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# Hyperbaric oxygenation alleviates chronic constriction injury (CCI)-induced neuropathic pain and inhibits GABAergic neuron apoptosis in the spinal cord

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ABSTRACT

**Background and aims:** Dysfunction of GABAergic inhibitory controls contributes to the development of neuropathic pain. We examined our hypotheses that (1) chronic constriction injury (CCI)-induced neuropathic pain is associated with increased spinal GABAergic neuron apoptosis, and (2) hyperbaric oxygen therapy (HBO) alleviates CCI-induced neuropathic pain by inhibiting GABAergic neuron apoptosis.
**Methods:** Male rats were randomized into 3 groups: CCI, CCI+HBO and the control group (SHAM). Mechanical allodynia was tested daily following CCI procedure. HBO rats were treated at 2.4 atmospheres absolute (ATA) for 60 min once per day. The rats were euthanized and the spinal cord harvested on day 8 and 14 post-CCI. Detection of GABAergic cells and apoptosis was performed. The percentages of double positive stained cells (NeuN/GABA), cleaved caspase-3 or Cytochrome C in total GABAergic cells or in total NeuN positive cells were calculated.
**Results:** HBO significantly alleviated mechanical allodynia. CCI-induced neuropathic pain was associated with significantly increased spinal apoptotic GABA-positive neurons. HBO considerably decreased these spinal apoptotic cells. Cytochrome-C-positive neurons and cleaved caspase-3-positive neurons were also significantly higher in CCI rats. HBO significantly decreased these positive cells. Caspase-3 mRNA was also significantly higher in CCI rats. HBO reduced mRNA expression of caspase-3.
**Conclusions:** CCI-induced neuropathic pain was associated with increased apoptotic GABAergic neurons induced by activation of key proteins of mitochondrial apoptotic pathways in the dorsal horn of the spinal cord. HBO alleviated CCI-induced neuropathic pain and reduced GABAergic neuron apoptosis. The beneficial effect of HBO may be via its inhibitory role in CCI-induced GABAergic neuron apoptosis by suppressing mitochondrial apoptotic pathways in the spinal cord.
**Implications:** Increased apoptotic GABAergic neurons induced by activation of key proteins of mitochondrial apoptotic pathways in the dorsal horn of the spinal cord is critical in CCI-induced neuropathic pain. The inhibitory role of HBO in GABAergic neuron apoptosis suppresses ongoing neuropathic pain.


## 1. Introduction

Chronic pain is a significant national public health problem. It is the most frequent reason for individuals to seek medical care, and accounts for millions of medical visits annually. The prevalence of neuropathic pain in the general population ranges from 6.9 to 14% [1]. Neuropathic pain usually results from a lesioned or diseased central or peripheral nervous system [2]. Currently, no definite treatment is available due to the heterogeneity of neuropathic pain syndromes that link to various mechanisms [3–5]. Different animal models have been used for understanding peripheral and central pathogenic mechanisms of neuropathic pain. Among these models, the chronic constrictive injury (CCI)-induced neuropathic pain model introduced by Bennet and Xie induces hyperalgesia and allodynia, and is similar to that seen in clinic patients [6].

A delicate balance between functions of excitation and inhibition in the dorsal horn of the spinal cord is essential to maintain adequate pain sensation. It has been suggested that the spinal GABAergic hypofunction resulting from the loss of GABA neurons, a reduction in spinal GABA content and its synthesizing enzymes,

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and a decrease in activity and expression of the $K^+$—$Cl^-$ cotransporter contribute to the development of neuropathic pain [7–10]. A significant reduction in GABAergic neurons in the superficial dorsal horn of the spinal cord has been observed in CCI-induced and partial sciatic nerve ligation induced neuropathic pain [11,12]. Apoptotic cell death in the dorsal horn of the spinal cord is believed to cause the reduction of GABAergic neurons [13–16]. Caspase-3-mediated cell apoptosis is involved in CCI-induced neuropathic pain [15]. In addition to activation of the TNF-α apoptosis-caspase signalling pathway in sensory neurons of dorsal root ganglion in the spinal nerve ligation (SNL) injury model [17], activation of mitochondrial apoptosis pathways is also involved in peripheral neuropathic pain [14,18–20]. Over-expression of NMDA receptors induce excessive $Ca_2$+ influx and generates NO and $O_2$ to form ONOO-, which results in an increase of mitochondrial membrane permeability. Cytochrome c (Cytc) is then released from the intermembrane space into the cytoplasm where the Cytc interacts with apoptotic protease-activating factor 1, triggering the caspase-independent cell death pathway [21,22]. Increased mitochondrial permeability plays an important role in activating apoptosis and inducing peripheral neuropathic pain [23]. However, the interaction between the loss of GABAergic neurons and the activated mitochondrial apoptosis pathway in CCI-induced neuropathic pain is unknown.

Hyperbaric oxygenation therapy (HBO), in which patients are exposed to 100% oxygen at above sea level atmospheric pressure in a cylindrical pressure chamber, has been used as a safe and effective treatment for a variety of clinical disorders. Recent research has suggested that HBO may have beneficial effects in the treatment of pain disorders, including delayed onset muscle soreness, fibromyalgia, inflammatory pain and neuropathic pain [24,25]. In this study, we examined our hypotheses that (1) excitotoxic apoptosis of GABA neurons in the dorsal horn of the spinal cord plays a key role in CCI-induced neuropathic pain, and (2) HBO may be beneficial in the treatment of neuropathic pain by reducing GABAergic neuron apoptosis through maintaining the integrity and function of the mitochondrial membrane.

