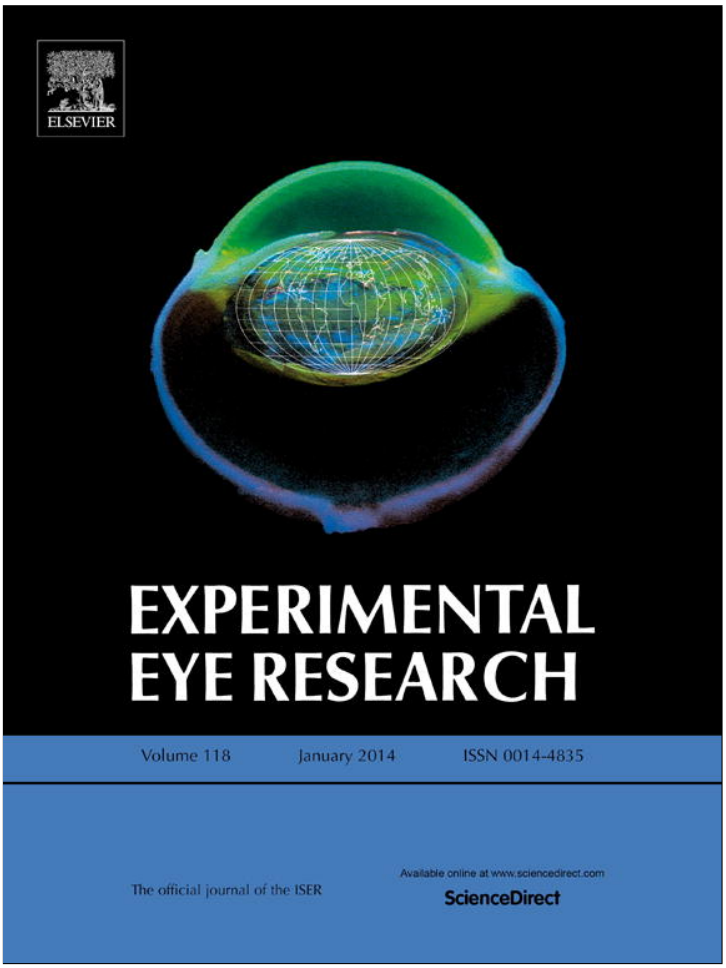
G-CSF對視神經病變具有神經保護效果



Experimental Eye Research 118 (2014) 109–116

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Neuroprotective effects of recombinant human granulocyte colony-stimulating factor (G-CSF) in a rat model of anterior ischemic optic neuropathy (rAION)

Chung-Hsing Chang [a], Tzu-Lun Huang [b,c], Shun-Ping Huang [b,d,e], Rong-Kung Tsai [b,c,f,*]

[a] Department of Dermatology, Graduate Institute of Medicine, School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Ophthalmology, Buddhist Tzu Chi General Hospital, Hualien, Taiwan
[c] Department of Ophthalmology and Visual Science, Tzu Chi University, Hualien, Taiwan
[d] Department of Medical Research, Buddhist Tzu Chi General Hospital, Hualien, Taiwan
[e] Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien, Taiwan
[f] Department of Ophthalmology, Kaohsiung Medical University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Received 14 October 2013
Accepted in revised form 21 November 2013
Available online 5 December 2013

Keywords:
rat anterior ischemic optic neuropathy
rAION
granulocyte colony stimulating factor
neuroprotection
retinal ganglion cells

ABSTRACT

The purpose of this study was to investigate the neuroprotective effects of recombinant human granulocyte colony stimulating factor (G-CSF), as administered in a rat model of anterior ischemic optic neuropathy (rAION). Using laser-induced photoactivation of intravenously administered Rose Bengal in the optic nerve head of 60 adult male Wistar rats, an anterior ischemic optic neuropathy (rAION) was inducted. Rats either immediately received G-CSF (subcutaneous injections) or phosphate buffered saline (PBS) for 5 consecutive days. Rats were euthanized at 4 weeks post infarct. Density of retinal ganglion cells (RGCs) was counted using retrograde labeling of Fluoro-gold. Visual function was assessed by flash visual-evoked potentials (FVEP) at 4 weeks. TUNEL assay in the retinal sections and immunohistochemical staining of ED1 (marker of macrophage/microglia) were investigated in the optic nerve (ON) specimens. The RGC densities in the central and mid-peripheral retinas in the G-CSF treated rats were significantly higher than those of the PBS-treated rats (survival rate was 71.4% vs. 33.2% in the central retina; 61.8% vs. 22.7% in the mid-peripheral retina, respectively; both $p < 0.05$). FVEP measurements showed a significantly better preserved latency and amplitude of the p1 wave in the G-CSF-treated rats than that of the PBS-treated rats (latency120 $\pm$ 11 ms vs. 142 $\pm$ 12 ms, $p = 0.03$; amplitude 50 $\pm$ 11 μv vs. 31 $\pm$ 13 μv, $p = 0.04$). TUNEL assays showed fewer apoptotic cells in the retinal ganglion cell layers of G-CSF treated rats [2.1 $\pm$ 1.0 cells/high power field (HPF) vs. 8.0 $\pm$ 1.5/HPF; $p = 0.0001$]. In addition, the number of ED1 positive cells was attenuated at the optic nerve sections of G-CSF-treated rats (16 $\pm$ 6/HPF vs. 35 $\pm$ 10/HPF; $p = 0.016$). In conclusion, administration of G-CSF is neuroprotective in the rat model of anterior ischemic optic neuropathy, as demonstrated both structurally by RGC density and functionally by FVEP. G-CSF may work via the dual actions of anti-apoptosis for RGC surviving as well as anti-inflammation in the optic nerves as evidenced by less infiltration of ED1-povitive cells.

