

Preconditioning with carbon monoxide inhalation promotes retinal ganglion cell survival against optic nerve crush via inhibition of the apoptotic pathway

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Abstract. Optic neurodegeneration, in addition to central nervous trauma, initiates impairments to neurons resulting in retinal ganglion cell (RGC) damage. Carbon monoxide (CO) has been observed to elicit neuroprotection in various experimental models. The present study investigated the potential retinal neuroprotection of preconditioning with CO inhalation in a rat model of optic nerve crush (ONC). Adult male Sprague-Dawley rats were preconditioned with inhaled CO (250 ppm) or air for 1 h prior to ONC. Animals were euthanized at 1 or 2 weeks following surgery. RGC densities were quantified by hematoxylin and eosin (H&E) staining and FluoroGold labeling. Visual function was measured via flash visual evoked potentials (FVEP). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and caspase-9 and caspase-3 activity in the retinas, were assessed at 2 weeks post-ONC. The RGC density of CO + crush rats was significantly increased compared with that of the corresponding crush-only rats at 2 weeks (survival rate, 66.2 vs. 48.2% as demonstrated by H&E staining, $P < 0.01$; and 67.6 vs. 37.6% as demonstrated by FluoroGold labeling, $P < 0.05$). FVEP measures indicated a significantly better-preserved latency and amplitude of the P1 wave in the CO + crush rats compared with the crush-only rats. The TUNEL assays demonstrated fewer apoptotic cells in the CO + crush group

compared with the crush-only group, accompanied by the suppression of caspase-9 and caspase-3 activity. The results of the present study suggested that inhaled CO preconditioning may be neuroprotective against ONC insult via inhibition of neuronal apoptosis.

Introduction

The optic nerve is an extension of the central nervous system (CNS). CNS trauma causes damage to neurons including inflammation, neuronal apoptosis and functional recovery inhibition (1,2). The impairment of the optic nerve results in degeneration of the axon and death of retinal ganglion cells (RGCs), as they convey visual signals from the retina along their axons through the optic nerve to the brain, and even irreversible loss of vision (3,4). Optic nerve crush (ONC) is a well-established model of axonal injury to the optic nerve, due to its accessibility, stability and reproducibility (5,6). Following ONC, the injured RGCs suffer from either early necrosis or delayed apoptotic death (7), and cellular apoptosis mainly occurred within the first 2 weeks (8,9).

Carbon monoxide (CO) is a colorless and odorless gas that has been known to be a toxic molecule for centuries, due to its potent affinity to hemoglobin which results in hypoxia (10). However a number of studies have demonstrated that low or near-physiological doses of CO may have anti-inflammatory (11), anti-proliferative (12) and anti-apoptotic (13) properties, and neuroprotective effects (14). In optic neuropathy, Biermann *et al* (15) demonstrated that preconditioning with CO may protect RGCs from retinal ischemic/reperfusion injury. A previous study indicated that inhaled CO exerts neuroprotective effect against ONC (16). The present study investigated the effects of preconditioning with CO inhalation on ONC-induced RGC loss and the possible mechanisms.

Materials and methods

Animals and groups. A total of 108 adult male Sprague-Dawley rats (6 weeks old, 150-200 g) obtained from the experimental animal center of Shanghai Jiao Tong University (certification:

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SCXK Shanghai 2012-0002) were used in the experiments. The animals were raised in 20 cages in a temperature (23°C) and humidity (50%) controlled room with a 12-h light/dark cycle. Standard animal chow and water were freely available. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals recommended by the National Institutes of Health (Bethesda, MD, USA), and were approved by the Ethics Committee for Animal Experimentation of Shanghai Jiao Tong University (Shanghai, China). Animals were randomly divided into three groups of 36: i) CO + crush group, in which ONC was performed on the right eyes immediately following exposure to 250 ppm CO for 1 h; ii) crush group, in which only ONC was performed on the right eye of each rat; and iii) sham group, in which sham surgery was performed on the right eye. When the rats had been anesthetized with 10% chloral hydrate solution (400 mg/kg), the eyeballs were rapidly removed in 2 min. CO₂ euthanasia (5 l/min) was subsequently administered to the rats. Other procedures, including ONC, flash visual evoked potential (FVEP) analysis and FluoroGold intravitreal injection, were performed under deep anesthesia with 10% ketamine (95 mg/kg) and 2% xylazine (7 mg/kg), injected intraperitoneally.