## 2. Material and methods

All experiments were approved by the Institutional Committee for the Humane Use of Animals, and were conducted in accordance with the guidelines established by the National Institutes of Health. Male Sprague-Dawley rats (Taconic, Hudson, NY) weighing 200–250 g were selected for the study and housed 2–3 per cage in controlled laboratory conditions (23 °C on a 12-h light/dark cycle with food and water ad libitum). One hundred and four rats were randomly divided into three groups: CCI ($n = 8$ at each point), CCI+HBO ($n = 8$ at each point) and the control group (SHAM) ($n = 7$ at each point).

### 2.1. Neuropathic pain model

Chronic constriction injury of the sciatic nerve was induced in the right hind limb in CCI and CCI + HBO rats. All surgeries were performed under general anaesthesia with the injection of ketamine and xylazine (150:30 mg/mL) at 0.7 mL/kg IM on the left buttock. In brief, the right common sciatic nerve of each rat was exposed at the mid-thigh level. Approximately 7 mm of nerve was freed and 4 ligatures (4.0 chromic gut) were loosely tied around the nerve with 1 mm of spacing between ligatures. The incision was then closed in layers with 3–0 monofilament nylon sutures. Animals in the SHAM group received surgery identical to that described but without nerve injury.

### 2.2. Behavioural test

Mechanical sensitivity was assessed using a von Frey filament in all rats for 3 consecutive days prior to any surgical procedures to establish baseline values. Mechanical allodynia was assessed daily post-surgery. According to pervious methods [26], rats were placed individually on a metal grid and covered with a Plexiglas box (10 cm × 12 cm × 30 cm). The middle-calibre filament (2.0 g) was pushed onto the plantar surface of the right hind paw 5 times (3 s separated each push) until the filament bent. The Up-Down method was used for mechanosensitivity testing with a series of von Frey filaments with logarithmically incremental stiffness from 0.04 to 26.0 g. The result of the test was determined to be the lowest force that provoked paw withdrawal at least twice in five von Frey filament presentations per test [27].

Three researchers participated in the management of experimental activities. One researcher performed the surgical procedures, the second performed the behavioural tests while blinded to the experimental treatment groups, and the third collected and analyzed the data.

### 2.3. Hyperbaric oxygen treatment

HBO was conducted based on our previous studies [15,26]. Calcium carbonate crystals were put on the bottom of the cylindrical chamber (Sechrist Model 1300B; Sechrist Industries, Inc., Anaheim, CA) to absorb exhaled carbon dioxide. The chamber was ventilated with 100% oxygen for 10 min before HBO-group rats were placed inside the chamber. Then, the pressure was increased at a rate of 0.1 atmospheres absolute (ATA)/minute to the desired pressure (2.4 ATA) and maintained for 60 min. Oxygen content was maintained at 98% and $CO_2$ at 0.03%. The HBO rats were treated once per day. The CCI and SHAM rats were placed into the chamber with room air without added pressure for 60 min once per day. The rats were euthanized and the spinal cord harvested on day 8 and 14 post-CCI procedure. The lumbar 4–5 region of the spinal cord was cryosectioned at 10 μm and immunohistochemical detection of GABAergic cells and apoptosis was performed.

### 2.4. Immunohistochemistry

The rats were transcardially perfused with 0.1 phosphate-buffered saline (PBS; pH 7.4; Sigma, St. Louis, MO, USA) followed by 10% neutral buffered formalin. The L4 and L5 lumbar segment was dissected out and post-fixed in 10% formalin (Sigma, St. Louis, MO, USA) overnight at 4 °C, then cryoprotected in 30% sucrose until sectioning. The lumbar 4–5 region of the spinal cord was embedded in optimum cutting temperature compound (Sakura Finetek, USA, Inc., Torrance, CA, USA) and snap frozen in liquid nitrogen. Coronal spinal cord sections were cut at a 10 μm thickness with freezing microtome (CM1850, Leica Microsciences, Mannheim, Germany) and processed for immunofluorescence. Sections were then permeabilized with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) and blocked in 0.3% horse serum (Sigma, St. Louis, MO, USA). Sections were then incubated with primary antibodies overnight at 4 °C: rabbit anti-Cleaved caspase-3 IgG (1:200; Cell Signalling Technology, Danvers, MA, USA), mouse anti-GABA IgG (1:1000; St. Louis, MO, USA), rabbit Anti-Cytochrome C IgG (1:100; Cambridge, MA, USA), mouse Anti-NeuN IgG (1:500; Millipore, Billerica, MA, USA), and rabbit anti-NeuN (1:500; Millipore, Billerica, MA, USA). After washing, sections were incubated with Alexa Fluor 594 donkey anti-Rabbit IgG (1:500; Invitrogen, Paisley, UK), Alexa Fluor 594 donkey anti-Mouse IgG (1:500; Invitrogen, Paisley, UK), Alexa Fluor 488 donkey anti-Mouse IgG (1:500; Invitrogen, Paisley, UK), and Alexa Fluor 488 donkey anti-rabbit IgG (1:500; Paisley, UK) for two hours in the dark at room temperature. Negative control sections

**Table 1**
Sequences of Primers for RT-PCR.

| | Forward Primers (5′ → 3′) | Reverse Primers (5′ → 3′) |
|---|---|---|
| Caspas3 | TCGATAAAAGCACTGGAATG | ACTGTGATCTTGGTTAGAAACAC |
| GAD1 | CTACTGGTTTGGATATCATTGG | GGAGAAAATATCCCATCACC |
| β-actin | AAGACCTCTATGCCAACAC | TGATCTTCATGGTGCTAGG |

underwent the same procedures without the presence of primary and secondary antibodies. Cyteblasts were counterstained with Hoechst 33342 (1:1000; Sigma, St. Louis, MO, USA). Samples were analyzed with confocal microscopy (Zeiss LSM780, Leica, Benshein, Germany) and an image analyzing system (Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA) by an investigator blinded to the experimental groups. Cell counts were performed within 1 mm diameter in the dorsal horn of the right spinal cord region; 3 sections were evaluated for each animal.