1. Introduction

Non-arteritic anterior ischemic optic neuropathy (NAION) is the most common type of ischemic optic neuropathy in people over 55 years old. The incidence of NAION is 2.3–10.3 per 100,000 in the US (Johnson and Arnold, 1994; Hattenhauer et al., 1997). The most common pathogenesis of NAION is caused by transient nonperfusion or hypoperfusion of the optic nerve disc head (ONH) and its risk factor is multifactorial (Hayreh, 1996, 2009, 2011). Risk factors that have been implicated include nocturnal hypotension, impaired autoregulation of the microvascular supply, vasculopathic occlusion, crowd disc appearance and venous insufficiency (Hayreh, 1996, 2009, 2011; Arnold, 2003; Kerr et al., 2009). Optic nerve ischemia will induce a chain reaction of edema, compartment and consequential tissue infarction and eventually result in retinal ganglion cells (RGCs) death and vision loss (Levin and Louhab, 1996). Currently, there is not effective treatment for NAION, especially insofar as the

* Corresponding author. Department of Ophthalmology, Buddhist Tzu Chi General Hospital, Tzu Chi University, 707 Sec.3 Chung-Yung Road, Hualien 970, Taiwan. Tel.: +886 3 8561825x2112; fax: +886 3 8577161.
E-mail addresses: rktsai@tzuchi.com.tw, tsai.rk@gmail.com (R.-K. Tsai).

0014-4835/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.exer.2013.11.012

role of systemic corticosteroid therapy in NAION is still controversial (Hayreh and Zimmerman, 2008; Rebolleda et al., 2013). And while the search for effective treatment strategies is ongoing, for pathogenetic studies of NAION, a non-fatal condition, few clinical specimens have been made available (Knox et al., 2000; Tesser et al., 2003). Therefore, establishing reliable animal models of NAION is an alternative research strategy for preclinical trials. Recent reports using photodynamic therapy (PDT) have demonstrated that observable optic nerve (ON) changes associated with rodent model of anterior ischemic optic neuropathy (*rAION*) and primate NAION (*pNAION*) are indistinguishable from that seen in clinical data of human NAION (Salgado et al., 2011; Bernstein et al., 2011).

Granulocyte colony-stimulating factor (G-CSF) is already used extensively for treating chemotherapy-induced neutropenia, bone marrow reconstitution and stem cell mobilization (Miser et al., 1993; Weaver et al., 1993). Administration of G-CSF results in the mobilization of hematopoietic stem cells (HSCs) from bone marrow into peripheral blood (Demetri and Griffin, 1991). Recently, neuroprotective effects of G-CSF have been reported in animal models of stroke (Shyu et al., 2004), Alzheimer's disease (Tsai et al., 2007) and spinal cord injury (Koda et al., 2007). G-CSF also has an anti-apoptotic effect through activating a variety of intracellular signaling pathways, including PI3K/Akt (Dong and Larner, 2000; Tsai et al., 2010). Our previous reports have demonstrated that administration of G-CSF is neuroprotective in a rat model of optic nerve crush both in functional assessment and RGC morphometry (Tsai et al., 2008, 2010). This protection is achieved through the dual actions of anti-inflammation and anti-apoptosis. Subsequent reports have also shown that G-CSF is neuroprotective in models of optic nerve axotomy (Frank et al., 2009), light induced retinal damage (Oishi et al., 2008), retinal ischemia and reperfusion (Bu et al., 2010; Shima et al., 2012) and oxygen-induced retinopathy (Kojima et al., 2011). Activation of PI3k/Akt is believed to have the most powerful anti-apoptotic effects upon administration of G-CSF (Schneider et al., 2005; Tsai et al., 2010). Taken together, these observations suggest that G-CSF is a potential neuroprotectant for optic nerve and retinal injury. To the best of our knowledge, G-CSF has never been evaluated in an animal model of anterior ischemic optic neuropathy. In this study, we thus examined the effects of G-CSF treatment in a rat model of anterior ischemic optic neuropathy. The results may provide some valuable pre-clinical data for NAION.

2. Materials and methods

2.1. Animals

Sixty adult male Wistar rats weighing 150–180 g (7–8 weeks old) were used in this study. The rats were obtained from the breeding colony of BioLASCO Co., Taiwan. Animal care and experimental procedures were performed in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research. In addition, the Institutional Animal Care and Use Committee at Tzu Chi Medical Center approved all animal experiments. All manipulations were performed with the animals under general anesthesia, brought about by intramuscular injections of a mixture of ketamine (40 mg/kg bodyweight) and xylazine (4 mg/kg bodyweight; Sigma, St. Louis, MO, USA), and by the use of topical 0.5% Alcaine eye drops (Alcon, Puurs, Belgium). The rats had free access to food and water, and were maintained in cages in an environmentally controlled room with a temperature of 23 ± 1 °C, humidity of 55 ± 5%, and a 12-h light/dark cycle (light period: 07:00–19:00) (Tsai et al., 2008).

2.2. Study design

In the following AION experiments, the rats received once daily subcutaneous injection of recombinant human G-CSF (100 μg/kg/day in 0.2 ml of saline, 20 rats) (Takasaki pharmaceutical Plant, Tokyo, Japan) or phosphate-buffer saline (PBS, serving as Control; 0.2 ml, 20 rats) immediately after rAION procedure for 5 days thereafter. The other 20 rats received sham-operation and served as normal. Rats were euthanized at the 4th week post- infarct by CO_2 insufflations. 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) at the 4th week post-infarct. TUNEL assays in the RGC layer and immunohistochemistry of ED1 (macrophage/microglia) expression in the optic nerve (ON) were also conducted.

2.3. Induction of rAION

Following a previous report (Bernstein et al., 2003) with modification, the ONH of rats were treated with an argon laser immediately after intravenous injection of photosensitizing agent Rose Bengal (RB; Sigma–Aldrich, St. Louis, MO, USA). Sham laser treatment consisted of illuminating the ON region with an argon laser without RB administration. Briefly, after general anesthesia, RB was administered intravenously through the tail vein using a 28-gage needle (2.5 mM RB in PBS/1 mL/kg animal weight). After administration of RB, the right optic discs were directly treated with an argon green laser 532-nm in wavelength, 500-μm in size and 80 mW in power (MC-500 Multi-color laser, Nidek Co.,LTD, Tokyo, Japan). There were 12 pulses of 1 s duration for each laser procedure. When RB is activated by green laser light, it glows a brilliant golden color, which can be evidenced as a successful PDT (Bernstein et al., 2011). Given that this rAION model produced superoxide radicals rather than direct thermal damage to the whole area of the ONH (Bernstein et al., 2003), this injury spared the larger caliber central retinal vessels which supply the inner retinal circulation. After this procedure, Tobradex eye ointment (Alcon, Puurs, Belgium) was administered. The rats were subsequently kept on electric heating pads at 37 °C for recovery.

