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ORIGINAL ARTICLE

Hyperbaric oxygenation treatment alleviates CCI-induced neuropathic pain and decreases spinal apoptosis

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Conflict of interest
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Abstract

Background: Increased apoptotic changes in the spinal cord may be responsible for the development of chronic constriction injury (CCI)-induced neuropathic pain. We previously reported the beneficial effect of hyperbaric oxygen (HBO) in the treatment of CCI-induced neuropathic pain. In this study, we tested our hypotheses that HBO may achieve its beneficial effect by inhibiting CCI-induced proapoptosis gene expression and apoptosis in the spinal cord.

Methods: Male rats were randomized into: SHAM, CCI and CCI + HBO groups. Mechanical hyperalgesia was tested daily following surgery. CCI + HBO rats were treated with HBO for 1 h daily. At 3 days post-CCI, the expression of tumour necrosis factor (TNF)-α and caspase-3 genes was detected. At 7 days post-CCI, apoptotic cells in the spinal cord were detected.

Results: Three days post-CCI, mechanical allodynia had developed in the ipsilateral paw compared with SHAM animals. HBO significantly alleviated CCI-induced mechanical allodynia. In comparison with SHAM, CCI-induced neuropathic pain was associated with higher mRNA levels of TNF-α and caspase-3. HBO significantly decreased CCI-induced mRNA levels of TNF-α and caspase-3. CCI-induced neuropathic pain was also associated with more apoptotic cells in the spinal cord 7 days post-CCI. HBO significantly reduced CCI-induced apoptosis to the level of SHAM animals.

Conclusions: Overly expressed proapoptosis genes, and subsequent increase in spinal apoptotic cells, seem to contribute to the development of CCI-induced neuropathic pain. The inhibitory role of HBO on spinal proapoptosis genes and apoptotic changes may contribute to its beneficial effect on CCI-induced neuropathic pain.

1. Introduction

Neuropath pain constitutes an enormous health problem because it is difficult to treat with conventional analgesics (Rose et al., 2011). Both peripheral and central mechanisms are involved in the development of neuropathic pain (Janig and Baron, 2002). Apoptosis is a process of programmed cell death. Physiological cell death that removes unwanted cells plays an important role in the development, tissue homeostasis and defence against viral infection and mutation (Kam and Ferch, 2000). Disordered apoptosis is implicated in a variety of pathologies, including neuropathic pain (Zimmermann, 2001) and neurodegenerative diseases (Kermer et al., 2004). Apoptotic changes have been observed following spinal cord injury-induced neurological pain (Zhang et al., 2011) and sciatic nerve chronic constriction injury (CCI)-induced neuropathic pain (Whiteside and Munglani, 2001). These apoptotic changes in the spinal cord have

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What's already known about this topic?

- Neuropathic pain constitutes an enormous health problem. Since mechanisms underlying this disorder are poorly understood, effective treatment leaves much to be desired. Hyperbaric oxygen (HBO) treatment has been shown to be beneficial in animal studies.

What does this study add?

- HBO can alleviate the neuropathic pain and suggests the following mechanisms.
- HBO may inhibit proapoptosis gene expression the spinal cord of CCI animals.
- HBO also inhibit neuron apoptosis in the spinal cord of CCI animals.

been hypothesized to be responsible for CCI-induced neuropathic pain (Whiteside and Munglani, 2001; Sekiguchi et al., 2003). It is logical to propose that if spinal cord apoptosis could be reduced or prevented after peripheral nerve injury, the development of neuropathic pain may be alleviated or even prevented. Therefore, measures to suppress apoptosis are critical to advance these areas of investigation (Fortini and Dogliotti, 2010).

Hyperbaric oxygen (HBO) treatment has been used as a safe and effective treatment in various clinical disorders (UHMS, 2014). Recently, the possible beneficial effect of HBO in the treatment of pain disorders has been suggested, including delayed onset muscle soreness (Germain et al., 2003; Bennett et al., 2005; Yildiz et al., 2006), inflammatory pain (Wilson et al., 2006), reduced onset of migraine headache (Wilson et al., 1998), fibromyalgia (Yildiz et al., 2004) and complex regional pain syndrome (Kiralp et al., 2004). However, further investigation is needed to define the role of HBO in pain treatment.