The stereological method was used to scan all samples and count neuronal cell deaths. The stereological method was also used to scan the figures presented in this manuscript.

### 2.5. Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR studies, the rats were perfused transcardially with 240 ml PBS at 4 °C. The spinal cords at the L4-5 level were harvested rapidly, cut into left and right parts and immediately frozen in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen, Paisley, UK) and purified using the RNeasy Mini Kit (Qiagen, Palo Alto, CA, USA). Total RNA concentration and the quantity of RNA was assessed using a Thermo Scientific NanoDrop ND 2000 Spectrophotometer. The integrity of total RNA was measured by agaros gel electrophoresis, and cDNA was synthesized using the High Capacity RNA-to-cDNA Kit according to the manufacturer's protocol (Catalogue number 4387406, Applied Biosystems). Primer sequences and amplification profiles used for caspase-3 and GAD67 (KiCqStart® SYBR® Green Primers, Sigma, St. Louis, MO, USA) are shown in Table 1. DNA amplification was carried out according to the manufacturer's protocol (LightCycler® 480 SYBR Green I Master, Roche Life Science, Indianapolis, IN, USA). The mixture was amplified for 40 cycles using qPCR instrumentation (Bio-Rad CFX480 touch, Bio-Rad Laboratories, Inc. Life Science Research, Hercules, CA, USA). Each cycle consisted of pre-incubation for 5 min at 95 °C, 15 s amplification at 95 °C, 15 s at 61 °C, 15 s at 72 °C, a melting curve for 5 s at 95 °C and 1 min at 65 °C, and cooling for 10 s at 40 °C. Amplification products were separated on 1.8% agarose gel. The intensity of each band was quantified using NIH Image software (version 1.60) and expressed in arbitrary units.

### 2.6. Statistical analysis

Numerical data are presented as the mean and standard deviation. Data analyses were performed using SPSS software (Ver. 11.5, IBM, Chicago, IL, USA). Statistical significance was determined using a two-way repeated-measures ANOVA and a two-way ANOVA, followed by Bonferroni post hoc analysis wherever appropriate. A value of $p < 0.05$ was considered statistically significant. Pearson's correlation coefficient was used for correlation analyses.

## 3. Results

### 3.1. HBO alleviates CCI-induced mechanical allodynia

The results of behavioural testing in the present study were in agreement with our previous reports [15,26]. The CCI rats showed ventroflexion of the right toe, walking with the medial edge of the right hind paw, shaking and licking of the right hind paw, and frequent holding of the affected hind paw off the floor, which are behaviours suggestive of spontaneous pain. The results of the behavioural test for mechanical allodynia of the hind paw are presented in Fig. 1. Following CCI, mechanical allodynia developed in the ipsilateral paw of CCI rats compared to SHAM rats ($1.42 \pm 1.24$ vs. $4.00 \pm 1.5$ on day 8; $0.65 \pm 0.36$ vs. $3.75 \pm 1.38$ on day 14; $p < 0.05$). HBO significantly improved mechanical allodynia ($2.8 \pm 1.3$ vs. $1.42 \pm 1.24$ on day 8; and $2.15 \pm 1.2$ vs. $0.65 \pm 0.36$ on day 14, $p < 0.05$).

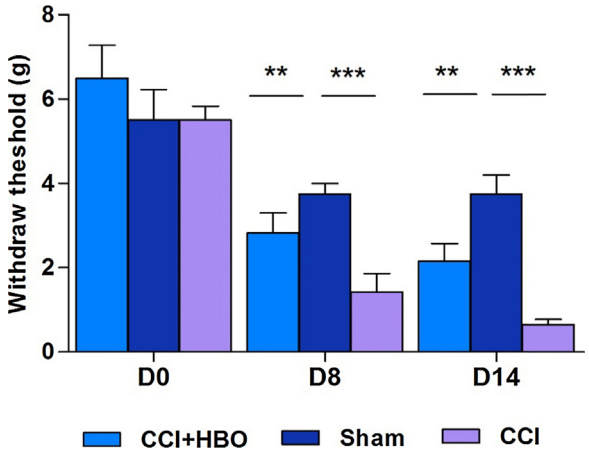



**Fig. 1.** Effect of HBO on mechanical allodynia 8 and 14 days post-surgery, and the baseline of mechanical allodynia. Following CCI, mechanical allodynia had developed in the ipsilateral paw compared to the SHAM rats ($1.42 \pm 1.24$ vs. $4.00 \pm 1.5$ at day 8; $0.65 \pm 0.36$ vs. $3.75 \pm 1.38$ at day 14; $p < 0.05$). HBO significantly improved mechanical allodynia ($2.8 \pm 1.3$ vs. $1.424 \pm 1.24$ on day 8; $2.15 \pm 1.2$ vs. $0.65 \pm 0.36$, on day 14; $p < 0.05$). The data are presented as mean ± SEM. HBO: Hyperbaric oxygenation treatment ($n = 16$); CCI: Chronic constriction injury induced neuropathic pain ($n = 16$); CCI+HBO: CCI rats treated with HBO; SHAM: Control group ($n = 12$). D0: Day zero, D8: Day 8, D14: Day 14. $^{*}p < 0.05$. $^{**}p < 0.01$, $^{***}p < 0.001$.