2.4. Retrograde labeling of RGCs with FluoroGold and morphometry of the RGCs

The detailed procedures have been described in our previous reports (Tsai et al., 2008, 2010; Huang et al., 2011). To avoid overcounting the RGCs by mixing labeled RGCs with dye engulfing macrophages and microglia, we performed retrograde labeling of the RGCs one week before the rats were euthanized. Briefly, the rats were anesthetized with a mixture of ketamine and xylazine, and placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). The skin covering the skull of the rats was opened, and 1.5 ul 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 eyeballs were placed in 10% formalin for 1 h, then the retina was carefully dissected and flat-mounted on a slide. The retinas were examined with a 400 × epi-fluorescence microscope (Axioskop; Carl Zeiss Meditech Inc., Thornwood, NY, USA) equipped with a filter set (excitation filter = 350–400 nm; barrier filter = 515 nm), as well as a digital camera (Axiocam MRm) and software (Axiovision 4.0). The retinas were examined for RGCs at a distance of 1 or 3 mm from the center of the ONH to provide the central and mid-peripheral RGC densities, respectively. We counted at least five randomly chosen areas of 62,500 μm^2 each in the central (about 40% of the central area) and mid-peripheral (about 30% of the mid-

periphery) regions of each retina, and their averages were taken as the mean density of RGCs per retina ($n \geqq 6$ in each group). RGCs survival percentage was defined as the number of RGCs in each treatment group divided by the number of RGCs in the sham-operated retinas, multiplied by 100.

2.5. Flash visual-evoked potentials (FVEP)

Visual-evoked potentials were recorded at 4 weeks after infarction. 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) (Tsai et al., 2008; Huang et al., 2011). We used a visual electrodiagnostic system (UTAS-E3000, LKC Technologies, Gaithersburg, MD, USA) to measure the FVEP. In brief, under general anesthesia, the recording electrodes in the occipital area and a reference electrode in the frontal area were separately connected with silver wires. The settings were background illumination off, a flash intensity of Ganzfeld 0 db, single flash with flash rate on 1.9 Hz, the test average at 80 sweeps, the threshold for rejecting artifacts at 20 mV, and a sample rate of 2000 Hz. We compared the latency and amplitude of the first positive going wavelet (P1) of FVEP among groups.

2.6. Optic nerve and retinal sample preparation

2.6.1. Optic nerves preparation

A segment of the ONs about 5–7 mm in length between the optic chiasm and the eyeball was harvested upon sacrifice of rats at 4 weeks. The nerves were immediately frozen at −70 °C for histological and immunohistochemical studies. None of the samples showed massive disc hemorrhage or retinal detachment on examination of the optic nerve histology. Cross sections of the optic nerve head were prepared and stained with hematoxylin-eosin.

2.6.2. Retinal section preparation

After animal sacrifice, the cornea, lens, and vitreous body were removed from the eyeball. The remaining eyecups, containing the sclera and the retina, were fixed in 4% paraformaldehyde for 2 h at room temperature. Each retinal cup was cut adjacent to the disc into two pieces. The tissues were then dehydrated in 30% sucrose overnight and kept at −20 °C until further processing. Some retinal cups were fixed in 4% paraformaldehyde for paraffin embedding and sectioning.

2.7. In situ nick end-labeling (TUNEL) assay

To ensure the use of equivalent fields for comparison, all retina frozen sections were prepared with retinas at 1–2 mm distance from the ONH. TUNEL reactions (DeadEnd™ Fluorometric TUNEL System, Promega Corporation, Madison, WI, USA) were performed to detect apoptotic cell. The TUNEL positive cells in the RGC layer of each sample were counted in ten high powered fields (HPF,×400 magnification), and three sections per eye were averaged (Tsai et al., 2008).

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

Longitudinal sections of ONs were stained with hematoxylin-eosin for histologic evaluation. ED1 antibodies react against extrinsic macrophages and intrinsic microglia (Milligan et al., 1991; Tsai et al., 2008). IHC of ED1 (CD68) using a monoclonal antibody (1:50, AbD Serotec, Oxford, UK) was performed (Huang et al., 2011). Briefly, the frozen ON sections were fixed with acetone at −20 °C for 30 min and blocked with 5% fetal bovine serum (FBS) containing 1% bovine serum albumin (BSA) for 15 min. The primary antibody 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 h. Counterstaining was performed using 4′,6-diamidino-2-phenylindole (DAPI,1:1000, Sigma, St Louis, MO, USA). For comparison, the ED1positive (+) cells were counted in six HPFs (×400 magnification) at the ON lesion site.

2.9. Fundus photography of rats

After pupil dilatation with Mydrin-P (Alcon, Puurs, Belgium) and applying topical anesthetics, a fundus contact lens for rats filled with 2% methylcellulose was put on the surface of the cornea. The optic discs of rats were observed and fundus photographs were taken through a slit-lamp biomicroscope (SL-D3, Topcon Inc., Tokyo, Japan) in the anesthetized rats.

2.10. Statistical analysis

All measurements in this study were performed in a masked fashion: mean values with standard deviations (S.D) were obtained. Statistical analysis was performed with commercial software (SPSS, Chicago, IL, USA). One-way ANOVA and post hoc Tamhane's T2 analysis was used to evaluate the differences among treated groups. Statistical significance was declared if a p value was <0.05.

3. Results

3.1. Morphometry of RGCs

The densities of RGC in the laser-controlled eyes (sham group) showed no significant difference with that of normal rats ($p > 0.05$, data not shown), which suggests that there was no thermal toxicity in the RGCs with laser treatment only. The densities of RGCs of central and mid-peripheral retinas in sham-operated rats were $2430 \pm 120/mm^2$ and $1600 \pm 150/mm^2$, respectively. Four weeks after induction of rAION, the RGC densities of the central and mid-peripheral retinas in the PBS-treated group were $810 \pm 230/mm^2$ and $360 \pm 20/mm^2$, respectively ($p = 0.009$ and 0.0001, respectively). In the G-CSF-treated and rAION-inducted group, the RGC densities of the central and mid-peripheral retinas were $1740 \pm 450/mm^2$ and $1010 \pm 410/mm^2$, respectively ($p = 0.014$ and 0.034, respectively, if comparing with the PBS-treated group; $p = 0.034$ and 0.047 respectively if comparing with the sham group) (Fig.1). In other words, the survival rate of RGCs in the central and mid-peripheral retinas in the G-CSF treated rats was significantly higher than those of the PBS-treated rats (71.4% *vs.* 33.2% in the central retina; 61.8% *vs.* 22.7% in mid-peripheral retina, respectively; both $p < 0.05$). The results demonstrate that RGC survival rate increases by approximately 38.2% in central retina and 39.1% in the mid-peripheral retina in the G-CSF-treated group, as compared to the PBS-treated group.