The beneficial effect of HBO may be achieved by decreased inflammation, oedema, oxidative burden, metabolic derangement and apoptotic cell death, as well as improved oxygenation and increased neural regeneration (Matchett et al., 2009). Our previous study suggests that HBO may alleviate CCI-induced neuropathic pain by reducing tumour necrosis factor (TNF)-α production in the affected sciatic nerve (Li et al., 2011). It is known that TNF-α is probably the most potent inducer of apoptosis (Rath and Aggarwal, 1999). We hypothesized that CCI-induced neuropathic pain may be associated with increased apoptosis in the spinal cord by activating proapoptosis genes, and HBO may inhibit the expression of these genes to reduce CCI-induced apoptosis. The present study was designed to examine the relationship between the expression of proapoptosis genes and apoptotic changes in the spinal cord and the development of neuropathic pain in CCI rats with or without HBO. The apoptotic changes were detected using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and cleaved caspase-3 immunofluorescent techniques. Meanwhile, the apoptotic cell types were also assessed using double staining of Nissl body with TUNEL. The effect of HBO on proapoptosis gene expressions (TNF-α and caspase-3) in the spinal cord during the development of CCI-induced neuropathic pain was also evaluated.

2. Methods

2.1 Experimental animals

The proposed study was approved by the Institutional Committee for the Humane Use of Animals. National Institutes of Health and International Association for the Study of Pain guidelines for animal experiments were strictly followed. Pathogen-free male Sprague Dawley rats (Taconic, Hudson, NY, USA) weighing 250–300 g were chosen. The rats were kept in standard solid-bottom 'shoebox' type caging with free access to water and coarsely ground Purina Chow (Ralston Purina, St. Louis, MO, USA) in a 12-h light/dark cycle. They were acclimated for at least a week before starting the study. Forty-eight rats, which appeared healthy and gained weight, were randomly divided into a CCI group (CCI, $n = 16$), a CCI+HBO group (CCI+HBO, $n = 16$) and a sham group (SHAM, $n = 16$). Eight rats in each group were sacrificed 3 days post-CCI for real-time polymerase chain reaction (real-time PCR) study. The rest were euthanized 7 days post-CCI for immunohistochemistry study.

2.2 Induction of mononeuropathic pain

Sciatic neuropathic pain was induced in the right hind limbs in CCI and CCI+HBO rats according to Bennett and Xie (1988). The surgical procedure was performed under general anaesthesia with the injection of ketamine and xylazine (90:9 mg/mL) at 0.7 mL/kg intramuscularly in the left buttock. The common sciatic nerve was exposed at the middle of the right thigh by blunt dissection through the biceps femoris. A 7 mm length of nerve was freed and four ligatures (4.0 chromic gut) were loosely tied (ligatures did not obstruct blood flow) with 1 mm of spacing. The incision was then closed in layers with 4-0 monofilament nylon. In the SHAM group, an identical dissection was performed; however, the sciatic nerves were not ligated.

2.3 HBO treatment

HBO has been described in our previous studies (Yang et al., 2006, 2010; Bosco et al., 2007). The CCI+HBO rats were

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exposed to 100% oxygen at a pressure of 2.4 ATA in a cylindrical pressure chamber (Sechrist Model 1300B, Sechrist Industries, Inc., Anaheim, CA, USA). When the designed pressure was reached, the flow of oxygen was reduced to maintain constant pressure while allowing the flow out of the chamber. Compression and decompression were maintained at a rate of 5 psi/min. A tray of calcium carbonate crystals was used to reduce the accumulation of CO_2 in the chamber environment. Temperature of the chamber was kept at a constant 22–24 °C. HBO preceded for 1 h once a day post-CCI for 7 days. The CCI and SHAM rats were placed in the same chamber breathing room air.

