

Hyperbaric Oxygen Treatment Produces an Antinociceptive Response Phase and Inhibits Astrocyte Activation and Inflammatory Response in a Rat Model of Neuropathic Pain

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Abstract Hyperbaric oxygen (HBO) treatment has been proven to be a promising candidate for protection of the nervous system after acute injury in animal models of neuropathic pain. The purposes of this study were to examine the antinociceptive response phase induced by HBO treatment in a model of neuropathic pain and to determine the dependence of the treatment's mechanism of alleviating neuropathic pain on the inhibition of spinal astrocyte activation. Neuropathic pain was induced in rats by chronic constriction injury of the sciatic nerve. Mechanical threshold and thermal latency were tested preoperatively and for 1 week postoperatively, four times daily at fixed time points. Methane dicarboxylic aldehyde (MDA) and superoxide dismutase (SOD) parameters were used as indices of oxidative stress response and tested before and after the treatment. The inflammatory cytokines interleukin (IL)-1 β and IL-10 were assayed in the sciatic

nerve were with enzyme-linked immunoassay. Glial fibrillary acidic protein activation in the spinal cord was evaluated immunohistochemically. The rats exhibited temporary allodynia immediately after HBO treatment completion. This transient allodynia was closely associated with changes in MDA and SOD levels. A single HBO treatment caused a short-acting antinociceptive response phase. Repetitive HBO treatment led to a long-acting antinociceptive response phase and inhibited astrocyte activation. These results indicated that HBO treatment played a dual role in the aggravation and alleviation of neuropathic pain, though the aggravated pain effect (transient allodynia) was far less pronounced than the antinociceptive phase. Astrocyte inhibition and anti-inflammation may contribute to the antinociceptive effect of HBO treatment after nerve injury.

A short note HBO treatment may be an important intervention for preventing neuronal damage. Recent studies have reported that HBO treatment can increase neuroplasticity leading to repair of chronically impaired brain functions and improved quality of life in patients who have experienced traumatic brain injury with prolonged post-concussion syndrome even in a late chronic stage. Furthermore, HBO can lead to significant neurological improvements in stroke patients, including chronic late-stage stroke patients. The observed clinical improvements suggest that HBO therapy after brain damage favors the repair of nervous tissue and function. The results presented in this paper may pave the way for future experimental studies, including research addressing HBO therapy efficacy in peripheral versus central nervous system damage.

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Abbreviations

HBO	Hyperbaric oxygen
TNF	Tumor necrosis factor
CCI	Chronic constriction injury
ATA	Atmospheres absolute pressure
MWT	Mechanical withdrawal threshold
TWL	Thermal withdrawal latency
AB	Avidin-biotin
GFAP	Glial fibrillary acidic protein
ROS	Oxygen species
CREB	cAMP response element-binding protein
MDA	Methane dicarboxylic aldehyde
SOD	Superoxide dismutase

Introduction

Neuropathic pain, induced by nerve injury, is not caused by a stimulus and is characterized by low nociceptive thresholds, even in response to a normal innocuous stimulus (Scholz and Woolf 2007). Although the etiology of neuropathic pain has not been established satisfactorily, several studies have revealed that algogenic substances released by activated glia play important roles in neuropathic and chronic pain mechanisms (Milligan and Watkins 2009; Tan et al. 2009). Activated astrocytes respond to pain by releasing signaling molecules and chemokines, which have pathological functions (Abbadie et al. 2009; Hald et al. 2009). Thus, astrocytes may be considered new targets for neuropathic pain control and should receive more attention.

Hyperbaric oxygen (HBO) treatment, in which 100 % oxygen with a pressure higher than atmospheric pressure is administered to the patient for a certain period of time, is used widely in clinical practice (Chung et al. 2010). Recent studies have reported that HBO treatment can increase neuroplasticity leading to repair of chronically impaired brain functions and improved quality of life in patients who have experienced traumatic brain injury with prolonged post-concussion syndrome, even in a late chronic stage (Boussi-Gross et al. 2013). Furthermore, HBO can lead to significant neurological improvements in stroke patients, including chronic late-stage stroke patients (Efrati et al. 2013).

This therapy has a reliable neuroprotective effect and can be used to reduce nerve injury via mechanisms including the enhancement of neuronal viability, reduction of edema, and prevention of permanent neurological injury (George Mychaskiw et al. 2005). HBO therapy is a classic treatment for neuropathic pain (Dayan et al. 2012; Tai et al. 2010), fibromyalgia (Wilson et al. 2006; Yildiz and Uzun 2006), and inflammatory pain (Sümen et al. 2001; Wilson et al. 2007). Thompson et al. (2010) found that HBO treatment successfully relieved neuropathic pain in two rat models of neuropathic injury. A recent study of the antinociceptive effect and mechanism of HBO treatment demonstrated that it mitigated neuropathic pain induced by cold and mechanical allodynia and reduced the endoneurial production of tumor necrosis factor (TNF) (Li et al. 2011).