### 3.2. HBO reduces CCI-induced increase in apoptotic cells and apoptotic GABAgeric neurons in the spinal cord

The development of CCI-induced neuropathic pain was associated with significantly increased apoptotic-positive neurons in the spinal cord compared to the SHAM group ($42 \pm 9\%$ vs. $13 \pm 5\%$, $p < 0.001$ on day 8; and $39 \pm 14\%$ vs. $14 \pm 7\%$, $p < 0.001$ on day 14). The ratio of apoptotic GABA-positive neurons in CCI rats was significantly higher compared to the SHAM rats ($48.0 \pm 10.0\%$ vs. $17.0 \pm 8.0\%$, $p < 0.001$ on day 8; and $48.0 \pm 11.0$ vs. $12.0 \pm 11.0$, $p < 0.001$ on day 14). HBO significantly reduced the occurrence of apoptotic cells ($26.0 \pm 5.0$ vs. $42.0 \pm 9.0\%$, $p < 0.001$ on day 8; and $25.0 \pm 9.0$ vs. $39.0 \pm 14.0$, $p < 0.001$ on day 14). Percentages of apoptotic GABA-positive neurons in CCI+HBO rats was significantly lower compared to CCI rats ($22.0 \pm 9.0\%$ vs. $48.0 \pm 10.0\%$ on day 8; and $20.0 \pm 11.0\%$ vs. $48.0 \pm 11.0\%$ on day 14, $p < 0.001$) (Fig. 2).

### 3.3. HBO decreased CCI-induced cleaved caspase-3-positive neurons in the dorsal horn of the spinal cord

The ratio of cleaved caspase-3-positive neurons was significantly higher in CCI rats than in SHAM rats ($20.0 \pm 5.0$ vs. $4.0 \pm 2.0$ on day 8; and $18.0 \pm 3.0$ vs. $4.0 \pm 2.0$ on day 14, $p < 0.001$). HBO significantly decreased the cleaved caspase-3-positive neurons in CCI rats ($10.0 \pm 4.0$ vs. $20.0 \pm 4.0$ on day 8, and $18.0 \pm 3.0$ vs. $8.0 \pm 3.0$ on day 14, $p < 0.001$) (Fig. 3).

Expression of caspase-3 mRNA was significantly elevated in the dorsal horn of the spinal cord in CCI rats compared to SHAM rats. Notably, mRNA expression of caspase-3 was higher in CCI rats