2.4 Behavioural tests

Mechanical allodynia was tested by von Frey filament daily before surgery to establish a baseline for comparison with post-surgical values and following surgery. Rats were placed individually in a clear plexiglass box ($10 \times 12 \times 30$ cm) on elevated wire mesh platforms to allow access to the ventral surface of the hind paws. In brief, a logarithmic series of von Frey filaments was sequentially applied to the left and right hind paws, each for 5 s at constant pressure. This was to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. The range of monofilaments used in these experiments (0.16 to 26.0 g) produced a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity [expressed as $\log_{10}$ (milligrams $\times 10$)]. The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function. This method normalizes the withdrawal threshold for parametric analyses (Harvey, 1997).

2.5 Spinal cord preparation, staining and analysis

2.5.1 Spinal cord preparation

For qPCR studies, on post-CCI day 3, the rats were subjected to a transcardial perfusion of 240 mL (4 °C) phosphate buffered saline (PBS). The spinal cords at L4–5 level were harvested, then cut into left and right parts and immediately frozen in liquid nitrogen. After harvesting, all samples were stored at −80 °C for future assay. For all other histological studies, on post-CCI day 7, the rats underwent transcardial perfusion with 240 mL of PBS followed by 300 mL of 4% paraformaldehyde. The spinal cords at L4–5 level were rapidly dissected and post-fixed for 24 h in the same paraformaldehyde at 4 °C, then cryoprotected in 30% sucrose until they sank. After immersion in embedding medium at −80 °C, 10 μm thick frozen sections were cut in the cryostat and mounted on glass slides. Five sections spaced 450 μm apart were selected and labelled for apoptosis staining. The number of apoptotic cells was qualitatively measured by an experimenter blinded to the study.

2.5.2 TUNEL staining

To analyse fragmented DNA, TUNEL was performed using an *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD, USA) as described in our previous study (Price et al., 2010). The fresh frozen samples were dried after sectioning overnight at room temperature. After rehydration in 100%, 95% and then 70% ethanol (5 min each), the samples were placed in PBS for 10 min at room temperature. The samples were covered with 50 μL of Cytonin™ (Trevigen, Gaithersburg, MD, USA) and incubated for 60 min at room temperature. Then, the samples were washed twice in deionized water for 2 min. The slides were immersed in 1 × terminal deoxynucleotidyl transferase(TdT) labelling buffer for 5 min. After, the samples were covered with 50 μL of labelling reaction mix and incubated at 37 °C for 1 h in a humidity chamber. Then to stop the labelling reaction, the samples were immersed in 1 × TdT stop buffer for 5 min at room temperature. The samples were washed two times in deionized water for 5 min each at room temperature. The samples were covered with 50 μL of Strep-Fluor solution and incubated for 20 min in the dark at room temperature. The samples were washed twice in 1 × PBS for 2 min each to get ready for analysis.

2.5.3 Cleaved caspase-3 staining

Caspase-3 analysis has been described in our previous study (Price et al., 2010). Briefly, the sections were incubated with cleaved caspase-3 antibody (Cell Signaling, Danvers, MA, USA) at a dilution of 1:400 in 0.25% Triton-X-100 and 0.25% bovine serum albumin (BSA) in PBS overnight at 4 °C. After rinsing three times in PBS (5 min per rinse), the sections were incubated with the secondary Alexa flour 555 conjugate goat anti-rabbit antibody (Cell Signaling) at a dilution of 1:500 in 0.25% Triton-X-100 and 0.25% BSA in PBS for 1 h in the dark at room temperature. All sections were mounted after washing and the nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole).

2.5.4 Co-staining: TUNEL with Nissl staining

The TUNEL sections were double-labelled with Nissl staining to determine if neurons underwent DNA damage. The NeuroTrace (Invitrogen, Carlsbad, CA, USA) stain was diluted in PBS to 300-fold. Approximately 200 μL of the diluted stain solution was applied to cover the entire slide and then incubated for 20 min. The sections were then washed for 10 min in PBS plus 0.1% Triton-X-100 and then washed in PBS for 2 h at room temperature.