ONC injury model. ONC was performed as previously described (5,8). To approach the optic nerve, an incision to the conjunctiva was made on the right eye with the guidance of a binocular-operating microscope. The optic nerve was exposed following separation of the retractor bulbi muscle. The crush was performed at 2 mm posterior the eyeball for 9 sec with a Sugita Titanium Aneurysm Clip II Applier (Mizuho Medical Co., Ltd, Tokyo, Japan). Care was taken to avoid affecting the retinal blood supply. An antibiotic ophthalmic ointment was applied to the models after surgery. The sham group received optic nerve exposure without the crush.

CO preconditioning. To examine the neuroprotective effect of inhaled CO, animals were randomized to receive treatment either with air, or air supplemented with 250 ppm CO (Shanghai Baosteel Gases Co., Ltd., Shanghai, China) for 1 h in an air-sealed chamber prior to ONC. The temperature in the chamber was maintained within the range of 22–25°C. During this procedure, the animals were awake and freely moving in the chamber. Anesthesia was performed (the procedure lasted ~10 min) immediately following gas inhalation, following the ONC approach.

Hematoxylin and eosin (H&E) staining. At 1 and 2 weeks post-surgery, six rats in each group were euthanized with excessive anesthesia and CO₂ inhalation. The eyeballs were dissected and fixed in 1% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 M PBS overnight at 4°C. Then the entire eyeballs were paraffin-embedded. H&E staining was performed using standard techniques for paraffin-embedded tissues at 25°C for 10 min. Sections of 4-μm were cut, and three sections with the sagittal plane through the optic nerve head area were selected for each eye. The H&E-stained retinas were imaged in 10 high power fields (HPF; magnification, x400) on a Nikon microscope (Nikon Eclipse E100; Nikon Corporation, Tokyo, Japan). Image Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to perform

quantitative analysis of cell numbers in the retina ganglion cell layer (GCL); six fields (two inner, two mid-periphery and two outer retinal eccentricity) per eye were averaged. There were six rats in every group at each time point.

RGC quantification. To evaluate the quantification of the RGCs at 2 weeks post-ONC, FluoroGold (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used 2 days prior to euthanasia, as previously described (16–18). The FluoroGold was dissolved in saline and injected into the intravitreal area using a Hamilton syringe, with a final concentration of 3% in 1 μl for each eyeball. At 2 weeks post-ONC, globes were removed and soaked in 4% PF for 2 h at 4°C. Following removal of the cornea and lens, the retinas were extracted and flattened onto microscope slides by making four radial cuts around the optic disc (19). We performed cell counting based on observation under a fluorescence microscope (Olympus BX-51; Olympus Corporation, Tokyo, Japan) at x20 magnification. A grid frame (0.5x0.5 mm) was used to choose the sample areas. The number of RGCs was counted and the density calculated. RGCs were sampled at the inner (1/6 retinal eccentricity), mid-periphery (1/2 retinal eccentricity) and outer (5/6 retinal eccentricity) of each quadrant of the retinal flat mount (20). RGC densities were quantified by calculating the mean of the samples. There were three animals in each group.

FVEP analysis. Visual electrophysiology was measured using an FVEP analyzing instrument (BMLab 4.0; Second Military Medical University, Shanghai, China) at 1 and 2 weeks post-ONC. Following anesthesia, each animal was secured to a stereotaxic frame. Subsequent to cutting the skin and drilling a hole above the visual cortex on the skull, an active electrode was placed on the pachymeninx and a passive electrode was placed on the skin in the interorbital region. The settings were background illumination off, 25 sweeps were added up for a test in a single flash (10 db; 1.0 Hz). Only the latency of the first positive (P) wavelet, and the amplitude between the first negative (N) and P wavelet of FVEP, were measured and compared among the three groups. There were six rats in every group at each time point.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) staining. TUNEL staining was performed on the cryosections using the *In Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA). A total of 2 weeks post-surgery, eyeballs were rapidly enucleated following sacrifice and postfixed in 4% PFA overnight at 4°C. Following dehydration with sucrose, the eyes were frozen and cut into 12-μm sagittal sections. Sections with the plane through the optic papilla were permeabilized with 0.1% Triton X-100 for 2 min. The TUNEL reaction was incubated in TdT enzyme and visualized by chromogenic staining with diaminobenzidine (10 mg/ml) at room temperature for 15 min (Sigma-Aldrich; Merck KGaA). TUNEL-positive (dark brown staining of the diaminobenzidine) cells in the retinal GCL were analyzed in 10 HPF (magnification, x400) and six fields were averaged for each eye. There were three animals in each group.