3.2. FVEP

The latency of P1wave at 4 weeks after rAION experiments was 76 ± 1 ms, 142 ± 12 ms and 120 ± 11 ms for the sham, PBS-treated and G-CSF-treated rats, respectively ($p = 0.03$ *vs.* PBS-treated rats; $p = 0.001$ *vs.* sham group). The amplitude of the P1wave was 74 ± 18 μv, 31 ± 13 μv and 50 ± 11 μv for the sham, PBS-treated and G-CSF-treated rats, respectively ($p = 0.04$ *vs.* PBS-treated rats; $p = 0.016$ *vs.* sham group). The FVEP results demonstrate that the G-CSF-treated group had significantly preserved partial visual function in comparison to the PBS-treated group after rAION induction ($n = 6$ in each group) (Fig. 2).

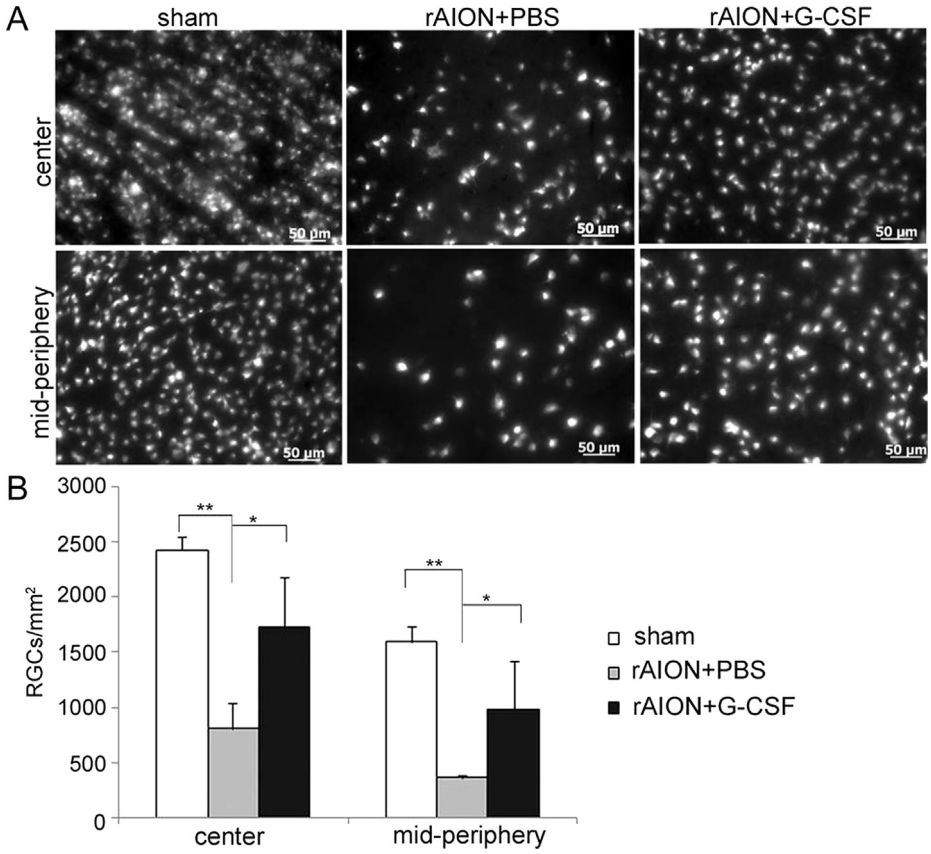


Fig. 1. Flat preparations of retinas and morphometry of RGCs by FluoroGold retrograde labeling at 4 weeks after rAION induction. A. Representative flat preparation of central and mid-peripheral retinas in normal (left), rAION with PBS-treated (middle) and rAION with G-CSF-treated rats. B. Morphometry of RGCs in the central and mid-peripheral retinas. RGC densities in the sham group were 2430 ± 120/mm^2 and 1600 ± 150/mm^2, respectively. Four weeks after rAION induction, RGC densities in the PBS-treated group were 810 ± 230/mm^2 and 360 ± 20/mm^2, respectively; RGC densities in the G-CSF-treated group were 1740 ± 450/mm^2 and 1010 ± 410/mm^2, respectively ($p = 0.014$ and 0.034, respectively, compared with RBS-treated retinas; $p = 0.034$ and 0.047, respectively, if compared with the sham group). RGC survival rates in the G-CSF-treated group were significantly higher than those in the PBS-treated group (71.4% vs. 33.2% in the central retina; 61.8% vs. 22.7% in mid-peripheral retina, respectively; both $p < 0.050$; One way ANOVA and post hoc analysis) [n ≧ 6 in each group]. $^*p < 0.05$; $^{**}p < 0.01$.