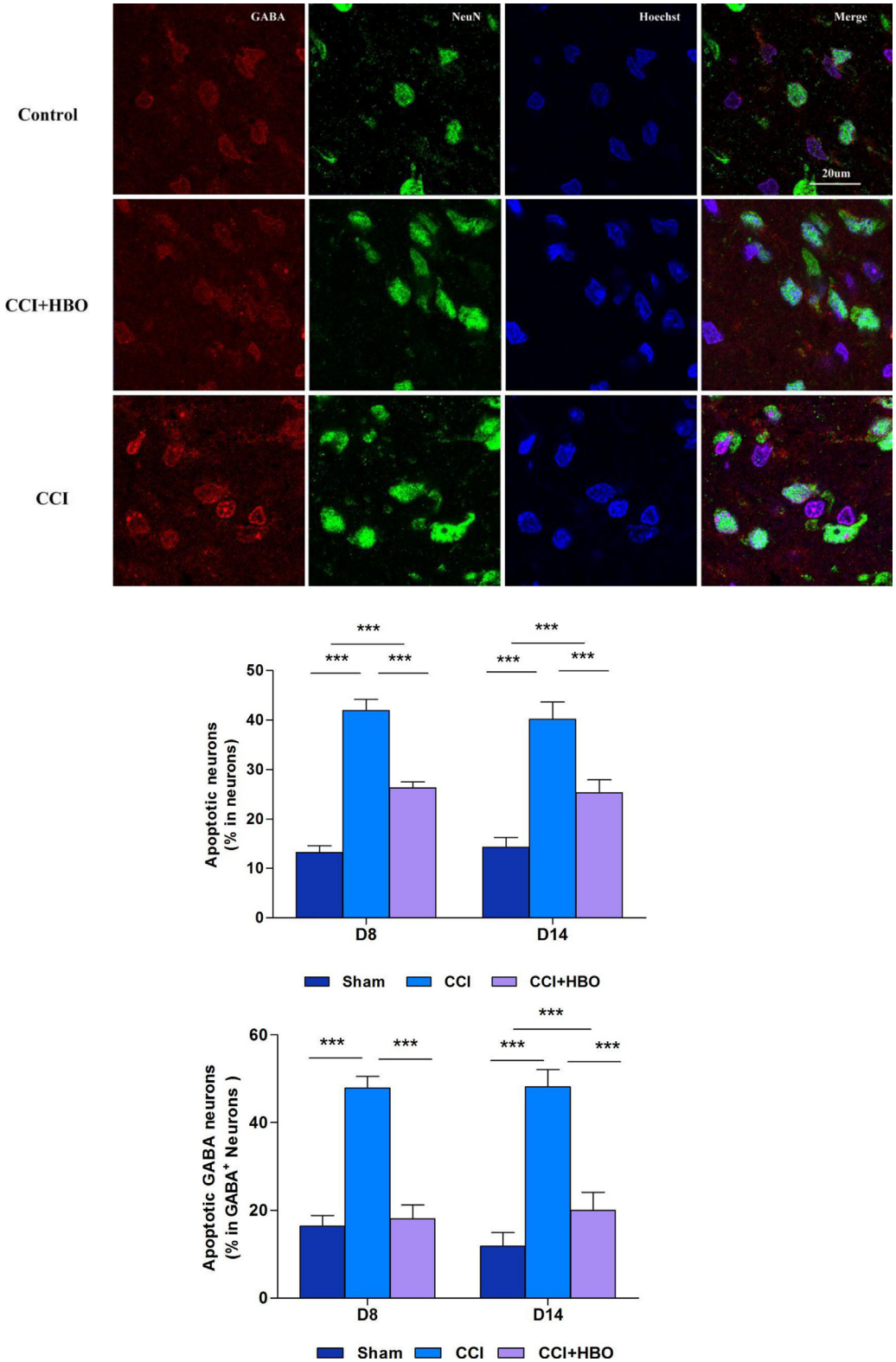



**Fig. 2.** Effect of HBO on apoptotic neurons and apoptotic GABAergic neurons on day 8 and day 14 post-surgery. The ratio of apoptotic neurons in CCI rats was significantly higher compared to SHAM rats ($42 \pm 9\%$ vs. $13 \pm 5\%$, $p < 0.001$ on day 8; $39 \pm 14\%$ vs. $14 \pm 7\%$, $p < 0.001$ on day 14). HBO significantly lowered apoptotic cells in CCI ($26.0 \pm 5.0$ vs. $42.0 \pm 9.0\%$, $p < 0.001$ on day 8; $25.0 \pm 9.0$ vs. $39.0 \pm 14.0$, $p < 0.001$ on day 14). Percentages of apoptotic GABA-positive neurons in CCI rats was significantly higher compared to SHAM rats ($48.0 \pm 10.0\%$ vs. $17.0 \pm 8.0\%$, $p < 0.001$ on day 8; $48.0 \pm 11.0$ vs. $12.0 \pm 11.0$, $p < 0.001$ on day 14, $p < 0.001$). HBO significantly lowered apoptotic cells in CCI ($22.0 \pm 9.0\%$ vs. $48.0 \pm 10.0\%$, on day 8; $20.0 \pm 11.0\%$ vs. $48.0 \pm 11.0\%$ on day 14, $p < 0.001$). GABA: GABA (red) positive cells; NeuN: NeuN (green) positive neurons; and Merge: GABA (red) expressed in neurons (NeuN green). HBO: Hyperbaric oxygenation treatment ($n = 16$); CCI: Chronic constriction injury induced neuropathic pain ($n = 16$); CCI+HBO: CCI rats treated with HBO; SHAM: Control group ($n = 12$). D8: Day 8, D14: Day 14. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

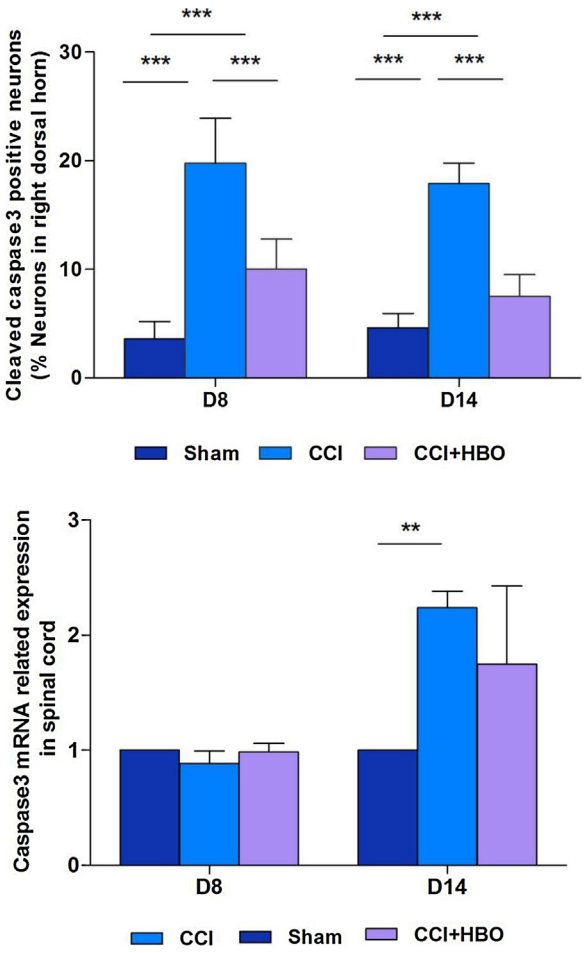



**Fig. 3.** Activated caspase-3 in neurons of spinal dorsal horn after CCI. The ratio of cleaved caspase-3-positive neurons was significantly higher in CCI rats than in SHAM rats ($20.0 \pm 5.0$ vs. $4.0 \pm 2.0$ on day 8; $18.0 \pm 3.0$ vs. $4.0 \pm 2.0$ on day 14, $p < 0.001$). HBO significantly decreased cleaved caspase-3-positive neurons in CCI ($10.0 \pm 4.0$ vs. $20.0 \pm 4.0$ on day 8; $18.0 \pm 3.0$ vs. $8.0 \pm 3.0$ on day 14, $p < 0.001$). After CCI, the related expression of caspase-3 mRNA was higher in CCI rats and CCI+HBO rats compared to SHAM rats ($2.24 \pm 3.0$ vs. $1.0 \pm 0.0$, $1.35 \pm 0.29$ on day 14, $p < 0.05$). Co-located staining of caspase-3 (red) and NeuN (green) demonstrated cleaved caspase-3 in neurons of the spinal dorsal horn. HBO: Hyperbaric oxygenation treatment ($n = 16$); CCI: Chronic constriction injury induced neuropathic pain ($n = 16$); CCI+HBO: CCI rats treated with HBO; SHAM: Control group ($n = 12$). D8: Day 8, D14: Day 14. $^{*}p < 0.05$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compare to SHAM rats ($2.24 \pm 0.29$ vs. $1.0 \pm 0.0$ on day 14, $p < 0.05$). HBO reduced mRNA expression of caspase-3 in the spinal cord ($1.75 \pm 1.25$ on day 14 (Fig. 3)).

### 3.4. HBO inhibits neuronal mitochondrial release of Cytc in the dorsal horn of the spinal cord

The ratio of Cytc-positive neurons was significantly higher in CCI rats than in SHAM rats ($43.0 \pm 7.0$ vs. $7.0 \pm 3.0$, $p < 0.001$ on day 8, and $40.0 \pm 7.0$ vs. $8.0 \pm 6.0$, $p < 0.001$ on day 14). HBO significantly decreased CCI-induced Cytc-positive cells ($16.0 \pm 6.0$ vs. $43.0 \pm 7.0$, $p < 0.05$ on day 8, and $16.0 \pm 4.0$ vs. $40.0 \pm 7.0$, $p < 0.001$ on day 14) (Fig. 4).