Caspase-9 and caspase-3 activity assay. To quantify the caspase-9 and caspase-3 activity among the groups, the retinal

caspase-9 and caspase-3 enzymatic activity was measured using a Caspase-9 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China) and a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) at 2 weeks post-surgery. Following measurement of the protein content using a protein assay kit (Beyotime Institute of Biotechnology), each sample was mixed with Ac-DEVE-pNA and reaction buffer to incubate at 37°C for 1 h. The retinal caspase-9 and caspase-3 activity was calculated as the results of the calibrated absorbance at 405 nm using a standard curve. There were three animals in each group for each measurement.

Statistical analysis. For all analysis, the analyzers were blinded to study group. All quantitative data are expressed as the mean \pm standard deviation. The results were analyzed by one-way analysis of variance and Tukey's post hoc multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhaled CO preconditioning increases RGC density after ONC. The decreased neuronal count in the retinal GCL following ONC was assessed by H&E staining (Fig. 1). The cell number in the GCL of the sham group was 37.2 ± 3.8 cells/HPF at 1 week and 34.5 ± 3.9 cells/HPF at 2 weeks post-surgery (Fig. 1B and E). A sequential decline of GCL neurons was observed as early as 1 week post-crush in the models, with aggravated decline by 2 weeks following ONC (Fig. 1C and F). More H&E-stained cells in the GCL were observed in the CO + crush group (Fig. 1D and G) compared with the crush-only group (29.0 ± 1.5 vs. 22.0 ± 1.2 cells/HPF at 1 week, $P < 0.001$; and 22.6 ± 3.0 vs. 16.6 ± 0.6 cells/HPF at 2 weeks, $P < 0.01$, respectively; Fig. 1H). It was apparent that preconditioning with inhaled CO exerted a marked rescue effect on the neurons of rat retinas following ONC insult.

Additionally, the RGC survival rate was assessed by FluoroGold labeling to count the surviving RGC number at 2 weeks post-ONC (Fig. 2). The average RGC density in the retinas of the sham-operated group was $3,236.2 \pm 360.0$ cells/mm² (Fig. 2A). At 2 weeks post-crush, the RGC densities decreased to $1,216.6 \pm 306.1$ and $2,186.1 \pm 394.0$ cells/mm² in the crush-only group and the CO + crush group, respectively (Fig. 2B and C; $P < 0.05$). Therefore, the RGC survival rates of the crush-only group were 48.2 and 37.6% as determined by H&E staining and FluoroGold labeling at 2 weeks, respectively. Increasing survival rates were observed in the CO + crush group of 66.2 and 67.6%, respectively. These results demonstrated that the RGC survival rate was increased in the CO + crush group compared with the corresponding crush-only group.

CO preconditioning ameliorated abnormal FVEP induced by ONC. In order to evaluate visual function among the groups, FVEP was tested at 1 and 2 weeks post-surgery (Fig. 3). The latency of the first P wave was 106.5 ± 8.6 msec (sham group), 181.2 ± 14.9 msec (crush-only group) and 156.3 ± 19.9 msec (CO + crush group) (crush-only group vs. sham group, $P < 0.001$; CO + crush group vs. crush-only group, $P < 0.05$; CO + crush group vs. sham group, $P < 0.001$; Fig. 3A and C) 1 week post-surgery. Simultaneously, the amplitude of the