To our knowledge, however, no study has examined the antinociceptive response phase of HBO treatment. The relationship between this therapy's antinociceptive effect and the mechanism of astrocyte inhibition should also be explored. Thus, the purposes of the present study were to examine the antinociceptive response phase induced by HBO treatment in a model of neuropathic pain and to determine the dependence of the treatment's mechanism of alleviating neuropathic pain on the inhibition of spinal astrocyte activation and the inflammatory response.

Methods and Materials

Animals

The Animal Ethics Committee of China Medical University (Shenyang, China), approved all protocols, which were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act. Experiments were performed on adult male Sprague–Dawley rats weighing 300–320 g, provided by the Laboratory Animal Center of China Medical University. The animals were housed individually in plastic boxes at 23–25°C, with food and water available ad libitum.

Experimental Protocol

The rats were randomly divided into four groups ($n=6$ each): the sham surgery, chronic constriction injury (CCI), HBO 2.0 [CCI+HBO treatment at 2 atm absolute pressure (ATA) daily for 7 days beginning on postoperative day 1], and HBO 2.5 (CCI+HBO treatment at 2.5 ATA, administered as in the HBO 2.0 group) groups. Baseline mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) testing was performed in all animals. All animals displaying baseline hyperalgesia were excluded from the study. Animals with MWT and TWL reductions >20 % compared with baseline values on the first postoperative day were included in subsequent experiments.

During the 7-day postoperative experimental period, MWT and TWL were measured four times per day in all animals. Testing was performed at the same timepoints (T_0 – T_3) every day: T_0 , immediately after removal from the hyperbaric chamber; T_1 , 1 h after removal from the chamber; T_2 , 2 h after removal from the chamber; and T_3 , before entering the hyperbaric chamber.

Induction of Neuropathic Pain

The CCI model of the sciatic nerve was used to induce the neuropathic pain condition (Bennett and Xie 1988). Rats were anesthetized with 10 % chloral hydrate (300 mg/kg i.p., supplemented as necessary). The left biceps femoris of each rat was bluntly dissected at the mid-thigh level to expose the sciatic nerve. Four 4–0 chromic cat gut sutures were loosely tied around the sciatic nerve at 1-mm intervals immediately proximal to the trifurcation. The wound was treated with antiseptic solution, and the incision was sutured in layers with 3–0 monofilament nylon sutures. In the sham surgery group, identical dissection was performed but the sciatic nerve was not ligated.

Hyperbaric Oxygen Treatment

The cylindrical HBO treatment chamber (DS400-IV; Weifang Huaxin Oxygen Industry Co., Ltd., Shandong, China) was ventilated with 100 % oxygen for 10 min, and soda lime was placed on the bottom of the chamber to minimize water vapor and carbon dioxide accumulation before the rats were placed in the chamber. After a rat was placed in the chamber, the pressure was increased at a rate of 0.1 ATA/min to the desired pressure (2.0 or 2.5 ATA in HBO treatment groups) and maintained for 60 min. The rats were allowed to breathe spontaneously during HBO treatment. The chamber was then decompressed to normal room pressure at a rate of 0.1 ATA/min. Rats in the control (sham surgery and CCI) groups were simply placed inside the HBO treatment chamber for approximately 100 min, but received no treatment.

Behavioral Testing

Mechanical Withdrawal Threshold Testing

MWTs were determined by measuring the incidence of left hind foot withdrawal in response to mechanical stimuli. Each animal was placed in a Plexiglas chamber on a screen and given a 10-min habituation period prior to each test session. MWTs were then determined using von Frey monofilaments (0.14–15 g), following the up-and-down procedure described by Song et al. (1999). Each trial began with the application of 0.2 g von Frey force for 3 s to the plantar surface of the left hind foot. If no withdrawal response was observed, then the next greatest force was applied; if a withdrawal response was observed, then a lesser force was applied. This protocol was continued through the greatest force causing no withdrawal or the administration of four stimuli following an initial withdrawal response. MWTs of 15 g were recorded for animals that did not respond to the greatest von Frey monofilament force. Threshold testing was performed ten times per session, and the MWT of each animal was recorded as the lowest stimulus force that provoked at least five hind foot withdrawal responses.

Thermal Withdrawal Latency Testing

To examine TWL, each rat was placed on the surface of a 3-mm-thick glass plate and covered with the same Plexiglas chamber to measure sensitivity to thermal stimuli using a BME-410C full-automatic plantar analgesia tester (Youer Equipment Scientific Co., Ltd., Shanghai, China). The radiant heat source was a high-intensity halogen projector bulb positioned at a fixed distance below the glass plate. The bulb was adjusted to project a circle of light with a 5-mm diameter on the surface of the floor. Heat stimuli were directed at the

exposure site on the left hind foot of each rat. The tester automatically determined TWL and cut off the heat stimulus upon hind foot withdrawal. Each test session comprised the delivery of five thermal stimuli at 5-min intervals. The stimulus was stopped when TWL exceeded 30 s to avoid excessive tissue injury, and the region was considered to be completely unresponsive.