The expression of GAD67 mRNA in spinal cord was lower in CCI rats than in SHAM rats ($0.57 \pm 0.39$ vs. $0.78 \pm 0.1$ on day 14), although this did not reach statistical significance. GAD67 mRNA did not change on day 8 after CCI (Fig. 5).

## 4. Discussion

The main findings of the present study are that (1) CCI-induced neuropathic pain was associated with increased apoptotic GABAergic neurons and decreased expression of GAD67 mRNA in the dorsal horn of the spinal cord; (2) the increase in apoptotic GABAergic neurons was associated with the increased apoptosis-associated mitochondrial outer membrane permeabilization; (3) HBO alleviated CCI-induced neuropathic pain and reduced apoptotic GABAergic neurons in the dorsal horn of the spinal cord; and (4) the beneficial effect of HBO in CCI-induced neuropathic pain may be due to its inhibitory role in CCI-induced GABAergic neuron apoptosis in the spinal cord.

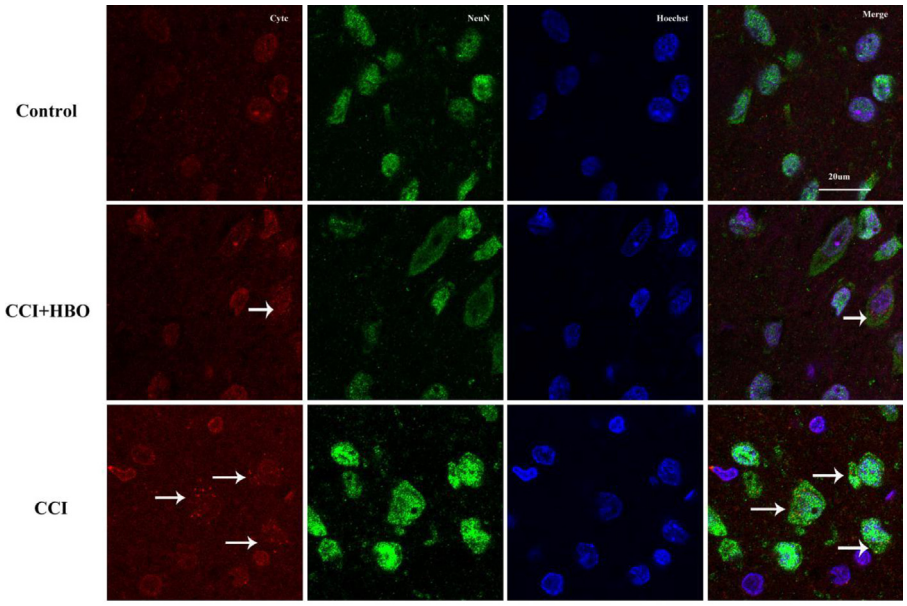
A delicate balance between excitation and inhibition in the dorsal horn of the spinal cord is essential for maintaining adequate pain sensation. In the superficial dorsal horn in the termination area of nociceptors, the majority of inhibitory neurons are purely GABAergic neurons [28]. It has been proposed that a profound dysfunction of these GABAergic neurons may be responsible for the development of nerve-injury-induced neuropathic pain [10,29,30].

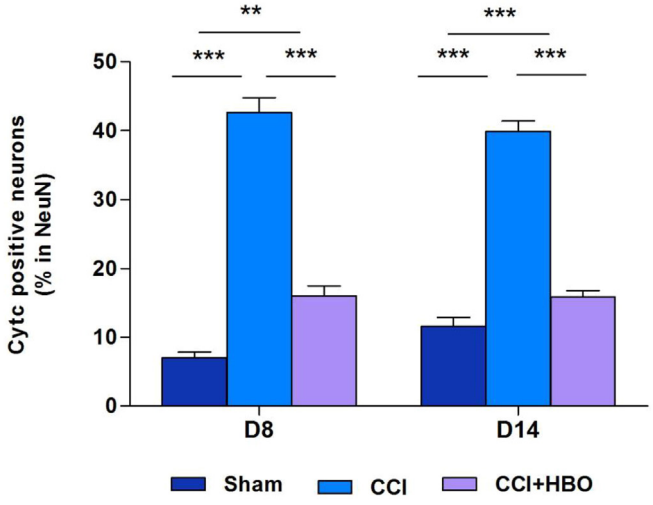
Reduction of GABAergic transmission may be caused by the loss of GABAergic neurons in the superficial dorsal horn and/or the decrease of glutamic acid decarboxylase (GAD) in the dorsal horn [7,9]. GAD is an enzyme that catalyzes the decarboxylation of glutamate to GABA. A reduction in GAD enzyme decreases GABA neurotransmitter products. Moore et al. found that partial nerve injury not only reduced presynaptic GABA release, but also decreased dorsal horn levels of the GABA-synthesizing enzyme GAD65 ipsilateral to the injury and induces neuronal apoptosis in identified neurons. Both of these mechanisms could reduce presynaptic GABA levels and promote a functional loss of GABAergic transmission in the superficial dorsal horn. Neuronal apoptosis has been attributed to the loss of GABAergic transmission [13].

We previously reported that CCI-induced neuropathic pain was associated with significantly increased neuronal apoptosis in the lumbar 4–5 spinal cord, and that these apoptotic cells were mainly distributed in the territory of the ipsilateral dorsal horn of the spinal cord. The present study provides new evidence that these apoptotic neurons are mainly GABAergic neurons. The present study suggests that reduced GABAergic neuron inhibitory function may play an essential role in CCI-induced neuropathic pain.