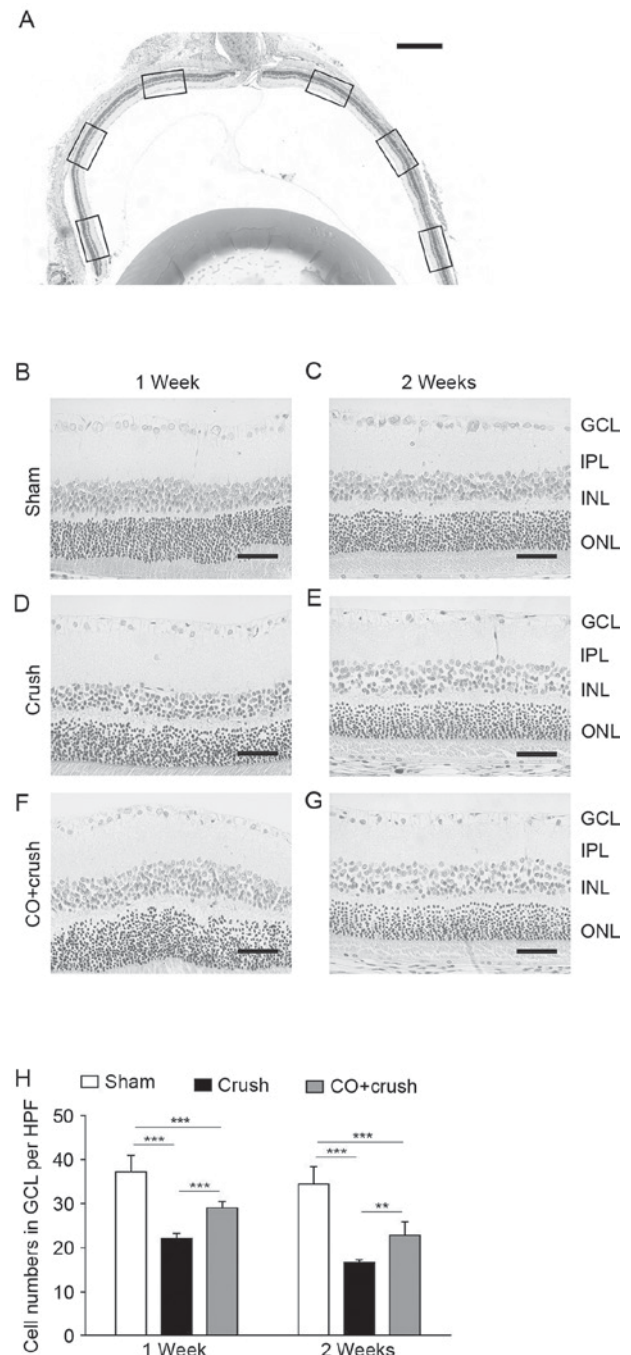


Figure 1. H&E staining of retinas. (A) Representative image of H&E of the retina with the optic nerve. Images were capture of the inner, mid-periphery and outer areas of the sections (scale bar, 500 μ m). Representative images are presented of retinas in the (B) sham group at 1 week, (C) sham group at 2 weeks, (D) crush group at 1 week, (E) crush group at 2 weeks, (F) CO + crush group at 1 week and (G) CO + crush group at 2 weeks (scale bars, 50 μ m). (H) The number of cells/HPF in the GCL of the CO + crush group was significantly increased compared with that of the crush-only group at the two time-points. ** $P < 0.01$, *** $P < 0.001$. n=6. HPF, high power field; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; H&E, hematoxylin and eosin.

N-P wave was $1,035.2 \pm 162.9$ μ V (sham group), 283.9 ± 67.3 μ V (crush-only group) and 599.0 ± 206.5 μ V (CO + crush group) (crush-only group vs. sham group, $P < 0.001$; CO + crush group vs. crush-only group, $P < 0.01$; CO + crush group vs. sham group, $P < 0.001$; Fig. 3A and D). A total of 2 weeks post-surgery, the visual function had deteriorated, as demonstrated by the

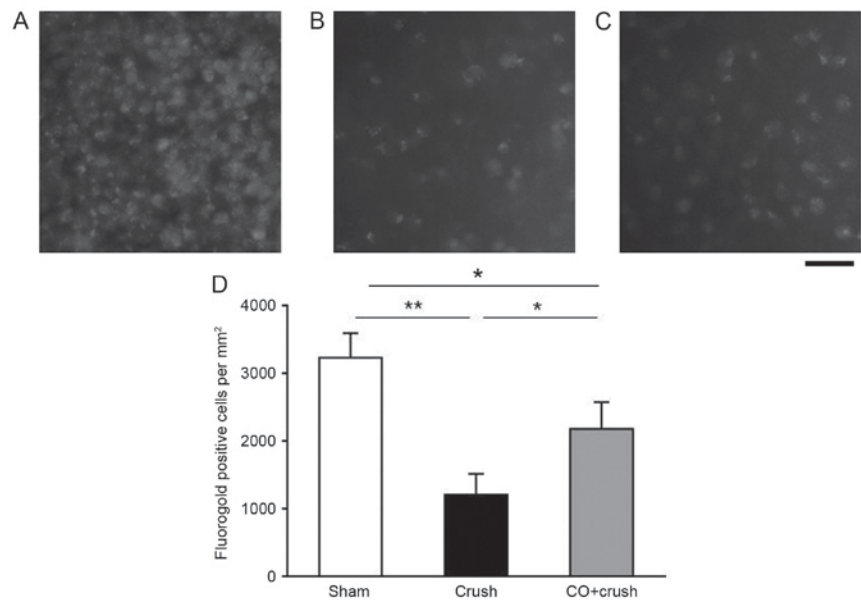


Figure 2. Representative images of flat-mounted retinas depicting FluoroGold-labeled RGCs 2 weeks post-surgery. Images are presented of (A) the sham group, (B) the crush-only group and (C) the CO + crush group. Scale bar, 50 μ m. (D) Quantification of FluoroGold-labeled RGC number/mm². CO preconditioning prior to crush exerted a significant neuroprotective effect. *P<0.05, **P<0.01. n=3. RGC, retinal ganglion cell.