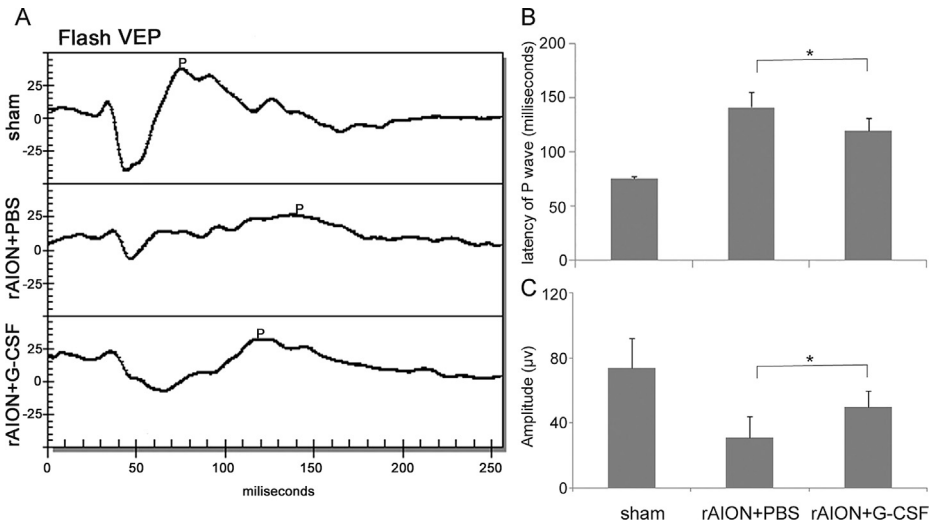


Fig. 2. FVEPs. A. Representative flash VEP tracings at 4 weeks after rAION inductions. B. The latency of the P1wave was 76 ± 1 ms, 142 ± 12 ms and 120 ± 11 ms in the sham, PBS-treated and G-CSF-treated rats, respectively ($p = 0.03$ *vs* PBS-treated rats; $p = 0.001$ *vs* sham group). The amplitude of the P1wave was 74 ± 18 μv, 31 ± 13 μv and 50 ± 11 μv in the sham, PBS-treated and G-CSF-treated rats, respectively ($p = 0.04$ vs PBS-treated rats; $p = 0.016$ *vs* sham group) [One way ANOVA and post hoc analysis, $n = 6$ in each group].

3.3. Histology of optic nerves and fundus photography after rAION

At 4 weeks after rAION induction, the optic disc showed edematous change with narrowing of vessels in the disc of PBS-treated rats, the disc appearance was less edematous and the architecture was much more preserved in the G-CSF-treated and rAION-inducted rats (Fig. 3). In the optic nerve sections, rAION induced disruption of the regular cellular columns, vacuolation of myelinated axons and accumulation of inflammatory cells in the PBS-treated rats; these pathologic changes were much more attenuated in the G-CSF-treated rats ($n = 3$ in each group) (Fig. 3).

3.4. In situ nick end-labeling (TUNEL) assay

TUNEL assays in the frozen retina sections demonstrated there were 1.3 ± 1.0 positive cells/HPF in RGC layers of retina in sham-operated rats, 8.0 ± 1.5 positive cells/HPF in the PBS-treated group and 2.1 ± 1.0 in the G-CSF-treated rats ($p = 0.0001$ *vs.* PBS-treated group, $p = 0.0465$ *vs. sham group;* $n = 6$ in each group) (Fig. 4). This observation indicated that G-CSF administration had anti-apoptotic effects on RGCs after rAION induction.

3.5. ED1 in the ON

At 4 weeks after rAION induction, the ED1 positive cells/HPF in the sham group, PBS-treated group and G-CSF-treated group were 3 ± 1, 35 ± 10 and 16 ± 6, respectively. The difference between treated groups was statistically significant ($p = 0.016$, $n = 6$ in each group) (Fig.5). These results indicate that G-CSF administration had anti-inflammatory effects at the ON after rAION, as demonstrated by little ED1-labeled macrophage/microglial accumulation at ONs.

4. Discussion

In the present study, our morphologic results demonstrate that G-CSF has a neuroprotective effect on ONs as well as on RGCs after induction of rAION in rats. In addition, the visual function demonstrated by FVEP was also better preserved in the G-CSF-treated rats compared to the PBS-treated rats, confirming the beneficial effects on the ocular structure.

rAION results in RGC loss by apoptosis, beginning at 7 days and the majority of RGC loss occur at 21 days post-induction (Slater et al., 2008; Bernstein et al., 2011). The rAION lesion specifically targets RGCs, and does not directly damage other retinal cell types (Bernstein et al., 2003). Our TUNEL assay results showed that G-CSF applications after rAION induction can rescue RGCs from apoptotic death and eventually preserve the survival rate of RGC, as evidenced by our results of the density measurements of RGCs. Our previous reports also indicated that G-CSF has anti-apoptotic effects on RGCs after optic nerve crush injuries in rats (Tsai et al., 2008, 2010). Possible rescuing mechanisms involving G-CSF administration in rAION models need further investigation.

Recent studies have shown that both G-CSF and its receptors are wildly expressed in the adult central nervous system and retinas (Hasselblatt et al., 2007; Oishi et al., 2008; Tsai et al., 2010). Given that exogenous G-CSF can penetrate the intact blood–brain barrier (BBB) (Schneider et al., 2005), exogenous G-CSF may penetrate retina-blood barrier (RBB) and bind with its receptors in the retinas. It has been suggested that autocrine protective mechanisms of G-CSF is one of the protective mechanisms for neurons (Schneider et al., 2005; Oishi et al., 2008; Tsai et al., 2010). Besides direct reaction with its receptors, G-CSF application results in the mobilization of HSCs to the peripheral blood. Trafficking of HSCs to the injured neurons mediated by the stromal cell-derived factor 1α (SDF-1α) can result in anti-inflammation and neurogenesis actions (Shyu et al., 2004; Schneider et al., 2005; Tsai et al., 2008).

The characteristics of VEP changes from rAION and human NAION, and specifically involves a reduction of amplitude instead of a prolongation of latency (Bernstein et al., 2003; Wilson, 1978). However, a decrease in amplitude and a lengthening of the P100 latency were observed in certain patients with NAION (Wildberger, 1984; Janáky et al., 2006). Our rAION model demonstrates a decreased amplitude and prolonged latency that occurred at 4 weeks after rAION induction. Prolongation of latency in VEP indicates post-infarct demyelination and axon loss in ON, and such histologic changes of demyelination have been confirmed in the