Determination of Serum MDA and SOD Levels

At T_0 or T_1 , after behavioral testing, blood was collected from the tail vein. Each sample was centrifuged and the supernatant was collected. The blood methane dicarboxylic aldehyde (MDA) level was determined by the TBA method using Type 721 spectrometry. The blood superoxide dismutase (SOD) level was evaluated by radioimmunoassay (RIA) with a kit, according to manufacturer's instructions (Anda Biological Co. Ltd.).

Determination of IL-1 β and IL-10 Levels in the Lumbar Spinal Cord with ELISA

Spinal cord specimens were weighed and then placed into homogenization buffer. Samples were centrifuged at 20,000 \times g for 30 min at 4 °C and the supernatant was collected. Enzyme-linked immunoassay (ELISA) was conducted according to manufacturer's instructions (Abcam, USA) and the A_{450} value was measured. Standard curves were drawn according to the absorption values of the standard samples, and curves were used to determine the concentration of IL-1 β and IL-10 in each in the lumbar spinal cord sample.

Immunohistochemistry

To examine astrocyte profiles, tissue samples were collected from the lumbar enlargements of the animals' spinal cords. After completion of the behavioral tests on day 7, the rats were deeply anesthetized with an intraperitoneal injection of 10 % chloral hydrate (300 mg/kg). The rats were sacrificed and perfused with 200 ml 0.9 % normal saline, followed by a fixative consisting of 4 % paraformaldehyde in 0.1 % phosphate-buffered saline (PBS; pH 7.4) was performed. Spinal cord segments L4 and L6 were isolated and postfixed overnight in the same fixative. After cryoprotection with 30 % sucrose in 0.1 % PBS at 4 °C for 24 h, 20- μ m sections of spinal cord were cut using a cryostat and mounted on microscope slides.

The spinal cord sections were washed in PBS and incubated with 0.1 % H₂O₂. They were then rinsed in PBS three times and pre-incubated for 30 min with 10 % normal goat serum in PBS containing 0.5 % Triton X-100. The sections were

incubated overnight at 4°C with rabbit anti-gial fibrillary acidic protein (GFAP) primary antibody [1:1,000](Z0334; Dako Co., Ltd., Denmark) diluted in PBS containing 1 % normal serum and 0.5 % Triton X-100. Bound primary antibodies were visualized with the avidin-biotin (AB) complex method (Chen et al. 2010) (Vectastain Elite ABC Kit; Vector Laboratories Co., Ltd., U.S.). The spinal cord sections were rinsed in PBS (5×10 min), incubated with secondary biotinylated antibody for 1 h, and then placed in AB complex for 30 min. Finally, after rinsing with PBS (5×10 min), peroxidase labeling was visualized with diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co., Ltd., USA). The reaction was stopped with distilled water and excess reaction product was rinsed away with PBS. The sections were dried overnight and then dehydrated with ascending concentrations of alcohol, followed by xylene.

Astrocyte Profile Counting

The number of astrocyte profiles in each spinal dorsal horn was examined using a confocal microscope (FluoView 1000; Olympus Co., Tokyo, Japan). The images were processed using a computer-based image analysis system. A minimum of six sections per rat that were not damaged, folded, or torn were used for the analysis. To ensure accuracy, the sections were examined under a Zeiss light microscope and images were captured with a digital camera (Nikon, Japan) at 400× magnification. A GFAP-immunoreactive cell profile consisted of a cell body with numerous radiating processes. The numbers of GFAP-immunoreactive astrocytes per 1-mm² region were calculated and compared with counts obtained for the sham surgery group.

Statistical Analysis

Changes in behavioral responses were compared among groups using two-way repeated-measures analysis of variance (ANOVA) with post hoc Bonferroni tests. One-way ANOVA with post hoc Bonferroni correction was used to identify differences in immunohistochemical results among groups. Data are presented as means±standard errors of the mean or *n* (%). *P* values<0.05 were considered significant.

Results

Transient Allodynia

MWT and TWL decreased significantly in the CCI group compared with the sham surgery group, indicating that CCI induced lasting mechanical and thermal hyperalgesia in the rats. Figure 1 presents the effects of HBO treatment at *T*₀, showing significantly lower MWT and TWL in the HBO 2.0

and 2.5 groups than in the CCI group. Transient allodynia faded within 1 h after HBO treatment (Fig. 2). These results indicate that HBO treatment induced transient allodynia, rather than exerting a measurable antinociceptive effect at the moment of treatment completion.

