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# Effects of low level laser treatment on the survival of axotomized retinal ganglion cells in adult Hamsters

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## Abstract

Injury to axons close to the neuronal bodies in the mammalian central nervous system causes a large proportion of parenting neurons to degenerate. It is known that optic nerve transection close to the eye in rodents leads to a loss of about half of retinal ganglion cells in 1 week and about 90% in 2 weeks. Using low level laser treatment in the present study, we demonstrated that treatment with helium-neon (660 nm) laser with 15 mW power could delay retinal ganglion cell death after optic nerve axotomy in adult hamsters. The effect was most apparent in the first week with a short period of treatment time (5 minutes) in which 65–66% of retinal ganglion cells survived the optic nerve axotomy whereas 45–47% of retinal ganglion cells did so in optic nerve axotomy controls. We also found that single dose and early commencement of laser irradiation were important in protecting retinal ganglion cells following optic nerve axotomy. These findings thus convincingly show that appropriate laser treatment may be neuroprotective to retinal ganglion cells.

***Key Words:*** *low level laser treatment; retinal ganglion cells; optic nerve injury; neuroprotection; microglial proliferation; optic nerve axotomy*

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## Introduction

Injury to axons in the central nervous system (CNS) initiates processes that cause primary degeneration of the nerve at the injury site and secondary degeneration along its axons, leading to the death of the neuronal body. The retina is regarded as a part of the CNS, and is an ideal model to study CNS degeneration and protection. Whereas the axons in optic nerve (ON) are easily accessible, their neuronal bodies of the retinal ganglion cells (RGCs) in the ganglion cell layer of the retina can be manipulated in the eye. In addition, RGC loss is also commonly seen in many clinical eye diseases such as glaucoma and age-related macular degeneration. This feature has also attracted extensive studies in RGC viability under various pathological conditions. A commonly used model to study RGC viability is ON transection approach, in which about half of RGCs were lost in 1 week and about 90% in 2 weeks following ON axotomy at the site close to the eye (Berkelaar et al., 1994).

Laser can produce different effects on the targeting tissues depending on the types of laser used; these effects include photochemical, thermal, ionization and mechanical ones. Low level helium neon laser irradiation was found to attenuate the posttraumatic damage in both peripheral nervous system (PNS) and CNS in mammals. For example, low level helium neon laser irradiation of crushed sciatic nerves in rats caused a significant increase in the amplitude of the compound action potential of the corresponding tibial nerve and diminished neural scar formation (Rochkind et al., 1987). Transcutaneous irradiation of crushed ON in rabbits with a low-energy helium-neon laser resulted in maintenance of their histological integrity (Schwartz et al., 1987).

Low energy laser (HeNe, ≤ 12 mW) is able to modify transport of ions and molecules across cell membranes and affects cytosolic and mitochondrial protein synthesis *in vitro* (Vacca et al., 1996). Using HeNe laser, Poon and Yew (1980) showed that 5-minute transocular irradiation caused a 2.5-fold increase in protein synthesis and enhanced phagocytic activity in mouse retina. Recently, a transpupillary thermotherapy towards ON head using diode laser at a much higher power (60 mW) achieved RGC protection, probably through heat shock proteins-related mechanisms (Ma et al., 2010).

Microglias are ubiquitously distributed in non-overlapping territories in the CNS and comprise up to 20% of the total glial cell population in the brain (Lawson et al., 1990). The function of the resting microglias and the factors keeping them in a quiescent state are not entirely clear. It is now