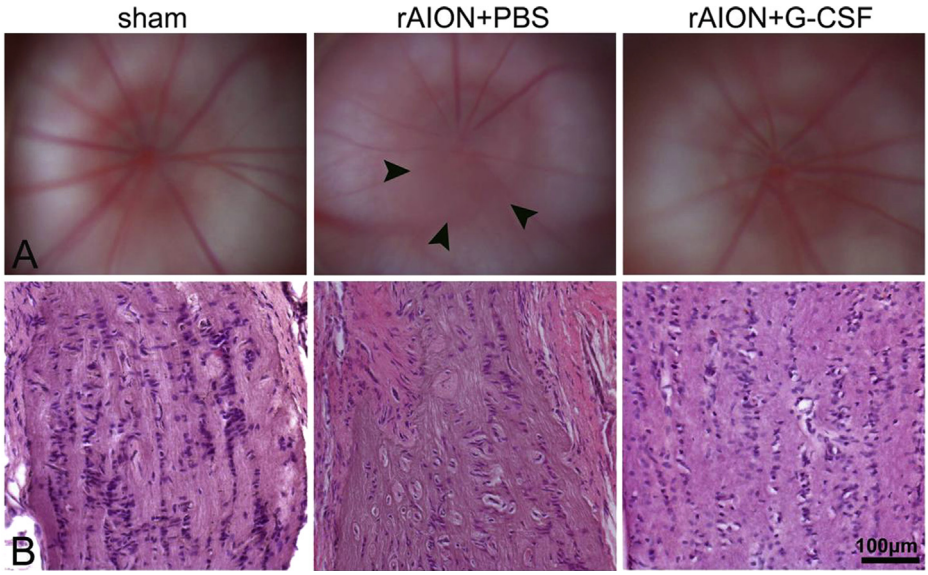


Fig. 3. Optic disc appearances and histology of optic nerve sections at 4 weeks after rAION inductions. A. The disc appearance showed little edema and the architecture was well-preserved in the G-CSF-treated and rAION-inducted rats as compared with PBS-treated rats (arrowhead indicate areas of blurry disc margin and edema). B. In ONs, rAION induced disruption of the cellular columns, vacuolation of myelinated axons and accumulation of inflammatory cells in the PBS-treated rats; these pathologic changes were greatly attenuated in the G-CSF-treated rats (Hematoxylin–eosin staining) [$n = 3$ in each group].

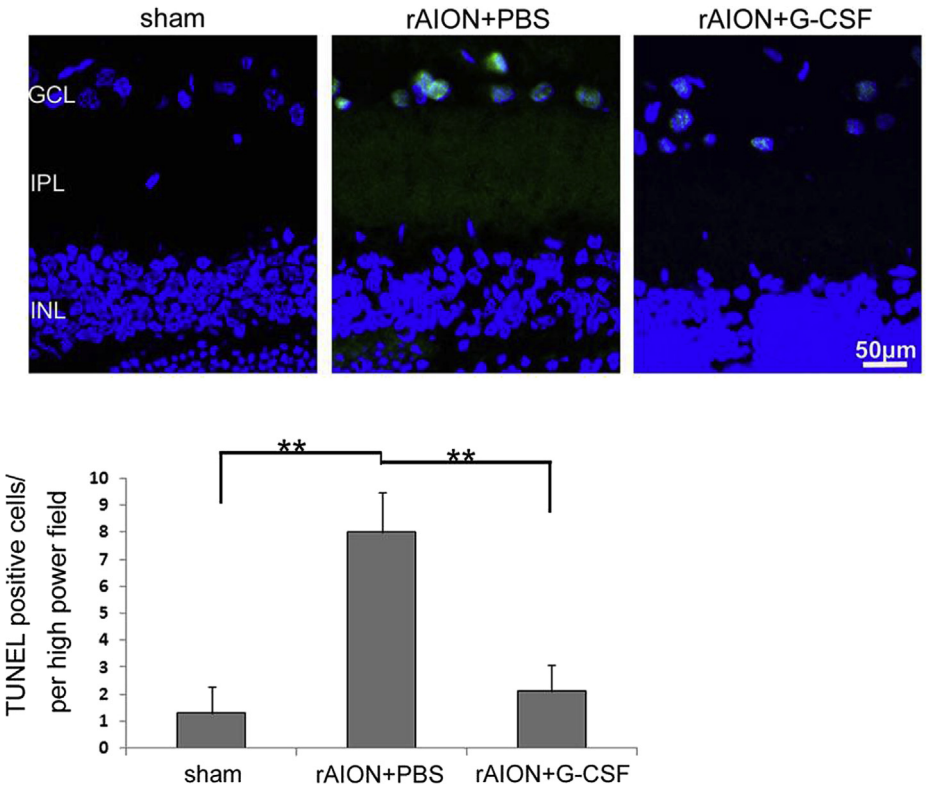


Fig. 4. TUNEL assays in retinal sections. The upper column was representative of the TUNEL in the retinas among the three groups. The lower column illustrates that there were 1.3 ± 1.0 positive cells/HPF in the RGC layers of retina in the sham-operated rats, 8.0 ± 1.5 positive cells/HPF in the PBS-treated group and 2.1 ± 1.0 in the G-CSF-treated rats ($p = 0.0001$ *vs.* PBS-treated group and $p = 0.0465$ *vs. sham group*) [One way ANOVA and post hoc analysis, $n = 6$ in each group].

rAION model (Goldenberg-Cohen et al., 2005; Bernstein et al., 2011). Although the latency prolongation of FVEP in our rAION model is somewhat different from other previous reports (Bernstein et al., 2003), the difference may reflect that severity of ON infarct by rAION induction in different species of rats is variable. Nonetheless, our results provide evidence that G-CSF application can preserve visual function both in amplitude and in latency of FVEP after rAION induction.

In a valuable report of human autopsy obtained 20 days after onset of NAION, accumulation of Iba1+/ED1+ cells (extrinsic

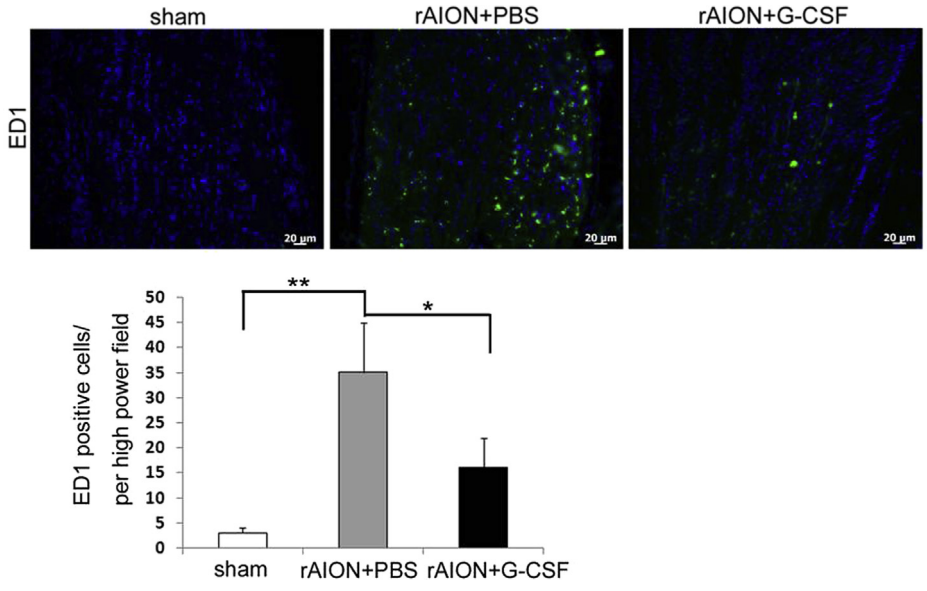


Fig. 5. Immunohistochemistry of ED1 in ONs at 4 weeks after rAION inductions. The upper column was representative of ED1 staining in the longitudinal sections of ON. The lower column indicates that the ED1+ cells/HPF in the sham group, PBS-treated group and G-CSF-treated group were 3 ± 1, 35 ± 10 and 16 ± 6, respectively. The difference between the treated groups was statistically significant ($p = 0.016$) [$n = 6$ in each group; One way ANOVA and post hoc analysis].