Short-Acting Antinociceptive Response Phase

A single HBO treatment generated a potent antinociceptive response phase and blocked hyperalgesia at *T*₁ (Fig. 2). Daily HBO treatment also inhibited mechanical and thermal hyperalgesia for a short period of time (Fig. 2), but this short antinociceptive response phase dissipated completely within 2 h after HBO treatment on postoperative days 1–5 (Fig. 3).

Long-Acting Antinociceptive Response Phase

A long-acting antinociceptive response phase began to emerge after five consecutive days of HBO treatment (Fig. 4). MWT and TWL values remained stable for ≥24 h (Figs. 3 and 4). No difference in this antinociceptive response phase or antinociceptive intensity was observed between the HBO 2.0 and 2.5 groups. These results indicate that repetitive HBO treatment may exert prolonged antinociceptive effects.

Changes in MDA Level and SOD Activity

At time point *T*₀ after HBO treatment, serum MDA levels in the HBO 2.0 or HBO 2.5 groups was significantly higher than the levels detected in the CCI and Sham groups. However, these MDA level elevations in the HBO 2.0 and HBO 2.5 groups were no longer significant. Conversely, serum SOD activity levels did not differ between the groups at time point *T*₀. But at *T*₁, SOD activity in the HBO 2.0 and HBO 2.5 groups was higher than that of the other two groups. Thus, from *T*₀ to *T*₁ (the HBO 2.0 and HBO 2.5 groups exhibited both decrease in MDA levels and a decrease in SOD activity (Fig. 5).

Expression of IL-1β and IL-10 in the Spinal Cord

ELISA experiments showed that IL-1β and IL-10 levels in the CCI group were significantly increased relative to the relatively low levels observed in the Sham group after 4 and 7 days of HBO treatment. After 4 days of hyperbaric oxygen treatment, IL-1β expression in the HBO 2.0 and HBO 2.5 groups was similar to that in the CCI group. However, after 7 days of hyperbaric oxygen treatment, IL-1β levels in the HBO 2.0 and 2.5 groups were greatly decreased compared to the CCI group. IL-10 expression in the HBO 2.0 or 2.5 groups did not differ from that in the CCI group after 4 days of hyperbaric oxygen treatment, but was greatly enhanced in the HBO 2.0 and the HBO 2.5 groups, relative to that in the CCI group, after 7 days of treatment (Fig. 6).

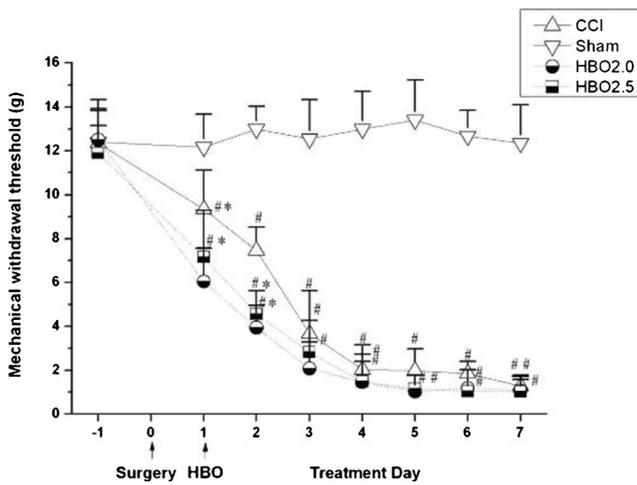
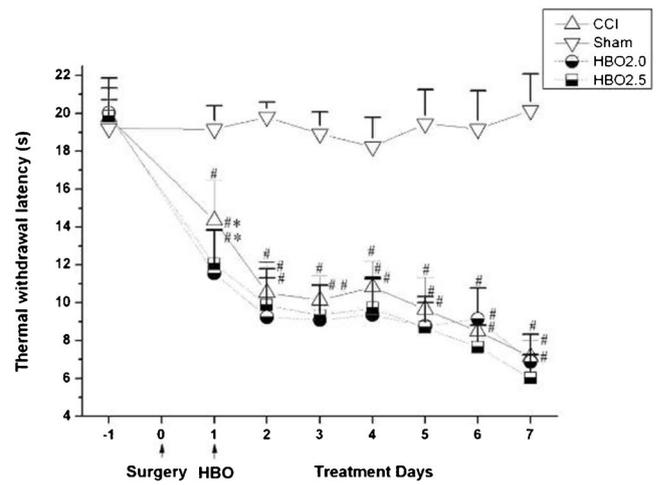