known that the resting microglias can rapidly be transformed from the resting to an activated state in response to a wide range of injuries. Activation of macrophages at appropriate times in the eye *via* lens injury or zymosan administration protected RGCs after ON injury (Leon et al., 2000; Yin et al., 2003). But activated microglias/macrophages release both beneficial and detrimental substances, *i.e.*, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), reactive oxygen species, nitric oxide and proteases (Thery et al., 1991; Banati et al., 1993a, b; Batchelor et al., 1999) and inflammatory cytokines, *i.e.*, interleukin 1 (IL-1), interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) (Giulian et al., 1986; Sawada et al., 1989; Dickson et al., 1993). Microglias also produce a large amount of glutamate and aspartate *in vitro* (Piani et al., 1991). The release of these excitatory amino acids thus points to a further role of microglias in N-methyl-D-aspartic acid (NMDA) receptor-mediated neuronal injury. An observation relevant to the present study is that injection of microglia inhibitory factor (MIF) into the vitreous of the eye during and after ON transection resulted in significant retardation of RGC death (Thanos et al., 1993). This indicates that suppression of microglial activities could protect RGCs after ON injury. In the present study, we investigated the effects of low level laser (HeNe, 15 mW) treatment on RGC survival as well as microglial proliferation in adult golden hamsters. We found that low laser treatment could protect RGCs after ON axotomy, but unlikely through mediation of microglial proliferation.

## Materials and Methods

### Animals

Adult golden hamsters (*Mesocricetus auratus*, 4–6 weeks old) were used in this study. All experiments were performed in compliance with European Union (EU) Directive 2010/63/EU for animal experiments and the Animal Experimentation Ethics Committee of The University of Hong Kong guidelines. All possible measures were taken to minimize suffering and limit the number of hamsters used in this study. Eighty-one hamsters were used for the effect of laser treatment on RGC viability whereas the other 16 were used for analysis of microglial proliferation. Additional animals were used for histological studies. All animals were anesthetized with sodium pentobarbitone (Nembutal, 50 mg/kg, i.p.) for all surgical procedures.

### Surgical procedures and laser irradiation

In the beginning of the study, six hamsters were used for examining the histology of parasagittal sections of the retinas after ON transection with or without laser irradiation. These hamsters were divided into three groups. Group 1 ($n$ = 2) received no laser treatment and served as controls. Group 2 ($n$ = 2) received a 5 minute laser treatment each day and lasted for 7 consecutive days. Group 3 ($n$ = 2) received a 35 minute laser treatment in the first day only.

For survival studies with ON transection, the left orbit was exposed, and the ON was cut with a pair of microscissors at 1.5 mm from the globe. The laser used immediately after ON transection was a helium neon (HeNe, 660nm; Omega Excel Laser, London, United Kingdom) type with 15 mW power. The irradiation was given for 1, 5 or 10 minutes each day, and for 1, 7 or 14 days starting immediately after ON axotomy through the cornea of the eyes. The laser probe was placed at the nearest distance from the cornea towards the retina but without touching the eye. Retinas were checked and found no clear damage after the laser treatment. The animals were allowed to survive for 7 or 14 days.

### Histological staining and quantitative analysis of the data

To examine the effect of the laser treatments on retinal structure, retinal sections were stained with Harris haematoxylin and eosin. The nuclei would be stained as blue with some metachromasia while cytoplasm would appear in pink.

To label the number of surviving RGCs, 2 days prior to animal sacrifice, a piece of gelfoam soaked with 3% of fluorescent dye granular blue (GB) was applied to the proximal end of the transected ON to retrogradely label the surviving RGCs. At appropriate time points, the animals were sacrificed with an overdose of pentobarbitone and then perfused with normal saline. The left retinas were dissected out and post-fixed in 4% paraformaldehyde (0.1mol/L, pH 7.4) for 1 hour at room temperature. To determine the number of RGCs in normal retinas, GB was applied to newly cut ON stump 2 days prior to animal sacrifice. RGCs only start to die 4–5 days after the ON axotomy (Berkelaar et al., 1994).

The number of GB-labeled RGCs was calculated, as previously published (Fu et al., 2009; Mi et al., 2012), using the systematic random sampling method under 40 × magnification. The number of GB-labeled RGCs was counted within the grid of an eyepiece (area = 200 μm × 200 μm) at 500 μm interval along the median line of four quadrants (superior nasal, superior temporal, inferior nasal and inferior temporal) of the retina from the optic disc to the peripheral border. Seven to eight grids were counted in each quadrant. Any GB-labeled RGCs overlapping the lower and the right margin of the grid was waived. The area of the retina was measured using a digital tablet coupled to a microcomputer. The estimated number of GB-labeled RGCs in the retina was calculated by multiplying the mean density of the GB-labeled cells by the area of the retina.

