layer, ONL; Outer nuclear layer, EPO; Erythropoietin, qRT-PCR; Quantitative real time polymerase chain reaction, **; P<0.01, and ***; P<0.001.

Effect of erythropoietin on astrocyte cells

To verify *in vivo* results, the EPO effect on primary mouse cortical astrocytes was studied. Isolated primary mouse cortical astrocytes had a polygonal to fusiform morphology. Moreover, we validated the efficiency of our isolation using immunofluorescence for the standard astrocyte marker, cytoskeletal protein *GFAP* (Fig.4A). As shown, on day 1, EPO in different concentrations did not have any significant effect on astrocyte cells survival rate. In addition, after seven days, number of the live cells was not changed in the 6 and 60 IU/ml EPO concentrations, in comparison with the control group (Fig.4B, C), suggesting that EPO was not toxic for these cells. Then, we questioned whether there was any EPO cytotoxic effect on the activated cells. To test, after reactivating these cells mechanically (Fig.4D), the effect of EPO was analyzed. Live and dead staining after 24 hours was validated while EPO was not differentially significantly changed number of the dead reactive astrocyte cells even in 60 IU/ml, compared to the control group (Fig.4E).

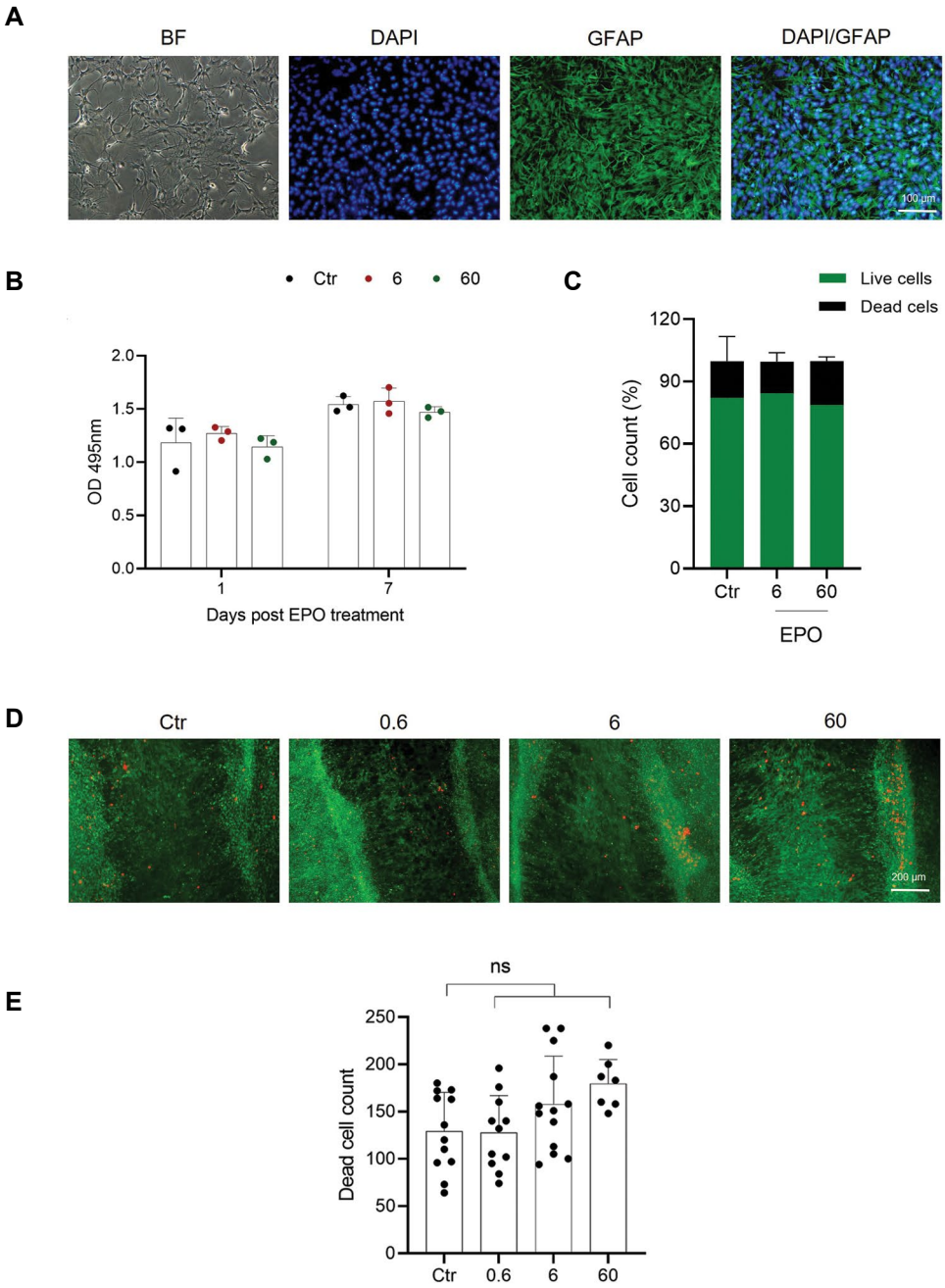

Fig.4: *In vitro* assessment of EPO toxicity. **A.** Characterization of astrocyte cells: bright field microscopy images of astrocyte cells and expression of astrocytic marker (*GFAP*) in the primary culture (scale bar: 100 µm). **B, C.** MTS assay as well as live and dead staining showed that number of astrocyte cells treated with 6 and 60 IU/ml of EPO was not significantly changed after days 1 and 7. **D, E.** Live and dead staining on reactive astrocyte cells showed no significant increase in the number of dead cells treated with 0.6, 6, and 60 IU/ml of EPO (scale bar: D: 200 µm). Ctr; Control, EPO; Erythropoietin, and ns; Not significant.

Discussion