Studies of Polgar et al. suggested that the reduction in GABA levels and the loss of GABAergic neurons were not detected in the spinal dorsal horn of CCI and SNL animals in the development of thermal hyperalgesia [31,32]. The reasons for the discrepancy remain unclear. As discussed by the authors, the explanation may lie in the use of inconsistent and/or different methods for immunolabeling GABAergic neurons and analyzing GABA levels. Further studies have shown that the number of enhanced green fluorescent protein (EGFP)+ GAD67 neurons and the density of axon terminals with GAD65 in the dorsal horn of spinal cord were found to be significantly decreased in SNL model mice and CCI model rats [33,34]. Furthermore, increasing the GABAergic neurons via the spinal cord transplantation of embryonic stem-cell-derived spinal GABAergic progenitor cells was proven successful in treating neuropathic pain [35–37]. These progenitor cells were activated by primary afferent and form GABAergic neurons integrating into host circuitry and recapitulating endogenous inhibitory circuits, which prevented the development of nerve-injury-induced mechanical hypersensitivity [36–38]. Our results support the conception that CCI-induced neuropathic pain was associated with increased apoptotic neurons, mainly GABAergic neurons, in the superficial dorsal horn of spinal cord as detected on days 8 and 14 post-surgery.

Apoptosis is initiated by a subfamily of cysteine proteases known as caspases. One of the pathways of cell apoptosis is



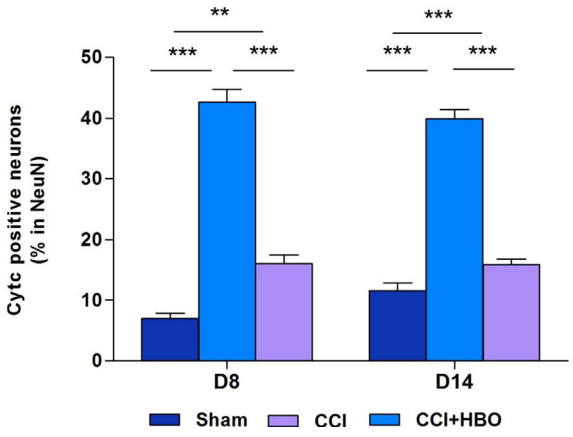




**Fig. 4.** Mitochondrial release of cytochrome C (Cytc) in neurons of the spinal dorsal horn. The ratio of Cytc-positive neurons was significantly higher in CCI rats than in SHAM rats ($43.0 \pm 7.0$ vs. $7.0 \pm 3.0$ on day 8; $40.0 \pm 7.0$ vs. $8.0 \pm 6.0$ on day 14, $p < 0.001$). HBO significantly decreased CCI-induced Cytc-positive cells ($16.0 \pm 6.0$ vs. $43.0 \pm 7.0$, $p < 0.05$ on day 8; $16.0 \pm 4.0$ vs. $40.0 \pm 7.0$, $p < 0.001$ on day 14). Cytc: Cytc was stained red; NeuN: NeuN was stained green. Merge: Cytc expression in neurons (NeuN stained green) of the spinal cord. HBO: Hyperbaric oxygenation treatment ($n = 16$); CCI: Chronic constriction injury induced neuropathic pain ($n = 16$); CCI+HBO: CCI rats treated with HBO; SHAM: Control group ($n = 12$). D8: Day 8, D14: Day 14. $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

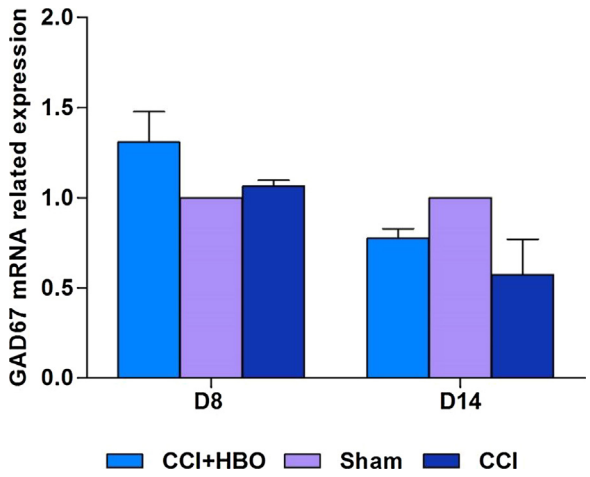



**Fig. 5.** mRNA expression of GAD67 in spinal dorsal horn after CCI. mRNA expression of GAD1 was lower in CCI rats than in SHAM rats ($0.57 \pm 0.39$ vs. $0.78 \pm 0.1$ on day 14). GAD67 mRNA did not change on day 8 after CCI. HBO: Hyperbaric oxygenation treatment ($n = 16$); CCI: Chronic constriction injury induced neuropathic pain ($n = 16$); CCI+HBO: CCI rats treated with HBO; SHAM: Control group ($n = 12$). D8: Day 8, D14: Day 14. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activated by the release of Cytc from the mitochondria into cytosol. In this pathway, a variety of apoptotic stimuli cause the release of Cytc from mitochondria; this in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death. The release of Cytc and activation of caspase-3 have been observed in CCI-induced neuropathic pain [39]. In nerve-injury-induced neuropathic pain, the synthesis of GAD67 and GAD65 proteins and mRNA was observed being down-regulated, which causes the hypofunction of GABAergic transmission [9,30,34,40–42]. Increasing the GAD67 level in GABAergic neurons by gene transfer technique alleviates neuropathic pain [41,43]. Therefore, the data showed that nerve injury not only significantly decreased GAD65/67 levels but also induced marked reductions in GABA levels in the superficial dorsal horn. Our result is similar to the results from Vaysse et al. [44] and Wang et al. [42] in that the reduction of GAD67 mRNA, but not GAD65 mRNA, was observed in the dorsal horn of the spinal cord in CCI group rats 14 days post-surgery. Nerve-injury-induced neuropathic pain is associated with mitochondrial dysfunctions that lead to the activation of the apoptotic cascade.