### Nucleoside diphosphatase (NDPase) staining and quantification of microglia

For nucleoside diphosphatase staining, the retinas were dissected and fixed in 19% ormalin-PBS for 1 hour and 15 minutes 1 week after ON transection. Normal control was also used for comparison. After several washes in PBS, retinas were incubated in 0.1 mol/L Tris-maleate buffer pH 7.2 at 4°C overnight. Inosine 5-diphosphate (IDP) was used to demonstrate NDPase activity (Schnitzer and Scherer, 1990). The reaction medium contained 0.1mol/L Tris-maleate (0.5 mL), 25 mmol/L distilled water (0.075 mL), manganese

chloride (0.25 mL), dimethylsulfoxide (DMSO; 0.0125 mL) and 10 mmol/L IDP (0.25 mL). After incubating the retinas in reaction medium at room temperature for 30 minutes, 1% lead nitrate (0.15 mL) was added and the retinas were incubated at 37°C for 30 minutes. Afterwards, retinas were rinsed in distilled water, immersed in 2% aqueous ammonium sulphide solution for 2 minutes, rinsed in distilled water, and mounted in glycerine jelly.

The number of microglias was counted in the four quadrants at 1, 2, 3, 4 mm with ocular grid size 0.245 × 0.245 $mm^2$ under 400 × magnification from the optic disc in the ganglion cell layer (GCL), inner plexiform layer (IPL) and outer plexiform layer (OPL). The three layers could be located by focusing the retina at different depths. The main branch of blood vessels can be found in the GCL. A network of blood vessels can be found in the OPL. The INL is located between the GCL and the OPL.

**Statistical analysis**

All data were expressed as the mean ± SD. All groups were compared statistically using one-way analysis of variance followed by Bonferroni multiple comparison test (Park et al., 2004; Luo et al., 2010). A level of $P < 0.05$ was considered significantly different.

## Results

**Effect of laser irradiation on the retinal structure**

We did not see any obvious change on the retinal structure in the parasagittal sections of the retinas that had received 5 minutes × 7 days or one 35 minute laser treatment (**Figure 1**). This suggests that our laser treatment approaches did not render toxic effect on the retina.

**Effect of laser irradiation on RGC survival at 7 days post-axotomy**

**Figure 2** shows the characteristics of photomicrographs of GB-labeled RGCs under different experimental conditions. The average number of GB-labeled RGCs in the GCL of normal hamsters ($n = 6$) was 78,711 ± 2,314 (**Figure 3**). After ON transection, the average number of RGCs was decreased to 35,945 ± 1,871/retina ($n = 6$) at 7 days post-axotomy and 10,608 ± 406/retina ($n = 5$) at 14 days post-axotomy (**Figure 3**). These were about 55% and 87% reductions in the normal RGC number. These results are consistent with previous reports that ON axotomy close to the eye led to a loss of about half of RGC population within 1 week and about 90% in 2 weeks (Berkelaar et al., 1994).

The low level laser treatment rendered a clear neuroprotection on RGCs at 7 days post-axotomy. One-, 5- and 10-minute laser treatments for 7 consecutive days resulted in average numbers of 48,462 ± 4,000/retina ($n = 6$), 52,238 ± 3,012/retina ($n = 6$) and 44,649 ± 1,202/retina ($n = 5$) of surviving RGCs, respectively (**Figure 3**). All were significantly higher ($P < 0.001$, $P < 0.001$, $P < 0.05$) than the axotomy only control; however, prolonged laser treatment duration (10 minutes) did not appear to have a better protective effect (**Figure 3**).