Fig. 1 Effects of daily hyperbaric oxygen (HBO) treatment on the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in rats at T_0 (allodynia phase). A single HBO treatment produced immediate mechanical and thermal allodynia. MWT and TWL values remained stable throughout the experimental period in the sham surgery



group; these values were significantly lower in the HBO 2.0 and 2.5 groups than in the sham surgery and chronic constriction injury (CCI) groups ($P < 0.05$). MWT and TWL values are means \pm standard errors of the mean for six rats in each group. * $P < 0.05$ vs. CCI group, # $P < 0.05$ vs. sham surgery group

Inhibition of Astrocyte Activation

CCI induced astrocyte activation in the spinal dorsal horn, and consecutive HBO treatments markedly inhibited this activation (Fig. 7). This inhibitory effect was stronger in the HBO 2.5 than in the HBO 2.0 group. These results suggest that HBO treatment produces an antinociceptive response due to the inhibition of astrocytes in the spinal dorsal horn.

single HBO treatment produced temporary allodynia and a short-acting antinociceptive phase. A 5-day course of HBO treatment produced a prolonged antinociceptive response and associated inhibition of astrocyte activation.

Discussion

This study demonstrated that HBO treatment could significantly alleviate neuropathic pain in rats with nerve injury. A

Antinociceptive Response of Rats with CCI to HBO Treatment

The animals exhibited allodynia immediately after the completion of HBO treatment, which disappeared within 1 h. Thus, the long-term antinociceptive efficacy of HBO treatment emerged gradually. These results were unexpected and inconsistent with those of some previous studies. For example, Li et al. (2011) reported that HBO treatment alleviated

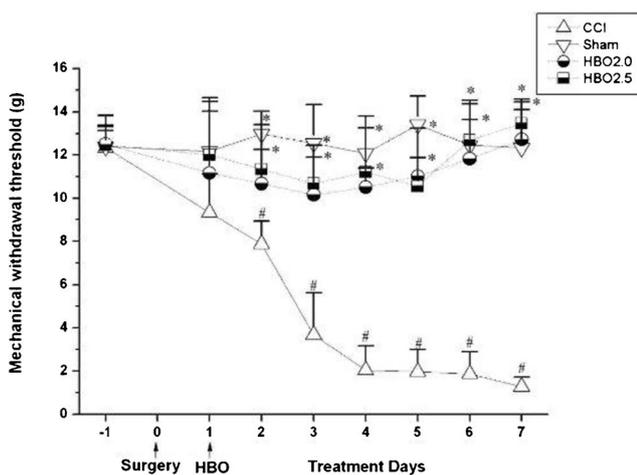
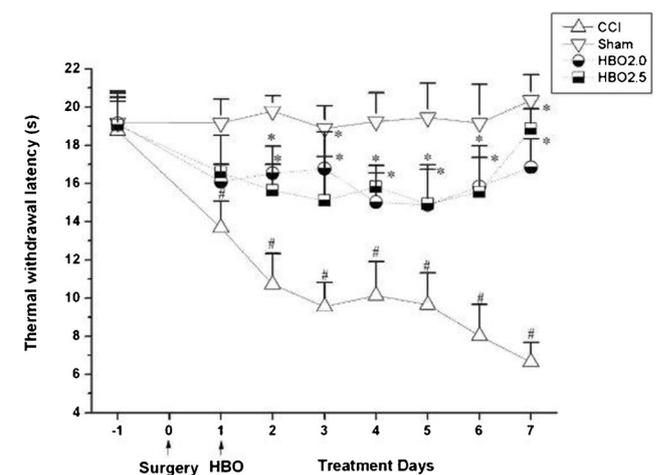


Fig. 2 At T_1 , hyperbaric oxygen (HBO) treatment produced a short antinociceptive phase affecting the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in rats. A single HBO treatment had a potent antinociceptive effect after 1 h. MWT and TWL values remained stable throughout the experimental period in the sham



surgery group; these values were significantly higher in the HBO 2.0 and 2.5 groups than in the chronic constriction injury (CCI) group ($P < 0.05$). MWT and TWL values are means \pm standard errors of the mean for six rats in each group. * $P < 0.05$ vs. CCI group, # $P < 0.05$ vs. sham surgery group