Lorenzo et al. showed that the density of GAD65 inhibitory terminals was reduced in lamina I and lamina II of the spinal cord after injury. The loss of GAD65 terminals was greatest in lamina II with the highest drop occurring at around 3–4 weeks post-injury, which correlated with the altered thresholds to mechanical and thermal stimuli [34]. The results from our study validate their findings that lower levels of GAD67 mRNA in the spinal cord occurs as detected on day 14 after surgery, which correlated well with CCI-induced behavioural changes.

Studies show that blocking the pathway of neuronal apoptosis prevents the loss of GABAergic neurons and the reduction of inhibitory currents, along with attenuation of pain hypersensitivity induced by peripheral nerve lesions. However, the mechanism of apoptosis of GABA neurons in the dorsal horn of the spinal cord during the development of neuropathic pain remains unclear. Excitotoxic apoptosis is a common pathway in neuronal apoptosis with excitatory amino acids as a final common pathway for neurologic disorders, including severe neuropathic pain, Alzheimer's disease, dementia, depression, and glaucoma [45].

In preclinical studies, the administration of NMDA receptor antagonists and GABA-A agonists can reverse allodynia and hyperalgesia [46,47]. Over-activation of glutamate receptor activates the intrinsic pathway that is the mitochondrial pathway of apoptosis [48]. In the intrinsic pathway, mitochondrial outer membrane permeabilization occurs. Cytc is released from mitochondria and binds to apoptotic protease-activating factor 1, leading to the formation of a caspase activation platform termed the apoptosome. The apoptosome activates an initiator caspase, caspase-9, which in turn cleaves and activates caspase-3 and caspase-7.

Apoptotic signalling is involved in neuropathic pain. Our previous studies showed that CCI-induced neuronal apoptosis might be associated with TNF-α-mediated activation of the caspase-3 protein and the increase of caspase-3 mRNA in the spinal dorsal horn. The present study suggests that CCI induced the increase in caspase-3 mRNA and activated the caspase-3 protein and the release of Cytc in spinal dorsal horn as detected on day 8 and 14 after surgery, which was in line with the results of behaviour tests and the increase of apoptotic GABAergic neurons. These data indicate that the mitochondrial pathway of apoptosis might be associated with CCI-induced apoptotic GABAergic neurons.

HBO refers to 100% oxygen provided at 2–3 times the atmospheric pressure at sea level [49]. HBO has been proposed to be a reliable option for neuroprotection [50]. HBO has been observed to prevent apoptosis after focal cerebral ischaemia by opening the mitochondrial ATP-sensitive potassium channel [51]. In addition, HBO therapy has been observed to reduce Cytc and decrease caspase-9 and caspase-3 in the hippocampus and ischaemic penumbra in MCAO models. Therefore, HBO protects brain tissues from ischaemia–reperfusion injury by suppressing mitochondrial apoptotic pathways [52]. The effect of HBO on the function of GABAergic neurons in nerve-injury-induced neuropathic pain has not been reported. In our previous study, HBO alleviated CCI-induced neuropathic pain via its inhibiting role in CCI-induced neuronal apoptosis in the spinal cord. HBO's inhibition of CCI-induced neuronal apoptosis might be due to the inhibition of endoneuronal TNF-α production [26].

The present study indicated that the beneficial effect of HBO in CCI-induced neuropathic pain may be due to its inhibitory role in CCI-induced GABAergic neuron apoptosis through the inhibition of mitochondrial apoptotic pathways in the spinal cord. In CCI-rats, increased release of Cytc and caspase-3 active fragments expressed in the sciatic nerve has been described. Treatment of animals with acetyl-l-carnitine prevents apoptosis induction and reduces cytosolic Cytc in a significant manner [53].

In diabetic neuropathic pain, the activation of caspase-3 and release of Cytc was significantly increased in the spinal cord of diabetic animals. Thymus caramanicus Jalas attenuates neural apoptosis and ameliorates diabetic neuropathy [54].

The hypothesis has been put forward that apoptotic inhibition via the mitochondrial pathway is involved in hyperbaric-oxygen-induced neuroprotection on ischaemia-reperfusion injury in rat brain. In traumatic brain injury, treatment with HBO was observed to alleviate neuronal apoptosis by reducing the release of Cytc and the dimers of Bax, and up-regulating the expression of Bcl-2 [55].

In a MCAO rat study, pre-treatment with HBO was also found to reduce cytoplasm Cytc levels, decrease caspase enzyme activity, upregulate the ratio of Bcl-2 and Bax expression, and abate the apoptosis of ischaemic tissue. The neuroprotection effect of HBO may be through suppression of the mitochondrial apoptotic pathways [52]. The present study provides further evidence that CCI-induced neuropathic pain is associated with the increased release of Cytc in the spinal cord. Alleviation of neuropathic pain by HBO may be associated with a decreased Cytc release from the spinal cord.

## 5. Summary

The present study shows that CCI-induced neuropathic pain was associated with an increase in apoptotic GABAergic neurons induced by the activation of key proteins of mitochondrial apoptotic pathways in the dorsal horn of the spinal cord. HBO alleviated CCI-induced neuropathic pain and reduced GABAergic neuron apoptosis in the dorsal horn of the spinal cord. The beneficial effect of HBO in CCI-induced neuropathic pain may be due to its inhibitory role in CCI-induced GABAergic neuron apoptosis via suppressing the mitochondrial apoptotic pathways in the spinal cord.

## Authors' contributions

ZY designed the experimental protocol. FQ conducted the experiments, performed chemical assays, collected and analyzed the data and performed statistical analysis. All authors participated in the experimental processes, data interpretation and manuscript preparation. All authors read and approved the final version of the manuscript.

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## Ethical issues

All experiments were approved by the Institutional Committee for the Humane Use of Animals, Upstate Medical University. Registration as IACUC#234. All experiments were conducted in accordance with the guidelines established by the National Institutes of Health.

## Conflict of interest

None declared.

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