**Effect of laser irradiation on RGC survival at 14 days post-axotomy**

The low level laser treatments also exerted neuroprotection on RGCs at 14 days post-axotomy; however, the protective effects were not as strong as at 7 days post-axotomy. Compared with the 14 days post-axotomy control group (10,608 ± 406/retina), One-, 5- and 10-minute laser treatments for 14 consecutive days resulted in average numbers of 15,943 ± 1656/retina ($n = 5$), 18,126 ± 2,155/retina ($n = 6$) and 13,969 ± 1,234/retina ($n = 5$) of surviving RGCs, respectively (**Figure 3**). Only 5-minute laser treatment regime showed a significant neuroprotection ($P < 0.05$) on RGCs as compared with the 14 days post-axotomy control group. Again these data show that prolonged laser treatment does not have a better neuroprotection on RGCs.

**Effect of single dose laser irradiation**

Since prolonged laser irradiation did not appear to have a better action on RGC survival, we next examined the effect of single dose laser treatment on RGC viability. As **Figure 4** shows, single dose of low level laser irradiation rendered similar neuroprotective effects to the 7 consecutive day treatment regimes, and there were no statistically significant difference between single dose and 7-day treatment of 1-, 5- and 10-minute groups (**Figure 4**). The average numbers of surviving RGCs in a single dose of 1- and 5-minute laser treatment groups were 51,257 ± 2,144/retina ($n = 6$) and 54,759 ± 5,895/retina ($n = 5$), respectively; both values were significantly ($P < 0.001$) higher than the axotomy control (**Figure 4**). Similar to what was observed in 7 consecutive day treatment group, 10-minute laser irradiation exerted poor neuroprotective effect, and the average number of GB-labeled RGCs was 42,580 ± 1,490/retina ($n = 5$). This value was no longer significantly higher than the axotomy control. These data indicate that a single dose of laser treatment for a short time duration (1–5 minutes) renders a similar neuroprotective effect on RGCs as 7 days of laser irradiation treatment.

**Effect of post-delayed laser irradiation**

We further studied the effect of delayed commencement of laser treatment. Since 5-minute laser treatment groups had the best neuropretective effect at both 7 and 14 days post-axotomy, the 5-minute laser treatment regime was selected as the optimal treatment duration for delayed treatment studies. As shown in Figure 5, the average number of GB-labeled RGCs after axotomy at 7 days post-axotomy declined with the increased time-delay of commencement of a 5-minute single dose laser irradiation. Though the 0.5 hour delay still exerted a neuroprotective action on RGCs (44,002 ± 1,203/retina, $n = 4$; $P < 0.05$; **Figure 5**), the average numbers of GB-labeled RGCs in 1, 3 and 12 hour delay treatment groups decreased to 40,339 ± 1,987/retina ($n = 4$), 36,029 ± 2,459/retina ($n = 4$) and 36,956 ± 1,741/retina ($n = 4$), respectively (**Figure 5**). There was no significant difference ($P < 0.05$) between these three delayed treatment groups and the axotomy control, or among these three groups. In other words, there will be

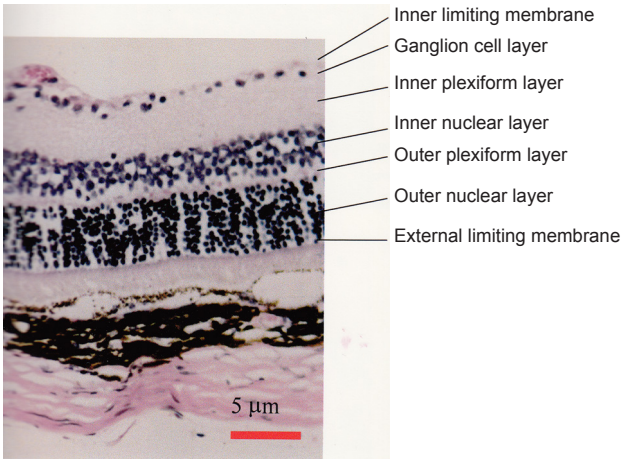


**Figure 1 Photomicrograph showing the retinal paraffin parasagittal section of a laser treated retina stained with Harris hematoxylin and eosin.**
There was no observable change in the retinal structure, suggesting that the laser approach did not render damage to the retina. Scale bar: 5 μm.

no protective effect if 5-minute laser irradiation treatment started 1 hour after ON axotomy.