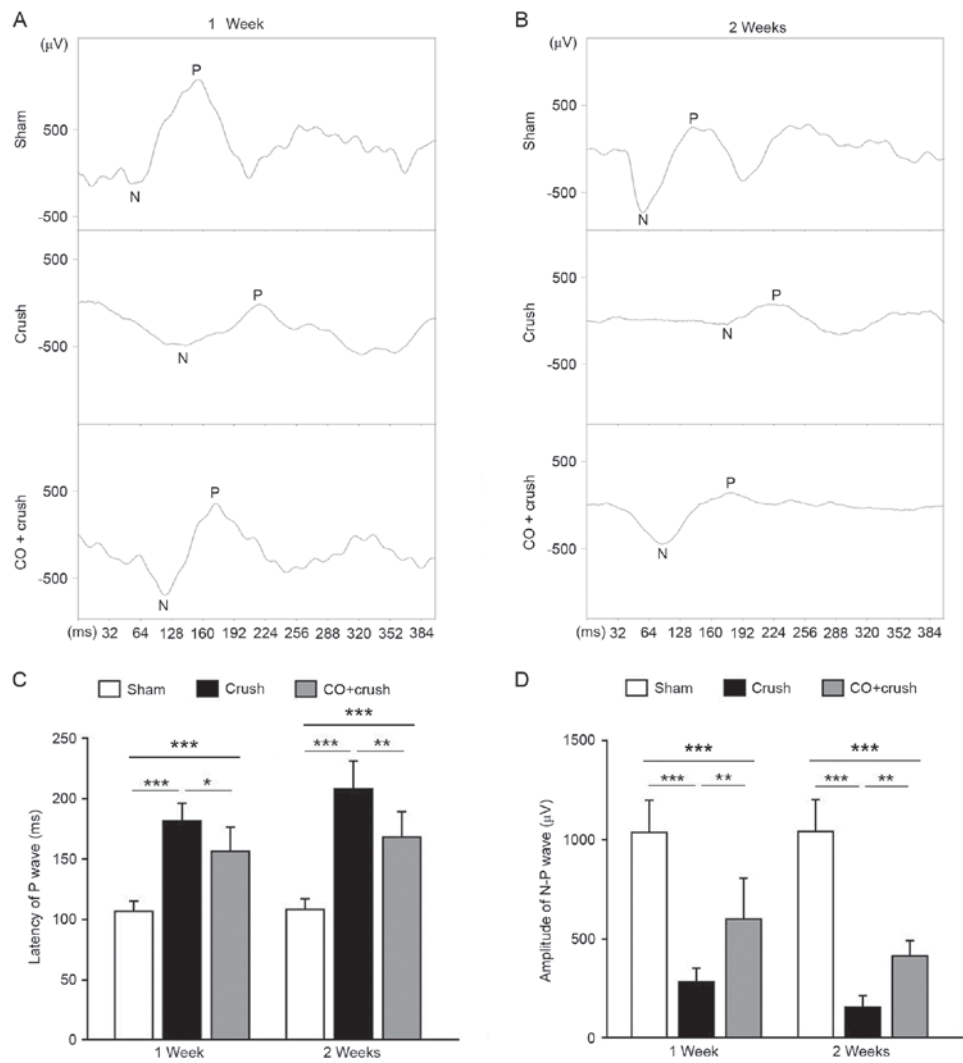


Figure 3. FVEP analysis. Representative FVEP tracings at (A) 1 week and (B) 2 weeks following surgery. (C) Latency and (D) amplitude of the P1 wave. The detection of FVEP in the CO + crush group exhibited a shorter latency and a higher amplitude compared with those in the crush-only group at the two time points. *P<0.05, **P<0.01, ***P<0.001. n=6. FVEP, flash visual evoked potential; P, positive; N, negative.

presentation of prolonged latency (207.8 ± 23.2 msec) and shortened amplitude (157.1 ± 55.9 μ V) in the crush-only group compared with the sham group (108.1 ± 8.8 msec of latency, $P < 0.001$; and $1,041.6 \pm 159.1$ μ V of amplitude, $P < 0.001$; Fig. 3B-D). CO preconditioning led to a shorter latency ($1,67.7 \pm 1.4$ msec; $P < 0.01$ CO + crush group vs. crush-only group; $P < 0.001$ CO + crush group vs. sham group; Fig. 3B and C) and a larger amplitude (414.1 ± 77.0 μ V; $P < 0.01$ CO + crush group vs. crush-only group; $P < 0.001$ CO + crush group vs. sham group; Fig. 3B-D) compared with the corresponding crush-only group at 2 weeks post-surgery. These data demonstrated that CO preconditioning preserved visual function to a certain degree following ONC injury.