macrophages/microglia) in ischemic areas of the optic nerve was observed (Salgado et al., 2011). ED1-positive phagocytes found in ONs after rAION include monocytes/macrophages of hematogenous origin as well as microglia. A portion of ED1- + cells concomitantly express the Ia antigen during Wallerian degeneration in the ON (Stoll et al., 1989). The presence of blood borne-ED1+ cells at ON after rAION indicates that the brain blood barrier is disrupted (Bernstein et al., 2011). Our results show that ED1-positive macrophage/microglia accumulation at the ON lesion site was attenuated in the G-CSF-treated rats and greatly preserved the architecture of ON in histologic studies, suggesting that immediate administration of G-CSF may have an anti-inflammatory effect on the injured ON after rAION induction.

G-CSF has been ascribed with anti-inflammatory actions mediated by inhibition of TNF-a, by decreasing inducible nitric oxide synthase (iNOS) activity (Görgen et al., 1992), and by a reduction of interleukin-1b expression (Gibson et al., 2005; Görgen et al., 1992). The optic disc appearance in the rAION-inducted and G-CSF-treated rats also revealed less edema in our observations of the fundus photography.

In conclusion, we demonstrated that immediate administration of G-CSF provides ocular neuroprotection, as indicated both by RGC density counts and by functional assessment using FVEP, after rAION induction in rats. The protective actions of G-CSF may involve both anti-apoptotic and anti-inflammatory processes.

Financial disclosure

CH Chang, None; TL Huang, None; SP Huang, None; RK Tsai, None.

Acknowledgments

The authors thank Mr. Nigel Daly for his English editing and Ms. Su Zen Chen for her excellent figure assistance. This study was supported by a National Science Council Grant from the Taiwanese Government NSC100-2314-B-303-005.