### Effect of laser treatment on microglial distribution and proliferation in the retina

**Figure 6** shows the characteristics of a photomicrograph of NDPase positive microglias in the GCL. After ON transection, a vast majority of microglial population (80%) were found in the GCL, with about 20% in the IPL and 1–2% in the OPL.

Since most of the microglias were found in the GCL after ON transection, we next investigated microglial proliferation in the GCL. One week after ON transection (control group), the average number of microglias/mm$^2$ in the GCL increased significantly ($P < 0.01$) from 211 ± 7.8/mm$^2$ ($n = 2$) in the normal group to 412 ± 13/mm$^2$ in the axotomy control group ($n = 4$; **Figure 7A**). Following a daily 5 minute laser irradiation for 7 days (axo_5 min group, $n = 4$), the average number of microglias/mm$^2$ dropped to 317 ± 11.7/mm$^2$, the latter was significantly less than the control group ($P < 0.05$). The average number of microglias/mm$^2$ in the axo_1 min group ($n = 4$) and axo_10 min group ($n = 4$) were 504 ± 11.5/mm$^2$ and 596.75 ± 11/mm$^2$ and were substantially higher than the control group (**Figure 7A**). There was no statistical difference between the axo_5 min group and the 5 minute laser protocol group which received noaxotomy (noaxo_5 min group, $n = 2$). Thus, the neuroprotective effect of 5-minute treatment was in parallel with the decline in the number of microglias presented in the GCL.

### Effect of laser on the number of microglias in the IPL

One week after ON transection, the average number of microglias/mm$^2$ in the INL increased significantly from 75.75 ± 6.24/mm$^2$ in the normal group to 148.25 ± 7.5/mm$^2$ in the control group ($P < 0.01$; **Figure 7B**). The 5 minute laser irradiation also significantly ($P < 0.001$) reduced the number of microglias in this layer when compared with the axotomy control group. A significant reduction in the number of microglias was also seen after 1-minute laser treatment ($P < 0.05$; **Figure 7B**). However, owing to large variation, no statistically significant difference was seen between axo_10 min groups and the axotomy control group. Since there were few microglias in the OPL, the data are not presented here.