Anti-apoptotic effect of CO preconditioning. TUNEL staining was performed to determine the extent of apoptosis in the retina. The nuclei of cells were clearly stained and few TUNEL-positive cells were observed in sham-operated rats (Fig. 4A). As illustrated in Fig. 4B, TUNEL-positive cells were markedly increased in the GCL of the crush-only group. CO preconditioning reduced the number of TUNEL-positive cells (Fig. 4C). The TUNEL assay demonstrated that the numbers of TUNEL-positive cells/HPF in the GCL were 4.1 ± 3.3 cells in the sham group, 16.4 ± 1.9 cells in the crush-only group and 10.6 ± 0.8 cells in the CO + crush group (sham group vs. crush-only group, $P < 0.01$; crush-only group vs. CO + crush group, $P < 0.05$; Fig. 4D) 2 weeks following surgery, demonstrating that CO exerted a significant anti-apoptotic effect on RGCs following ONC.

As presented in Fig. 5, Caspase-9 and Caspase-3 Activity Assay kits were used to measure the caspase-9 and caspase-3 activity in the retina. The caspase-9 activity was 0.8 ± 0.2 , 2.8 ± 0.4 , 1.8 ± 0.3 μ mol/grams of protein in the sham, crush-only and CO + crush groups, respectively ($P < 0.05$, CO + crush vs. crush-only group). The caspase-3 activity was 2.4 ± 0.2 , 8.7 ± 1.0 , 6.0 ± 0.8 μ mol/gprot in the sham, crush-only and CO + crush group, respectively ($P < 0.05$, CO + crush vs. crush-only group). The results demonstrated that the reduction of caspase-9 and caspase-3 activity may be involved in the mechanism of CO-induced neuroprotection.

Discussion

The principal findings of the present study regarding inhaled CO preconditioning were as follows: i) Preconditioning significantly rescued the loss of RGCs due to ONC injury, as evidenced by the improvement in RGC density in H&E-stained and FluoroGold-labeled flat-mounted retinas; ii) preconditioning ameliorated visual function, as demonstrated by the shortened latency of P waves and increased amplitude of N-P waves of FVEPs at 1 and 2 weeks post-ONC; and iii) preconditioning significantly inhibited the apoptotic process in the retina, as demonstrated by the reduction in TUNEL staining and caspase-3/9 expression. The results of the present study demonstrated that CO inhalation had a neuroprotective effect on RGCs in a rat ONC model.

RGCs are well-characterized CNS neurons, with the soma located in the inner part of the retina and the axon processed along the optic nerve that reach the superior colliculus in the brain (21,22). The crush operation directly