2.5.5 Prepare for viewing

Two drops of fluorescence mounting medium (Trevigen) were spotted onto each sample and a coverslip was positioned on top of the sample. For extended storage, the samples were dried overnight at room temperature in the

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dark with the border of the coverslip sealed by nail polish. The samples were viewed under fluorescence microscope directly.

2.5.6 Photography and quantification of apoptotic cells

The sections were photographed using a fluorescence microscope (Eclipse, Nikon, Tokyo, Japan) equipped with a digital camera system (Digital sight DF-U1, Nikon). The total number of TUNEL or caspase-3 positive cells in five randomly selected sections of each rat was obtained, respectively. Caspase-3 positive cells of CCI were used to observe and compare the distribution of apoptosis in different areas of spinal cord which was divided into ipsilateral dorsal horn, ipsilateral central area, ipsilateral ventral horn and contralateral half area.

2.5.7 Spinal cord total RNA extraction and real-time PCR

It has been reported that TNF-α gene levels began to increase on day 1 and showed a maximal increase on day 3 after CCI, then decreasing to control levels by day 7. Therefore, TNF-α and caspase-3 gene expression was only measured on day 3 in our study. Total RNA was isolated by the TRIzol method (GIBCO BRL, Life Technologies, Grand Island, NY, USA). Total RNA (2 μg) was converted to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR was performed with the StepOnePlus Real-Time PCR System with StepOne software V2.0 (Applied Biosystems, Forster City, CA, USA) according to the manufacturer's guidelines. iQ SYBER Green Supermix (Bio-Rad Laboratories) was used to detected TNF-α and caspase-3. The primers utilized were 5′- AGA ACT CCA GGC GGT GTC TGTG -3′ (Sense) and 5′- GTG GCA AAT CGG CTG ACG GTGT -3′ (Anti-sense) for TNF-α, 5′- CAT GAC CCG TCC CTT GAA -3′ (Sense) and 5′- CCG ACT TCC TGT ATG CTT ACT CTA -3′ (Anti-sense) for caspase-3, 5′- ATG GAT GAC GAT ATC GCT GCG -3′ (Sense), 5′- CGT CAT TCA CTT CGA GCA GA -3′ (Sense) and 5′- AAA ATG AGG TCC TGC AGT GG -3′ (Anti-sense) for superoxide dismutase (SOD).

2.6 Statistical analysis

All data are expressed as mean ± standard error. Paw withdrawal threshold data are analysed by two-way (time and treatment) analysis of variance (ANOVA), followed by the Tukey's post hoc test for significant differences between appropriate groups. One-way ANOVA was used to test for statistical difference of TUNEL or caspase-3 positive cell counting or gene expression among groups. $p < 0.05$ was considered statistically significant. The statistical analyses were done by the 19.0 version of SPSS (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Behavioural tests

Studies have shown that CCI on the sciatic nerve induces spontaneous pain behaviours, long-lasting allodynia and hyperalgesia in the affected paw (Bennett and Xie, 1988; Dowdall et al., 2005). All the CCI rats showed shaking and licking behaviour, often holding the affected hind paw off the floor. These behaviours are suggestive of spontaneous pain. As