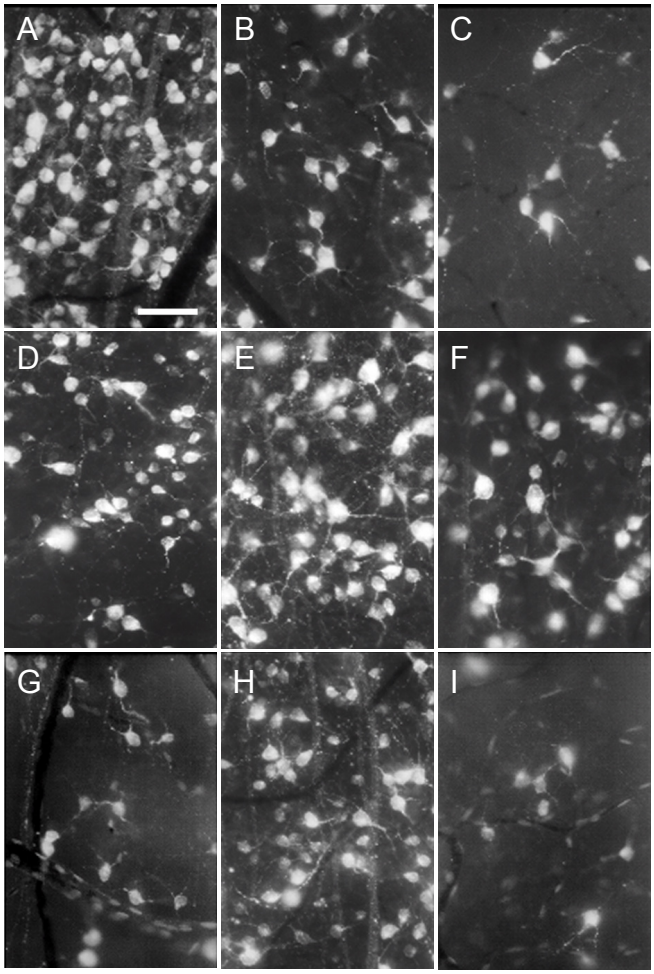


**Figure 2 Photomicrographs of fluorescent dye granular blue (GB)-retrogradely labeled retinal ganglion cells (RGCs) taken from superior-temporal quadrants of the retinas with 3 mm from the optic disc in animals treated with or without laser.**
(A) Normal, (B) control group on 7 days post-axotomy (dpa), (C) control group on 14 dpa, (D) 1-minute (min) laser treatment group on 7 dpa, (E) 5-min laser treatment group on 7 dpa, (F) 10-min laser treatment group on 7 dpa, (G) 1-min laser treatment group on 14 dpa, (H) 5-min laser treatment group on 14 dpa, and (I) 10-min laser treatment group on 14 dpa. Scale bar: 10 μm.

## Discussion

The results of the present study indicate that low energy laser (HeNe, 15 mW) irradiation delays degeneration of RGC in adult rodents after ON transection. Most of the effect is achieved after a single dose of laser irradiation. The effect is maximal when the time duration of laser irradiation is 5 minutes rather than 1 minute or 10 minutes and the irradiation is initiated within 0.5 hour after axotomy. Our

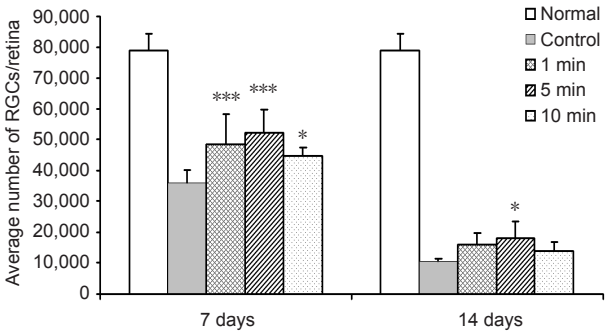


**Figure 3 Effects of various low level laser irradiation treatment regimes on the survival of retinal ganglion cells (RGCs) after optic nerve (ON) axotomy at 7 and 14 days post-axotomy in hamsters.**
1-min: 1-minute laser treatment; 5-min: 5-minute laser treatment; 10-min: 10-minute laser treatment. Data are expressed as the mean ± SD. $*P < 0.05$, $***P < 0.001$ (one-way analysis of variance followed by Bonferroni multiple comparison test).

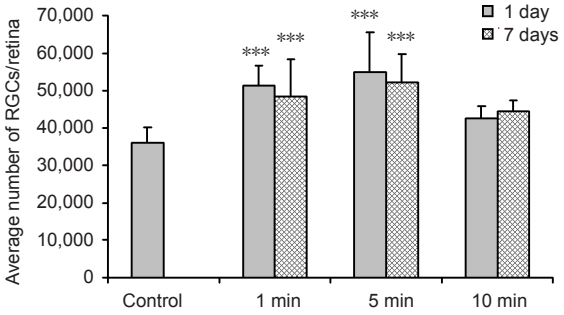


**Figure 4 Effect of single dose laser irradiation treatment (1 day) on the day of optic nerve injury compared with 7 consecutive treatment days (7 days).**
The average numbers of the granular blue-labeled retinal ganglion cells (RGCs) in the laser groups with single dose laser irradiation are not statistically different from those in the corresponding groups with 7 consecutive days of laser irradiation ($P > 0.05$), but both 1-minute (min) and 5 min groups irrespective of single dose or 7 consecutive days of laser treatment are neuroprotective ($P < 0.001$). Data are expressed as the mean ± SD. $***P < 0.001$ (one-way analysis of variance followed by Bonferroni multiple comparison test).