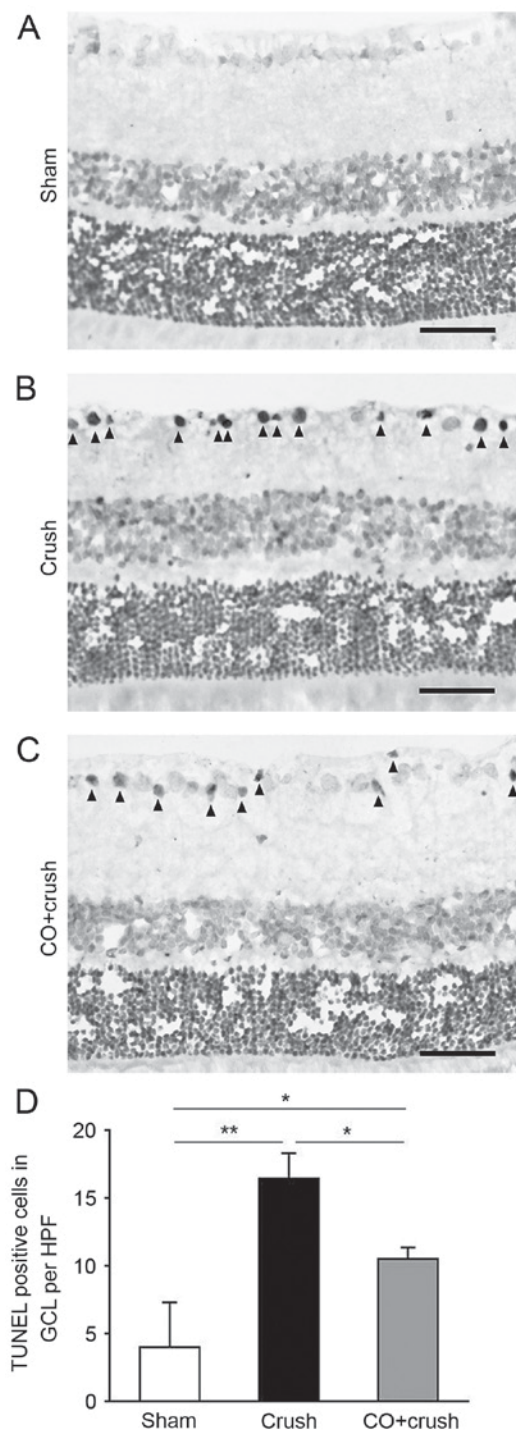


Figure 4. TUNEL staining of representative retinas at 2 weeks post-surgery. Images are presented of the (A) sham, (B) crush-only and (C) CO + crush groups. Scale bar, 50 μ m. (D) The number of TUNEL-positive cells in the GCL of the three groups was calculated. There was a significant increase in the crush-only group compared with the sham group, and the CO + crush group had fewer apoptotic cells compared with the crush-only group. * $P < 0.05$, ** $P < 0.01$. n=3. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; GCL, ganglion cell layer; HPF, high power field.

damages the optic nerve, in addition to causing rapid injury to the RGCs (23). ONC is suitable for studying the neurodegeneration of traumatic optic neuropathy and glaucomatous damage. The development of strategies for neuroprotection in response to neuropathy has involved gaseous therapies, including CO.

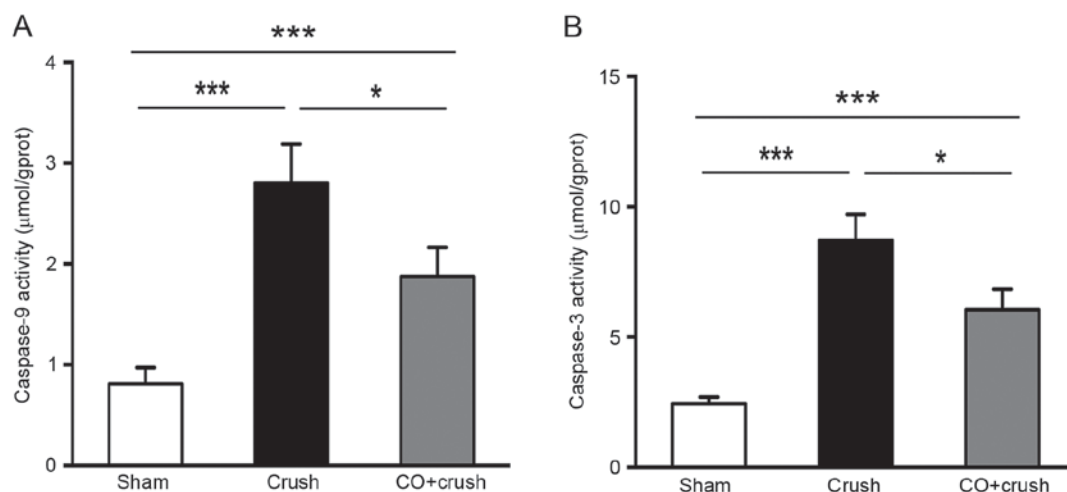


Figure 5. Caspase-9 and caspase-3 activity. The (A) caspase-9 and (B) caspase-3 enzymatic activity in the retinas at 2 weeks post-surgery. The caspase-9 and caspase-3 activity in the retinas of CO + crush group were significantly attenuated compared with those in the crush-only group. * $P < 0.05$, *** $P < 0.001$. $n = 3$.

Similar to nitric oxide (NO), CO is regarded as a neurotransmitter and a signal molecule which mediates vasodilation, although its capacity to activate soluble guanylyl cyclase is markedly lower compared with NO (24). Inhaled CO, in addition to CO donors, including HO, or CO releasing molecules (CORMs), has been investigated for its potential neuroprotective effect in a number of studies. *Ex vivo*, CO derived from heme protects against amyloid β peptide toxicity following inhibition of 5'-AMP-activated protein kinase catalytic subunit α -1 activation, and this protective effect exists concurrently in CORM-2 in a concentration-dependent manner (25). The study of Wang *et al* (14) demonstrated a benefit of decreasing infarct size using 250 ppm CO inhalation immediately following permanent middle cerebral artery occlusion. In the present study, the morphological results demonstrated that inhaled CO preconditioning attenuated RGC loss due to ONC injury. In addition, FVEP measurements demonstrated that visual function was better-preserved in the CO + crush group compared with the crush-only group, indicating a protective effect on the ocular structures. These results indicated that CO preconditioning may prevent RGC damage due to ON injury. CO or its donors may be applied in sophisticated retinal or ON surgery to protect against ON damage. The present study was different from previous work that focused on precautions to be taken prior to neuronal injury to avoid increased damage.