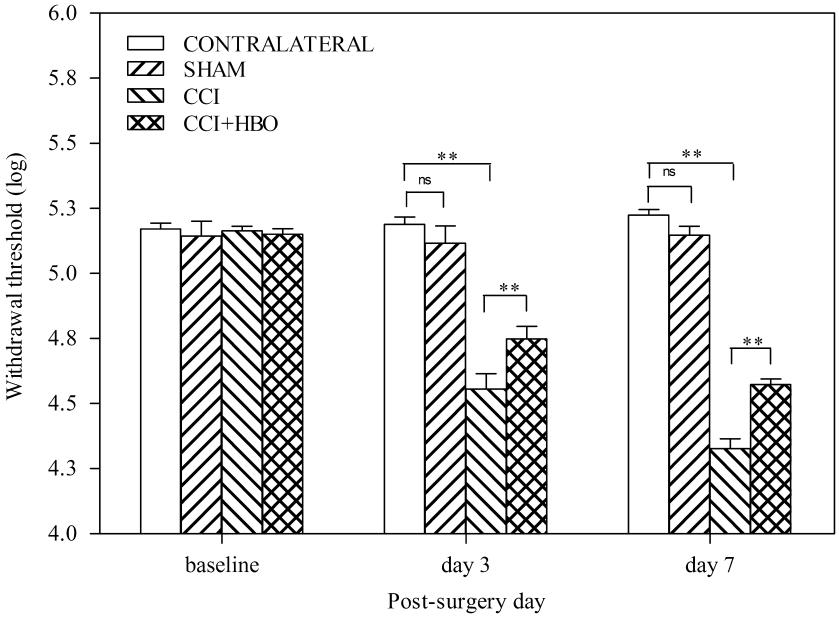

Figure 1 The effect of daily hyperbaric oxygenation (HBO) therapy on mechanical allodynia in SHAM and chronic constrictive injury (CCI)-induced neuropathic pain rats for 7 days after the surgery. The threshold of foot withdrawal to repeated mechanical stimuli applied to the hind paw with von Frey filaments is expressed as logarithm and data represent mean ± standard error. No significant change in mechanical allodynia was observed in sham-operated rats throughout the experiment. Significant mechanical allodynia had developed in all CCI rats ($n = 8$) as shown on post-operative days 3 and 7. HBO significantly alleviated CCI-induced mechanical allodynia ($n = 8$); ** $p < 0.01$, ns = no significance.

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shown in Fig. 1, the mechanical hypersensitivity in SHAM rats remained stable throughout the test period, the threshold of mechanical allodynia significantly decreased in CCI rats compared with SHAM rats on day 3, the value was 4.56 ± 0.06 versus 5.12 ± 0.07 ($p < 0.01$) and 4.33 ± 0.04 versus 5.15 ± 0.04 ($p < 0.01$) on day 7. HBO significantly increased the threshold of mechanical allodynia in comparison with CCI rats on day 3, with the value being 4.75 ± 0.05 versus 4.56 ± 0.06 ($p < 0.01$) and 4.57 ± 0.02 versus 4.33 ± 0.04 ($p < 0.05$) on day 7. No significant mechanical hypersensitivity was observed on the left side (contralateral, nonoperative side).

3.2 Apoptotic cells counting

As shown in Supporting Information Fig. S1, the number of TUNEL-positive apoptotic cells in CCI rats was significantly higher than those in SHAM rats, as the value was 19.25 ± 2.45 versus 10.0 ± 0.45 ($p < 0.01$). HBO significantly decreased CCI-induced apoptotic cells to the level in SHAM rats. The values were 8.25 ± 0.9 (CCI+HBO) versus 19.25 ± 2.45 (CCI; $p < 0.05$). As also shown in Supporting Information Fig. S1, the number of caspase-3 positive apoptotic cells in CCI rats was significantly higher than that in SHAM, as the value was 45.52 ± 5.48 versus 8.71 ± 2.21 ($p < 0.05$). HBO significantly decreased CCI-induced apoptotic cells to the level in SHAM rats, being 45.52 ± 5.48 (CCI) versus 11.13 ± 3.90 (HBO+CCI; $p < 0.05$).

3.3 Distribution of apoptotic cells in CCI group

As shown in Supporting Information Fig. S2, the number of caspase-3 positive cells was significantly higher in the ipsilateral dorsal horn of the spinal cord than in the ipsilateral central area, 16.5 ± 3.4 versus 5.7 ± 2.5 ($p < 0.05$); ipsilateral ventral horn 16.5 ± 3.4 versus 3.3 ± 1.14 ($p < 0.05$) and contralateral half area 16.5 ± 3.4 versus 2.9 ± 1.0 ($p < 0.05$).