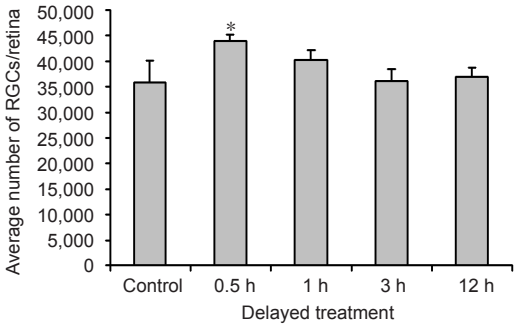


**Figure 5 Effects of delayed laser irradiation on the survival of retinal ganglion cells (RGCs) after optic nerve axotomy.**
The average number of granular blue-labeled RGCs after axotomy at 7 days post-axotomy was declined with the increased time from the commencement of a 5-minute single dose laser irradiation. Laser treatment was no longer neuroprotective if the commencement of the treatment was delayed 1 hour (h) or more. Data are expressed as the mean ± SD. $*P < 0.05$ (one-way analysis of variance followed by Bonferroni multiple comparison test).

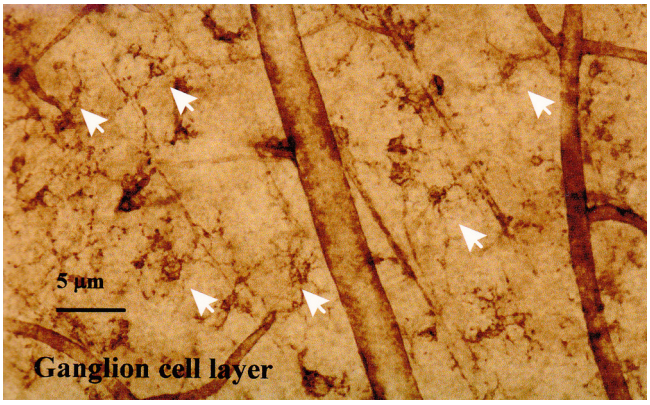


**Figure 6 A photomicrograph of NDPase positive microglias in the ganglion cell layer in optic nerve-axotomized retina.**
Arrowheads point to some NDPase positive microglias. Scale bar: 5 μm.