The mechanisms behind low concentration CO-induced protective effects are various. One possibility is promoting the anti-apoptotic pathway to prevent cell and tissue injury (26-28). The present study used TUNEL staining, a method involving an *in situ* test with TdT-specific binding to the 3'-OH ends of fragment DNA (29), to investigate the apoptotic alterations following ONC. The results of the present study suggested that the neuroprotection of CO preconditioning was mediated by a reduction in apoptosis of the retina in the rat model of ONC. The apoptotic pathway remains complex and poorly understood; it is considered that the caspase cascade executes this process, in which caspase-9 is an important amplifier of the mitochondrial signaling downstream and caspase-3 acts

as the trigger of the apoptosis pathway (30). Inhibition of excessive caspase activity has been demonstrated to be neuro-protective for RGCs (31). In the present study, compared with the crush-only group, the attenuated caspase-9 and caspase-3 expression in the CO + crush group demonstrated that inhaled CO preconditioning suppressed caspase-9 and caspase-3 enzymatic activity, thereby protecting retina by mitigating apoptosis.

There are various pathways underlying CO-mediated protection against apoptosis. Brouard *et al* (28) reported that 10,000 ppm CO preconditioning protected endothelial cells from tumor necrosis factor- α -induced apoptosis by the activation of nuclear factor- κ B-dependent genes, including baculoviral IAP repeat-containing protein 3 and Bcl-2 family member A1. Overexpressing heme oxygenase 1 (HO-1) had ameliorating effects on amyloid β 1-42 toxicity in Alzheimer's disease via the production of CO (25). Mitochondria triggered the anti-apoptotic function of mesenchymal stem cells, and inhibiting HO activity prevented mitochondrial biogenesis and attenuated the protective response in a co-culture of mesenchymal stem cells and distressed somatic cells (32). These beneficial effects of HO-1 and CO suggested that CO and HO-1 may interact with each other and the underlying mechanism for its precondition neuroprotection will be explored. In addition, Abe *et al* (33) applied high-pressure CO in kidney transplantation and demonstrated its anti-apoptotic effects by increasing phosphatidylinositol-3 kinase and p38-mitogen-activated protein kinase. The study used mixed gas composed of 2,000 hPa CO and 1,500 hPa O_2 to precondition the isolated kidney prior to transplantation, which led to improved renal function and tubular epithelial cellular apoptosis compared with 2,000 hPa N_2 and 1,500 hPa O_2 . Therefore, methods of applying CO, and parameters including pressure and dosage, are considerations for future research.

CO has a primarily negative connotation as a poisonous gas, which is acknowledged so widely that working against this dogma difficult. However, CO gas and its molecular release have exhibited protective effects in a number of animal models (34-37). CO has also demonstrated

benefits in suppressing proliferation and inflammation in cell cultures (11,12). Being a well-known toxic gas, safety and efficacy are considerations when translating the use of CO to a therapeutic setting. A single-blind crossover study of 12 volunteers indicated that the increase in COHb level following 1,200 or 1,500 ppm CO administration was comparable to smoking 20 cigarettes per day, and CO inhalation had no significant effect on blood pressure or heart rate (38). Given that CO has a potent influence on the delivery of oxygen, partial pressure of oxygen and oxygen saturation were measured by Liu *et al* (39), and the results demonstrated that 250 ppm CO did not affect oxygenation, although COHb significantly increased by 5.5, 2.8 and 1.5% following CO saline intraperitoneal administration for 1, 3 and 6 h respectively. In addition, CO application alone did not affect intestinal cellular apoptosis. In a study of CO preconditioning with retinal ischemic/reperfusion injury, there was no difference in FluoroGold retrograde labeling RGC densities between the control group and the CO group (15). In the present study experiments, all animals were free and active during the CO exposure; it therefore appears that the dose of CO was safe for rats.

In conclusion, the results of the present study suggested that preconditioning with a low concentration CO inhalation provided neuroprotection following ONC, with an increased RGC survival rate, and ameliorated visual function via a caspase-dependent anti-apoptotic pathway.

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