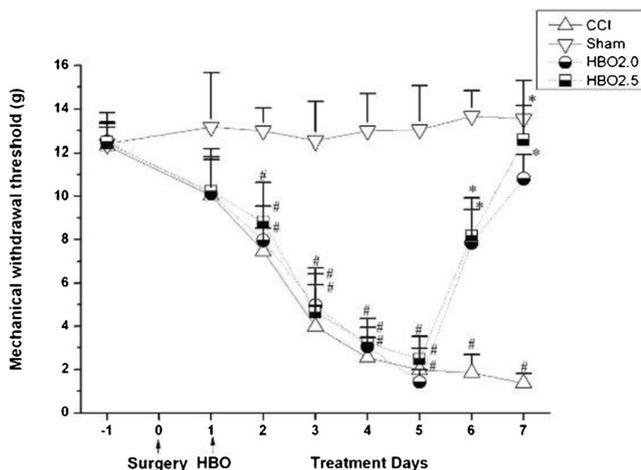
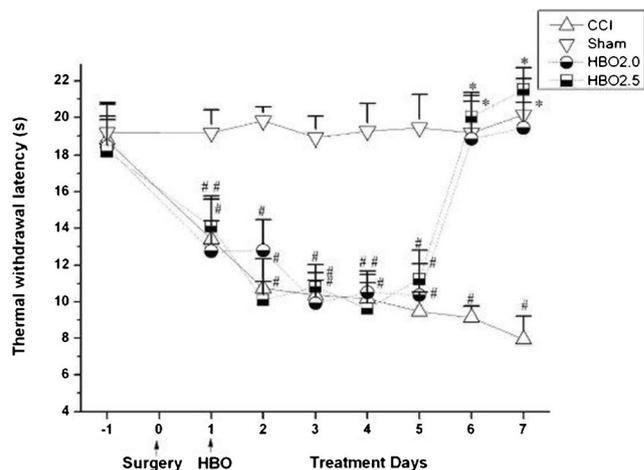


Fig. 3 At T_2 , hyperbaric oxygen (HBO) treatment produced a long antinociceptive phase affecting the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in rats. The short antinociceptive phase dissipated within 2 h of treatment on postoperative days 1–5, but five consecutive days of HBO treatment produced a long-



acting antinociceptive response phase. MWT and TWL values were significantly higher in the HBO 2.0 and 2.5 groups than in the chronic constriction injury (CCI) group on days 6 and 7 ($P < 0.05$). MWT and TWL values are means \pm standard errors of the mean for six rats in each group. * $P < 0.05$ vs. CCI group, # $P < 0.05$ vs. sham surgery group

neuropathic pain. Some possible explanations for the phenomenon observed in the present study may be due, at least in part, to temporary allodynia. Our results showed that the transient allodynia induced by hyperbaric oxygen treatment in CCI rats in the behavioral test was closely associated with the changes in MDA and SOD levels. When the transient allodynia occurred, the rats' serum MDA levels were elevated. It is possible that, following peripheral nerve injury, their bodies underwent the normal condition-hyperbaric high oxygen-normal condition process after hyperbaric oxygen treatment. Thus, oxidative injury greatly increased MDA generation, and moderate hyperbaric oxygen may enhance levels of nonlethal reactive oxygen species (ROS) (Gim et al. 2007; Yogaratnam et al.

2006). When ROS levels exceed the activity of endogenous antioxidant enzymes, it may aggravate oxidative injury of nerve tissues (Bubici et al. 2006). When the transient allodynia disappeared, serum SOD levels were increased, confirming that hyperbaric oxygen serves as an oxidative stimulant to damaged nerve tissues, producing protection of damaged neurons by increasing the production of endogenous antioxidant enzymes (such as SOD), enhancing the clearance of free radicals and thereby decreasing injury induced by oxygen free radicals (Kiralp et al. 2004). Hence, our results indicate that alternation of MDA and SOD levels may be a major mechanism underlying the transient allodynia induced by hyperbaric oxygen treatment. Upon removal from the hyperbaric chamber

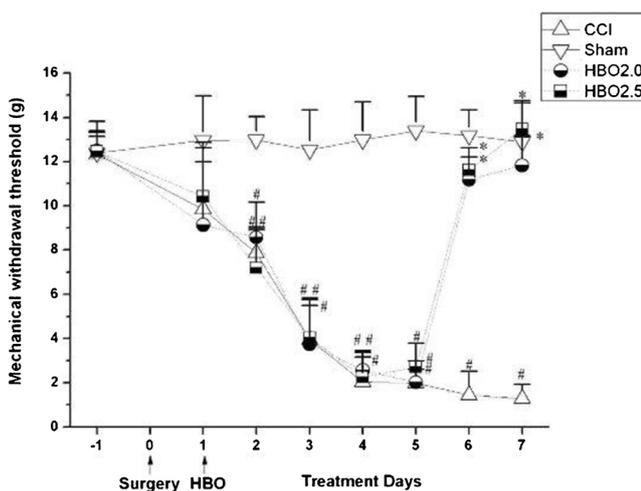
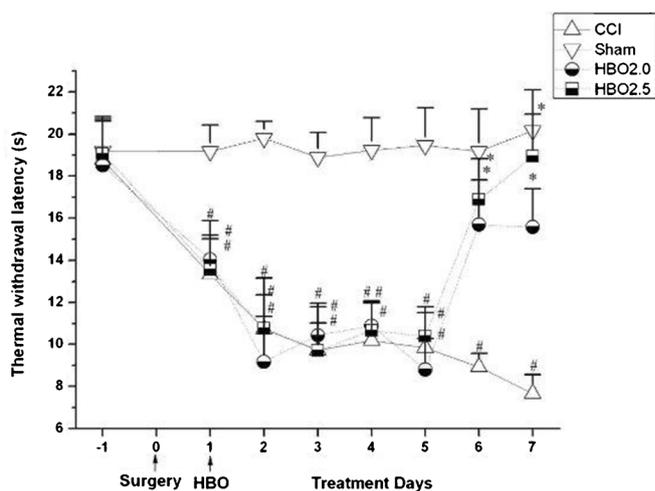