References

Arnold, A.C., 2003. Pathogenesis of nonarteritic anterior ischemic optic neuropathy. J Neuroophthalmol. 23, 157–163.
Bernstein, S.L., Guo, Y., Kelman, S.E., Flower, R.W., Johnson, M.A., 2003. Functional and cellular responses in a novel rodent model of anterior ischemic optic neuropathy. Invest. Ophthalmol. Vis. Sci. 44, 4153–4162.
Bernstein, S.L., Johnson, M.A., Miller, N.R., 2011. Nonarteritic anterior ischemic optic neuropathy (NAION) and its experimental models. Prog. Retin. Eye Res. 30, 167–187.
Bu, P., Basith, B., Stubbs Jr., E.B., Perlman, J.I., 2010. Granulocyte colony-stimulating factor facilitates recovery of retinal function following retinal ischemic injury. Exp. Eye Res. 91, 104–106.
Demetri, G.D., Griffin, J.D., 1991. Granulocyte colony-stimulating factor and its receptor. Blood 78, 2791–2808.
Dong, F., Larner, A.C., 2000. Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF); evidence of the role of a tyrosine kinase activity distinct from the janus kinases. Blood 95, 656–1662.
Frank, T., Schlachetzki, J.C., Göricke, B., Meuer, K., Rohde, G., Dietz, G.P., Bähr, M., Schneider, A., Weishaupt, J.H., 2009. Both systemic and local application of granulocyte-colony stimulating factor (G-CSF) is neuroprotective after retinal ganglion cell axotomy. BMC Neurosci. 10, 49–58.
Gibson, C.L., Jones, N.C., Prior, M.J., Bath, P.M., Murphy, S.P., 2005. G-CSF suppresses edema formation and reduces interleukin1beta expression after cerebral ischemia in mice. J. Neuropathol. Exp. Neurol. 64, 763–767.
Goldenberg-Cohen, N., Guo, Y., Margolis, F.L., Miller, N.R., Cohen, Y., Bernstein, S.L., 2005. Oligodendrocyte dysfunction following induction of experimental anterior optic nerve ischemia. Invest. Ophthalmol. Vis. Sci. 46, 2716–2725.
Görgen, I., Hartung, T., Leist, M., Niehörster, M., Tiegs, G., Uhlig, S., Weitzel, F., Wendel, A., 1992. Granulocyte colony-stimulating factor treatment protectsrodents against lipopolysaccharide-induced toxicity via suppression of systemic tumor necrosis factor-alpha. J. Immunol. 149, 918–924.
Hasselblatt, M., Jeibmann, A., Riesmeier, B., Maintz, D., Schäbitz, W.R., 2007. Granulocyte-colony stimulating factor (G-CSF) and G-CSF receptor expression in human ischemic stroke. Acta Neuropathol. 113, 45–51.
Hayreh, S.S., 1996. Acute ischemic disorders of the optic nerve: pathogenesis, clinical manifestations and management. Ophthalmol. Clin. North Am. 9, 407–442.
Hayreh, S.S., Zimmerman, M.B., 2008. Non-arteritic anterior ischemic optic neuropathy: role of systemic corticosteroid therapy. Graefes Arch. Clin. Exp. Ophthalmol. 246, 1029–1046.
Hayreh, S.S., 2009. Ischemic optic neuropathy. Prog. Retin Eye Res. 28, 34–62.
Hayreh, S.S., 2011. Management of ischemic optic neuropathies. Indian J. Ophthalmol. 59, 123–136.
Hattenhauer, M.G., Leavitt, J.A., Hodge, D.O., Grill, R., Gray, D.T., 1997. Incidence of nonarteritic anterior ischemic optic neuropathy. Am. J. Ophthalmol. 123, 103–107.
Huang, T.L., Chang, C.H., Lin, K.H., Sheu, M.M., Tsai, R.K., 2011. Lack of protective effect of local administration of triamcinolone or systemic treatment with methylprednisolone against damages caused by optic nerve crush in rats. Exp. Eye Res. 92, 112–119.
Janáky, M., Fülöp, Z., Pálffy, A., Benedek, K., Benedek, G., 2006. Electrophysiological findings in patients with nonarteritic anterior ischemic optic neuropathy. Clin. Neurophysiol. 117, 1158–1166.
Johnson, L.N., Arnold, A.C., 1994. Incidence of nonarteritic and arteritic anterior ischemic optic neuropathy. Population-based study in the state of Missouri and Los Angeles County, California. J. Neuroophthalmol. 14, 38–44.
Kerr, N.M., Chew, S.S., Danesh-Meyer, H.V., 2009. Non-arteritic anterior ischaemic optic neuropathy: a review and update. J. Clin. Neurosci. 16, 994–1000.
Knox, D.L., Kerrison, J.B., Green, W.R., 2000. Histopathologic studies of ischemic optic neuropathy. Trans. Am. Ophthalmol. Soc. 98, 203–220.
Koda, M., Nishio, T., Kamada, T., Someya, Y., Okawa, A., Mori, C., Yoshinaga, K., Okada, S., Moriya, H., Yamazaki, M., 2007. Granulocyte colony-stimulating factor (G-CSF) mobilizes bone marrow-derived cells into injured spinal cord and promotes functional recovery after compression-induced spinal cord injury in mice. Brain Res. 1149, 223–231.
Kojima, H., Otani, A., Oishi, A., Makiyama, Y., Nakagawa, S., Yoshimura, N., 2011. Granulocyte colony-stimulating factor attenuates oxidative stress-induced apoptosis in vascular endothelial cells and exhibits functional and morphological protective effect in oxygen-induced retinopathy. Blood 117, 1091–1100.
Levin, L.A., Louhab, A., 1996. Apoptosis of retinal ganglion cells in anterior ischemic optic neuropathy. Arch. Ophthalmol. 114, 488–491.
Milligan, C.E., Cunningham, T.J., Levitt, P., 1991. Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain. J. Comp. Neurol. 314, 125–135.
Miser, J., Storb, R., Hansen, J.A., Bensinger, W., 1993. Syngeneic transplantation with peripheral blood mononuclear cells collected after the administration of recombinant human granulocyte colony-stimulating factor. Blood 82, 1981–1984.
Oishi, A., Otani, A., Sasabara, M., Kojima, H., Nakamura, H., Yodoi, Y., Yoshimura, N., 2008. Granulocyte colony-stimulating factor protects retinal photoreceptor cells against light-induced damage. Invest. Ophthalmol. Vis. Sci. 49, 5629–5635.
Rebolleda, G., Perez-Lopez, M., Casas-Llera, P., Contreras, I., Munoz-Negrete, F.J., 2013. Visual and anatomical outcomes of non-arteritic anterior ischemic optic neuropathy with high-dose systemic corticosteroids. Graefes Arch. Clin. Exp. Ophthalmol. 251, 255–260.
Salgado, C., Vilson, F., Miller, N.R., Bernstein, S.L., 2011. Cellular inflammation in nonarteritic anterior ischemic optic neuropathy and its primate model. Arch. Ophthalmol. 129, 1583–1591.
Schneider, A., Kruger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., Aronowski, J., Maurer, M.H., Gassler, N., Mier, W., Hasselblatt, M., Kollmar, R., Schwab, S., Sommer, C., Bach, A., Kuhn, H.G., Schäbitz, W., 2005. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J. Clin. Invest. 115, 2083–2098.
Shima, C., Adachi, Y., Minamino, K., Okigaki, M., Shi, M., Imai, Y., Yanai, S., Takahashi, K., Ikehara, S., 2012. Neuroprotective effects of granulocyte colony-stimulating factor on ischemia-reperfusion injury of the retina. Ophthal. Res. 48, 199–207.
Shyu, W.C., Lin, S.Z., Yang, H.I., Tzeng, Y.S., Pang, C.Y., Yen, P.S., Li, H., 2004. Functional recovery of stroke rats induced by granulocyte colony-stimulating factor stimulated stem cells. Circulation 110, 1847–1854.
Slater, B.J., Mehrabian, Z., Guo, Y., Hunter, A., Bernstein, S.L., 2008. Rodent anterior ischemic optic neuropathy (rAION) induces regional retinal ganglion cell apoptosis with a unique temporal pattern. Invest. Ophthalmol. Vis. Sci. 49, 3671–3676.
Stoll, G., Trapp, B.D., Griffin, J.W., 1989. Macrophage function during Wallerian degeneration of rat optic nerve: clearance of degenerating myelin and la expression. J. Neurosci. 9, 2327–2335.
Tesser, R.A., Niendorf, E.R., Levin, L.A., 2003. The morphology of an infarct in nonarteritic anterior ischemic optic neuropathy. Ophthalmol 110, 2031–2035.
Tsai, K.J., Tsai, Y.C., Shen, C.K., 2007. G-CSF rescues the memory impairment of animal models of Alzheimer's disease. J. Exp. Med. 204, 1273–1280.
Tsai, R.K., Chang, C.H., Wang, H.Z., 2008. Neuroprotective effects of recombinant human granulocyte colony-stimulating factor (G-CSF) in neurodegeneration after optic nerve crush in rats. Exp. Eye Res. 87, 242–250.
Tsai, R.K., Chang, C.H., Sheu, M.M., Huang, Z.L., 2010. Anti-apoptotic effects of human granulocyte colony-stimulating factor (G-CSF) on retinal ganglion cells after optic nerve crush are PI3K/AKT-dependent. Exp. Eye Res. 90, 537–545.

Weaver, C.H., Buckner, C.D., Longin, K., Appelbaum, F.R., Rowley, S., Lilleby, K., Miser, J., Storb, R., Hansen, J.A., Bensinger, W., 1993. Syngeneic transplantation with peripheral blood mononuclear cells collected after the administration of recombinant human granulocyte colony-stimulating factor. Blood 82, 1981–1984.

Wildberger, H., 1984. Pattern-evoked potentials and visual field defects in ischaemic optic neuropathy. Doc. Ophthalmol. 40, 193–201.

Wilson, W.B., 1978. Visual-evoked response differentiation of ischemic optic neuritis from the optic neuritis of multiple sclerosis. Am. J. Ophthalmol. 86, 530–535.