3.4 Identification of apoptotic cells

Positive co-staining of TUNEL and Nissl body cells as showed in Supporting Information Fig. S3 indicate that these apoptotic cells are neurons.

3.5 Real-time PCR

In comparison with SHAM rats, CCI-induced neuropathic pain was associated with higher mRNA levels of TNF-α (16.9 ± 0.9 vs. 12.0 ± 1.5, $p < 0.05$; Supporting Information Fig. S4), caspase-3 (17.7 ± 3.4 vs. 12.7 ± 3.0, $p < 0.05$; Supporting Information Fig. S5) and SOD (3.3 ± 0.5 vs. 1.4 ± 0.4, $p < 0.05$; Supporting Information Fig. S6), measured 3 days post-CCI. HBO significantly decreased CCI-induced mRNA levels of TNF-α (11.7 ± 1.8 vs. CCI, $p < 0.05$), caspase-3 (11 ± 1.0, $p < 0.05$ vs. CCI, $F = 13.50$, $p < 0.05$) and SOD (1.2 ± 0.1 vs. CCI, $p < 0.05$).

4. Discussion

The main findings are that (1) the development of CCI-induced neuropathic pain was associated with overly expressed proapoptosis genes (TNF-α and caspase-3) and the subsequently increased neuronal apoptosis in the spinal cord; (2) HBO alleviated CCI-induced neuropathic pain; and (3) HBO inhibited CCI-induced proapoptosis gene expression and apoptosis.

Apoptotic death of spinal neurons has been hypothesized to be a pathological mechanism in peripheral nerve injury-induced neuropathic pain (Azkue et al., 1998). Sugimoto et al. were the first to describe that the incidence of degenerated neurons, recognized as dark-staining cells ('dark neurons'), had increased significantly in the lumbar dorsal horn 8 days after CCI (Sugimoto et al., 1990). Later, Maione et al. identified these 'dark neurons' as apoptotic cells (Maione et al., 2002). The development of CCI-induced neuropathic pain has also been observed to be associated with increased apoptosis in the superficial laminae of the spinal cord (Siniscalco et al., 2008). Our data provides new evidence that CCI-induced neuropathic pain was associated with significant apoptotic changes in the L4–5 spinal cord. We also showed that these apoptotic cells were mainly distributed in the territory of the ipsilateral dorsal horn of the spinal cord, which was in accordance to the result of Sekiguchi et al. (2003). Controversy exists regarding the types of the apoptotic cells. Some previous morphological studies suggested the apoptotic cells were neurons in the spinal cord following peripheral nerve insult (Kawamura et al., 1997; Whiteside and Munglani, 2001). There is also evidence that astrocytes undergo apoptosis in the spinal cord of neuropathic rats (Woolf et al., 1992). In our study, positive co-staining result of TUNEL with Nissl body and negative co-staining result of TUNEL with glial fibrillary acidic protein suggest that these apoptotic cells are neurons.

There are several possible explanations for the existence of apoptosis in the spinal cord in the development of neuropathic pain. Firstly, tissue injury or nerve damage can induce long-lasting sensitization in dorsal horn neurons (McMahon et al., 1993). In the