The present study demonstrated that EPO could promote, under *in vivo* assay, protection of RGCs in ONC animal models via suppressing the reactive retinal astrocytes. Pigmented Lister Hooded rats were selected for this study because of their higher visual acuity, which is important for performance visual behavioral tasks (24). We found that EPO administration in optic nerve injury (ONI) led to certain benefits. First, it promoted axonal protection and regeneration in RGCs and improved axonal function, which was supported by the better results of visual function tests in EPO recipients compared to the vehicles. Studies have established that two weeks after ONI models, about 90% of RGCs were lost through apoptosis (25). However, our study was in accordance with the other studies showing that EPO treatment resulted in retinal cells survival and regeneration (26, 27); for review, see (7). Following ONI, several physiological and biochemical events and signaling pathways led to the apoptosis of RGCs (28). Histopathological analysis indicated that axonal degeneration and RGC death resulted in structural and functional loss of ON (29), but our study on EPO was able to preserve nerve function. Interestingly, in presence of the EPO, amplitude potential of RGCs was increased even higher than day 0. In fact, two EPO injections occurred on days 2 and 9 after a crush, leading to high amplitude recovery. This increase at VEP was significantly different than the control -from day 14 (after the second EPO injection) and consisted of a 2-months study. Here two indices were calculated for these phenomena: 1) bursting activity of RGCs was enhanced and long-term potentiating was induced via EPO effect, 2) EPO-induced influx of Ca^{2+} occurred via voltage-activated Ca^{2+} channels. In this regard, one study indicated selective enhancing effect of EPO on rat hippocampus cells and showed that neuroplasticity might be based on a more efficient bursting activity (30). Additionally, Assandri et al. (31), demonstrated that EPO could interact with neuronal cells by affecting Ca^{2+} homeostasis through an increase in Ca^{2+} influx via plasma membrane t-type voltage-dependent Ca^{2+} channels. In this study, they showed that the super fusion of rh-EPO led to an increase in peak Ca^{2+} current.