Fig. 4 At T_3 , hyperbaric oxygen (HBO) treatment produced a prolonged antinociceptive phase affecting the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in rats. Before HBO treatment on days 6 and 7, rats in the HBO 2.0 and 2.5 groups sustained



the increased MWT and TWL values observed after the previous treatment ($P < 0.05$). MWT and TWL values are means \pm standard errors of the mean for six rats in each group. * $P < 0.05$ vs. chronic constriction injury (CCI) group, # $P < 0.05$ vs. sham surgery group

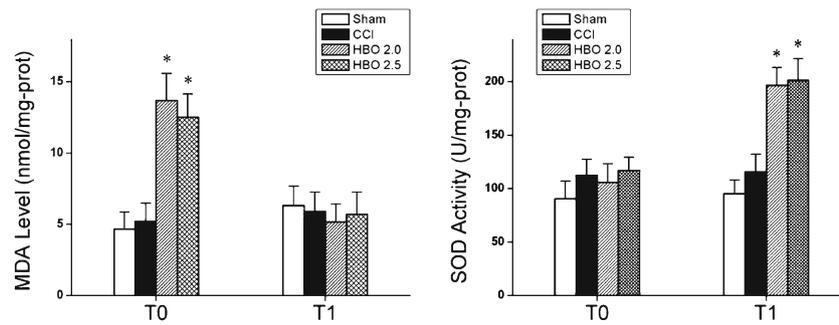


Fig. 5 The content of MDA and activity of SOD in plasma. MDA content and SOD activity in the Sham and CCI groups did not differ significantly from each other at either T_0 or T_1 ($P > 0.05$). At T_0 , the serum MDA content of the HBO 2.0 and HBO 2.5 groups was significantly higher than that of the CCI group ($*P < 0.05$). However, at T_1 , there was no

significant difference in serum MDA content between the experimental groups ($P > 0.05$). At T_0 , there was no significant difference in serum SOD activity between the experimental groups ($P > 0.05$). Conversely, at T_1 , the serum SOD activity measured for the HBO 2.0 or HBO 2.5 groups was significantly higher than that measured for the CCI group ($*P < 0.05$)

and return to the normal environment, animals are exposed to reduced environmental oxygen pressure. Hypoxia and reoxygenation are pathogenic factors in many diseases due to ROS accumulation (Fan et al. 2012; Gao et al. 2007). Excessive levels of ROS may induce pain by interfering with inhibitory transmission in the form of reduced synaptic gamma-aminobutyric acid release. ROS are involved in *N*-methyl-D-aspartate receptor phosphorylation, leading to central sensitization and in turn contributing to acute inflammatory pain and persistent neuropathic pain (Bhogal et al. 2012; Guedes et al. 2008; Yowtak et al. 2011). The oxidative pressure produced by HBO may upregulate antioxidant enzymes, catalases, and superoxide dismutase against ROS (Nie et al. 2005). Thus, the generation and disappearance of ROS may underlie the temporary allodynia observed in the present study.

We found that the antinociceptive response phase occurred very rapidly, within 1 h after HBO treatment. However, the antinociceptive effect gradually disappeared within 2 h after a single HBO treatment. Zelinski et al. (2009) reported that naltrexone antagonized the antinociceptive effect after a single HBO treatment, suggesting that rapid antinociception was

mediated by opioid receptors. HBO treatment does not likely directly affect opioid receptors, but it may be related in some way to endogenous opiate release.

Our results clearly indicate that repetitive HBO treatment tended to stabilize and prolong the antinociceptive effect. Clinically, the long-acting antinociceptive response phase has more practical value than the short-acting phase. Lasting endogenous mechanisms must be activated to sustain the pharmacological effect of HBO treatment. Li et al. (2011) found that a 7-day course of 1-h HBO treatment at 2.4 ATA reduced CCI-induced neuropathic pain in association with significantly reduced TNF- α production in the affected sciatic nerve. A DNA microarray analysis demonstrated HBO-induced neuroprotection against ischemic injury at 6, 12, and 24 h after treatment, and the authors suggested that genes relevant to the neurotrophin and inflammatory systems were involved in this neuroprotection (Hirata et al. 2007). Several cistrons related to the neuroprotective effect were found to be upregulated, where gene regulation had possibly occurred under HBO treatment. Mu et al. (2012) found that delayed daily HBO treatment had a potent neuroprotective effect,