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condition of neuropathic pain, central sensitization is an important part of pathologic changes of neurons in the spinal cord, which means the dorsal horn neuron pain threshold is reduced and the receptive field of the dorsal horn neuron grows (McMahon et al., 1993). The process of central sensitization may cause the neurons in the spinal cord to undergo pathologically high performance and high metabolism condition, which is one of the reasons of ageing and programmed death (apoptosis). Secondly, injury of the peripheral nerve leads to a substantial degeneration and loss of the central terminals of C fibres in lamina II in the spinal cord (Castro-Lopes et al., 1990), then the central projection of surviving A-β fibres in lamina III and IV may be stimulated by growth factors to sprout into the 'foreign' territory vacated by C fibre terminals from lamina II (Woolf et al., 1992). Thus, the reorganization and phenotypic changes associated with nerve injury allow innocuous input to reach lamina, a pain transmission region of the spinal cord. These sprouts may even establish contacts with deafferentated pain transmission neurons (Woolf et al., 1995). These morphological changes provide a possibility of retrograde axonal transport of some proinflammatory cytokines, i.e., TNF-α and interleukin (IL)-1β, which is well known that they take part in the regulation of cellular apoptosis (Shubayev and Myers, 2001; Schafers et al., 2002). In the present study, the overly expressed TNF-α and caspase-3 genes, during the development of CCI-induced neuropathic pain, provide further evidence that apoptosis plays an essential role in neuropathic pain. As pointed out by a recent review, TNF-α and other mediators form a network to interact with downstream signalling mechanisms (Leung and Cahill, 2010). The effect of HBO on these downstream signalling systems warrant further investigation. The neuroprotective effect of HBO in traumatic brain injury has been explained molecularly by the prevention of cell apoptosis (Vidigal et al., 2007). Our study suggests that HBO may alleviate neuropathic pain by decreasing apoptotic changes in the spinal cord. There are several possible explanations for beneficial effects of HBO against apoptosis in neuropathic pain. CCI-induced neuronal injury, similar to other acute peripheral nerve lesions, has an important inflammatory component (Sanchez, 2007). Many of the axons undergo Wallerian degeneration (Myers et al., 1993; Ramer and Bisby, 1997). HBO has been reported to decrease paw oedema in an acute inflammatory condition (Eliav et al., 2009) and increases oxygen content in circulation and tissue perfusion. This may have a direct role in the prevention of spinal cord neuronal apoptosis. IL-10 is capable of inhibiting synthesis of proinflammatory cytokines such as TNF-α. IL-10 has also been reported to have anti-apoptotic effects by down-regulation of Bax, caspase-3 and up-regulation of Bcl-2 (Londono et al., 2011). HBO has been reported to enhance IL-10 activity in traumatic brain injury (Chen et al., 2014). In the present study, HBO may also enhance the IL-10 activity to antagonize CCI-induced apoptotic changes. The data from our study, as well as others, show that a reduced sciatic endoneurial TNF-α level by administration of HBO, thalidomide or sirolimus was associated with an improvement of neuropathic pain in the CCI model of rats (George et al., 2000; Orhan et al., 2010; Li et al., 2011). In the present study, CCI-induced overexpression of TNF-α gene in the spinal cord provides convincing evidence to support that TNF-α plays a critical role in the development of neuropathic pain. The reduction of TNF-α may inhibit not only the apoptosis of first order (dorsal root ganglion neuron) and second order neurons (spinal cord neuron), but also the synaptic connection, depending on retrograde axonal transport of TNF-α between the first and second order neurons, which would prevent spinal cord neuron from further apoptosis (Londono et al., 2011).

The dose and time-dependent characteristics of neuroprotective HBO effects suggest that there may be other roles in the mechanism of HBO (Narkowicz et al., 1993; Brvar et al., 2010). As pointed out by Chung and Yowtak et al., reactive oxygen species (ROS) have been implicated in the development of persistent pain states that result from nerve injury or inflammatory insult (Chung, 2004; Yowtak et al., 2011). Studies show that the removal of the excess production of ROS by scavengers reduced the expression of proapoptotic genes, thereby preventing dorsal horn apoptosis (Scholz et al., 2005; Naik et al., 2006; Siniscalco et al., 2007). Dayan et al. observed that HBO significantly decreased injury-induced SOD level increase following acute thoracic spinal cord injury (Dayan et al., 2012). This significantly decreased SOD level was associated with markedly reduced formation of ROS. In the present study, production of ROS was not measured. However, significantly increased SOD gene expression in CCI rats suggests that there may have been a markedly increased production of ROS. Reduced CCI-induced SOD gene expression in HBO rats may indicate reduced ROS formation. The present study suggests that reduced SOD gene expression may contribute to the beneficial effect of HBO in CCI-induced neuropathic pain.