In addition, it is well established that microglia are the resident immune cells of the retina. Neuronal survival depends on the astrocyte presence, especially upon injury (32). Microglial activation is one of the earliest events and a crucial contributor to retinal diseases, and a correlation between reactive microglia and axonal loss has been demonstrated in glaucoma (33). While reactive astrocytes can respond to treat and maintain homeostasis, they can produce an environment prohibiting axonal regeneration and it might cause secondary neuronal damage (32). In this line, Cooper et al. (34) showed that reactive astrocyte gliosis contributed to remodel optic nerve, as a primary inducer of the progression of axon pathology in glaucoma. The relationship of EPO with axon protection and regeneration, following optic nerve injury, is unclear. Given the EPO impact on astrocytes, we evaluated microglia in control and EPO-treated retina. EPO significantly reduced *GFAP* expression as a glial marker, which was higher than the normal retina in the vehicle group. In our study, the positive *GFAP* was an indicator of astrocytes among the other retinal glial, such as muller cells, because of expression location in the RGC layer. In addition to diminishing the *GFAP* expression under effect of EPO during 7- and 60-days post-injury, we found that mean cross-sectional area of glia, increased as the injured nerve, was evaluated on the 7th day post-crushed. Perhaps our most important and novel result was that many nerves retained their high axon packing density when EPO was applied. For these nerves, high axon density was matched with suppression of glial scars. We now interpret the lower *GFAP* expression, noted following EPO administration, is not directly related to the toxic effect of EPO. For killing the nerve resident astrocytes, our *in vitro* study on EPO-treated non-reactive and reactive mouse astrocyte cells led us to believe that the mechanism of glial suppression was not related to substantial axon loss. Even so, we can conclude that EPO promoted neuron regeneration by inhibiting astrocyte reactivity, which is in line with the previous studies (35). For example, Hu et al. (15) confirmed that exogenous EPO was able to reverse diabetes-induced reactive gliosis and acted indirectly to protect neurons by promoting production of neurotrophic factors in Muller cells.

Besides, EPO may alter different neurotrophic factor expressions in astrocytes. Our study showed that expression of *CNTF* in the retina was extremely higher in the EPO recipient group. *CNTF* is a multipotent neurotrophic factor that, in high concentrations, indorses axonal regeneration and accelerates retinal neuroprotection (8). A study demonstrated that neuro-regenerative effects of *CNTF* are due to raising the number of glial cells after ONI (36). In fact, *CNTF* is the neuroprotective and axon-growth-promoting cytokine releasing from retinal astrocytes and strongly activating regenerative pathways in RGCs (10).

In contrast, in our study, expression of *BDNF* was lower in the EPO group. Several studies indicated that presence of *BDNF* increased RGC survival and promoted axonal regrowth (37, 38). However, another study established that *BDNF* promoted RGC survival, while preventing its outgrowth (39).

A major limitation of our study is that the adverse effects of EPO usage were not monitored. As known, EPO is a hematopoietic agent and its systemic administration can lead to certain adverse effects, such as an abnormal increase in hematocrit, pyrexia, headache, nausea, generalized weakness, superficial phlebitis, thromboembolic events, and even death. However, studies have shown that administration of low doses of EPO was safe and did not raise the chances for serious adverse effects (40).

In summary, we demonstrated that intravenous administration of EPO in ONC presented neuroprotective and neuro-regenerative aspects through different mechanisms, such as anti-apoptosis, an increase in

neurotrophic agents (like *CNTF*), and neural and axonal regenerations. It also restored the optic nerve function and improved visual acuity in rats after nerve transection.

Different studies done over the years and the results of EPO from basic research and even some clinical studies, for more information review (7), supported the possibility of integrating EPO therapeutic effects on optic nerve protection and retinal regeneration.

Conclusion

This study provided a better understanding of multiple potential protective mechanisms of EPO in TON and suggested that astrocyte cells may be an important target in the therapy of traumatic retina, perhaps in addition the other neurodegenerative retinal diseases to prevent visual impairment and improve patients' quality of life.

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Authors' Contributions

A.E., M.Se.; Data collection, analysis, interpretation, and manuscript writing. Y.A.; Data analysis and manuscript writing. F.H.M., L.Z., M.Z.-Kh.; Data analysis, interpretation, and manuscript writing. M.S.Sa., F.P., L.S.; Conducting experiments, conception and design, data analysis and interpretation, administrative and financial support. L.S.; Manuscript proof. All authors read and approved the final manuscript.

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