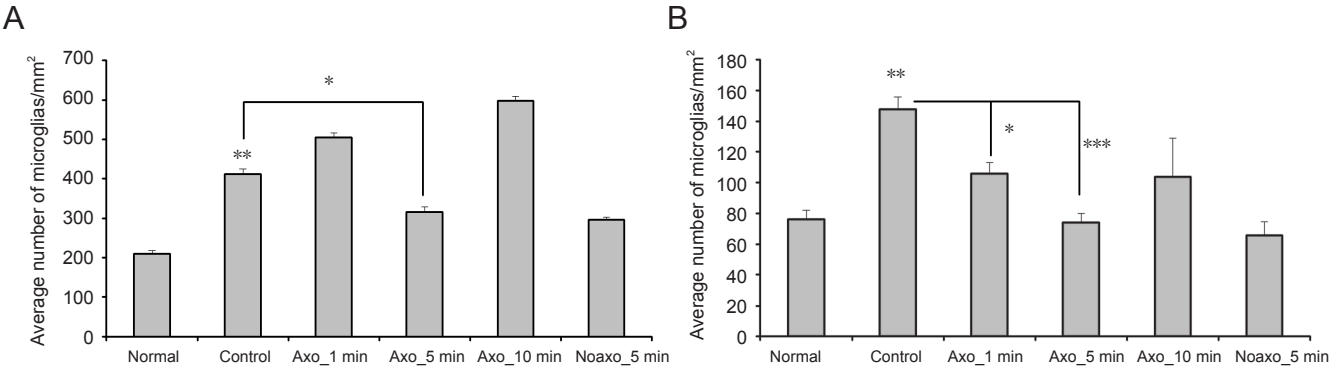


**Figure 7 Effects of laser on the number of microglias in the optic nerve-axotomized retina.**
(A, B) Effects of laser treatments on the number of microglias in the ganglion cell layer (A) and inner plexiform layer (B). The number of microglias increased in both layers after optic nerve axotomy but the extent of the increase was the least in the group receiving 5 minute (min) laser irradiation (axo_5 min group) in the two layers. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way analysis of variance followed by Bonferroni multiple comparison test).

results were supported by other experiments in rabbits and rats which showed that delayed degeneration of peripheral or central nerve was evident histologically (Schwartz et al., 1987; Menovsky et al., 2000) and electrophysiologically (Rochkind et al., 1987; Assia et al., 1989; Ebert and Roberts, 1997) after low energy laser irradiation. Recently, pre-exposure to low power diode laser irradiation was shown to promote cytoprotection in the rat retina (Sun et al., 2014).

The present results showed that laser treatment might reduce the proliferation of microglias in the retina following axotomy provided that an appropriate length of laser irradiation (5-minute) was used. In light of protective effect of MIF on RGCs after ON axotomy (Thanos et al., 1993), the observed laser treatment protection in the 5-minute group might be the suppression of both microglia activities and proliferation, leading to reduced production of pro-inflammatory molecules. This is compatible with an earlier experiment in which HeNe laser irradiation of the brain significantly increased the level of superoxide dismutase, an important enzyme in the antioxidant defense system and decreased the level of aspartate transferase, an enzyme yielding pro-inflammatory glutamate and oxaloacetate (Rossetti et al., 1991). In addition, Leung et al. (2002) reported that low energy laser (660 nm) could suppress the activity of nitric oxide synthase, an enzyme that produces neurotoxic molecule nitric oxide. However, macroglial activities rather than the number of microglias may play a critical role in mediating RGC protection, because all treatments render certain extents of RGC protection, but the number of microglias declined only in the 5-minute, not 1- or 10-minute, treatment group (**Figure 7A**).

Wollman and Rochkind (1998) also demonstrated that low energy laser irradiation applied to the brain tissue of rats might induce neurite process sprouting, thereby improving nerve tissue recovery. On the other hand, low energy laser irradiation of eyes with crushed ON prevented the enhanced production of prostaglandin E2 and leukotriene B4 *in vitro* after trauma (Naveh et al., 1990). The suppressive effect of low energy laser irradiation on arachidonic acid metabolism is reminiscent of steroidal and non-steroidal anti-inflammatory action, and might indicate the possible biochemical mechanisms associated with the clinical anti-inflammatory effect of low energy laser irradiation. The anti-inflammatory actions rendered by low energy laser treatment could thus explain how it delays the degeneration of RGCs in adult rodents in the present study, and why the laser irradiation should be commenced as soon as possible after ON injury. However, in our study, the 10 minute laser irradiation treatment had no beneficial effect. An earlier study showed that laser 670 nm with a much higher power (200 mW) produced cytotoxic singlet oxygen and led to neuronal death (Macklis, 1993). The absence of RGC protection in our prolonged treatment group may be the overproduction of singlet oxygen.

In conclusion, low-energy helium-neon (660 nm, 15 mW) laser treatment could delay the degeneration of RGC after ON transection in adult hamsters, provided that earlier commencement at appropriate duration was used. The protection may be achieved through reduction of pro-inflammatory microglial activities, but not microglial proliferation. Our results thus demonstrate that low energy laser treatment could be a tool to protect RGCs under certain conditions.

**Author contributions:** *So KF designed the work, supervised the experiments and analyzed the data. Leung MCP performed hamster experiments, analyzed the data and participated in the manuscript preparation. Cui Q analyzed the data and participated in the manuscript preparation. All authors approved the final version of this paper.*
**Conflicts of interest:** *None declared.*

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