HBO has also been observed to selectively increase Spp1 and Birc4 gene expression in brain injury. Birc4

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is a naturally occurring inhibitor of apoptosis proteins. It can bind to and deactivate already active caspases (Deveraux and Reed, 1999). We previously observed that HBO reduced brain damage and selectively decreased expression of proinflammatory cytokine genes, including Cxcl2, IL-1 alpha and IL-6 (Mahajan et al., 2008). The present study suggests that HBO may inhibit proapoptotic gene expression to achieve its beneficial effect in CCI-induced neuropathic pain.

In conclusion, our study suggests that the overly expressed proapoptosis genes and the subsequent increase in apoptotic cells in the spinal cord may contribute to the development of CCI-induced neuropathic pain. The inhibitory role of HBO on spinal apoptotic changes may be responsible for its beneficial effect in CCI-induced neuropathic pain.

Author contributions

Q.H. helped in conducting the study, analysing the data and preparing the manuscript. L.F. helped in conducting the study, analysing the data and preparing the manuscript. F.L. helped in designing the study and preparing the manuscript. S.T. helped in designing the study. Z.Y. helped in designing the study, analysing the data and preparing the manuscript. All authors have read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Effect of daily hyperbaric oxygenation (HBO) therapy on apoptotic cell counts 7 days post-surgery. Apoptotic cells are significantly higher in the spinal cord in chronic constrictive injury (CCI)-induced neuropathic pain rats than that in sham-operated rats. HBO significantly decreased CCI-induced apoptotic cells in the spinal cord to the level of SHAM group detected using both TUNEL staining and caspase-3 staining. $^{**}p < 0.01$.
Figure S2. The distribution of caspase-3 positive apoptotic cells in different areas of the spinal cord in chronic constrictive injury (CCI)-induced neuropathic pain group. The numbers of apoptotic cells are mainly distributed in the ipsilateral dorsal horn. $^{**}p < 0.01$.
Figure S3. TUNEL and Nissl body co-staining in the spinal cord 7 days post-surgery in different groups. The slides were viewed and photographed using a fluorescence microscope at 400 × magnification. Double-labelled coronal sections revealed that some of the TUNEL-positive cells (green) were also positive to Nissl staining (red) in all of the three groups simultaneously. Example of positive co-staining is indicated with arrows. The vast majority of cells engaging in the apoptosis in our study are neurons.
Figure S4. Effect of hyperbaric oxygenation (HBO) therapy on TNF-α gene expression 3 days post-surgery. Chronic constrictive injury (CCI)-induced neuropathic pain increased TNF-α gene expression in L4–5 spinal cord 3 days post-surgery. HBO significantly reduced CCI-induced overexpression of TNF-α gene to the SHAM group level. The mRNA levels (mean ± standard error) of the TNF-α gene under analysis were measured and reported by real-time polymerase chain reaction (PCR) amplification. Each real-time PCR was repeated at least four times. $^{*}p < 0.05$.
Figure S5. Effect of hyperbaric oxygenation (HBO) therapy on caspase-3 gene expression 3 days post-surgery. Chronic constrictive injury (CCI)-induced neuropathic pain increased the caspase-3 gene expression 3 days post-surgery in L4–5 spinal cord. HBO significantly reduced CCI-induced overex-

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pression of the caspase-3 gene to the SHAM group levels. The mRNA levels (mean ± standard error) of the caspase-3 gene under analysis were measured and reported by real-time polymerase chain reaction (PCR) amplification. Each real-time PCR was repeated at least four times. *$p < 0.05$.

Figure S6. Effect of hyperbaric oxygenation (HBO) therapy on SOD gene expression 3 days post-surgery. Chronic constrictive injury (CCI)-induced neuropathic pain increased the SOD gene expression 3 days post-surgery in L4–5 spinal cord. HBO significantly reduced CCI-induced overexpression of the SOD gene to the SHAM group levels. The mRNA levels (mean ± standard error) of the gene under analysis were measured and reported by real-time polymerase chain reaction (PCR) amplification. Each real-time PCR was repeated at least four times. **$p < 0.05$.

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