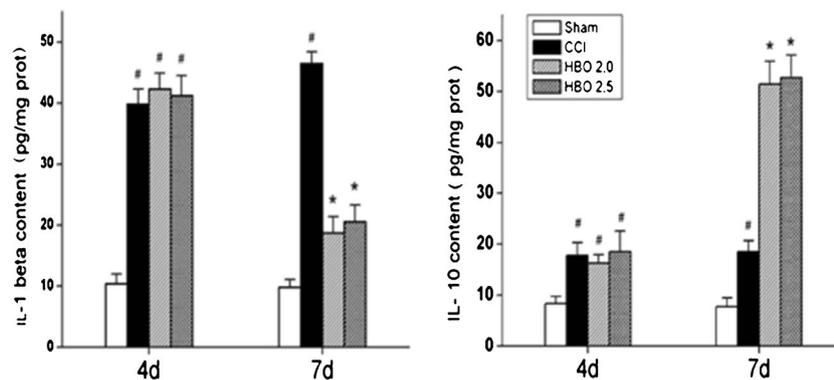
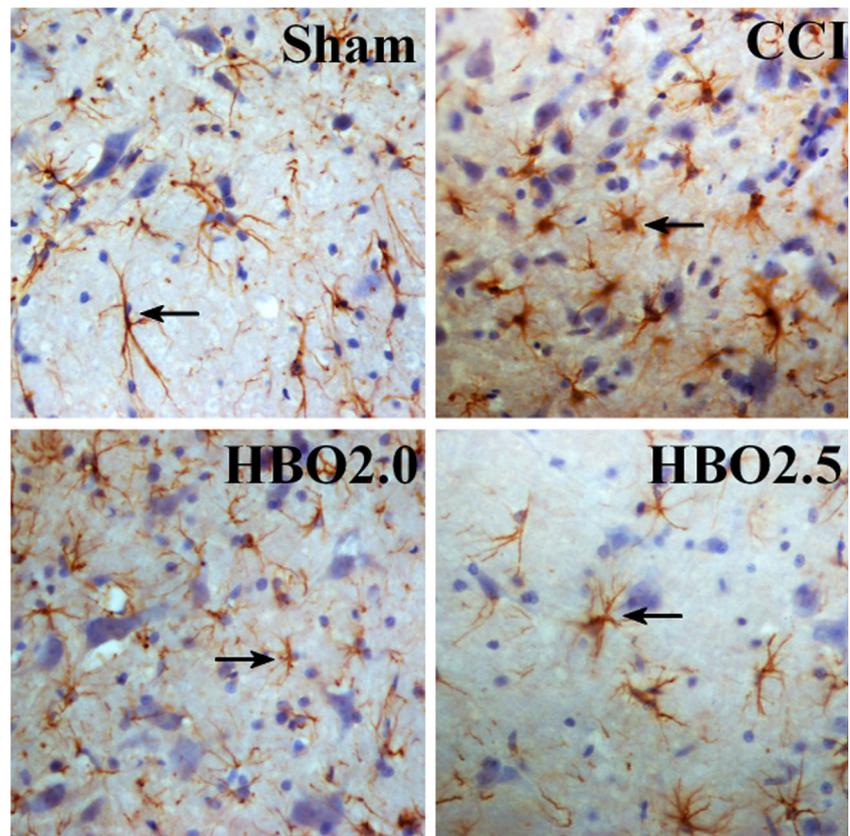


Fig. 6 Expression of IL-1 β and IL-10 in the spinal cord after HBO treatment. On day 4 after CCI surgery, spinal cord IL-1 β was significantly upregulated in the CCI, HBO 2.0, and HBO 2.5 groups compared to the Sham group ($^{\#}P < 0.05$). On day 7 after surgery, spinal cord IL-1 β was significantly reduced in the HBO 2.0 and HBO 2.5 groups compared to

the CCI group ($*P < 0.05$). On day 4 after CCI surgery, spinal cord IL-10 was significantly upregulated in the CCI, HBO 2.0, and HBO 2.5 groups compared to the Sham group ($^{\#}P < 0.05$). On day 7 after surgery, spinal cord IL-10 was significantly increased in the HBO 2.0 and HBO 2.5 groups compared to the CCI group ($*P < 0.05$)

Fig. 7 Photomicrographs and bar graph showing astrocytic responses in the spinal dorsal horn on postoperative day 7 (blue areas in photomicrographs are neuronal nuclei and thus are not indicators). Little glial fibrillary acidic protein (GFAP) immunoreactivity was observed in the sham surgery group, but the chronic constriction injury (CCI) group showed markedly increased GFAP immunoreactivity ($P<0.05$). GFAP-immunoreactive astrocytic profiles were characterized by small, compact cell bodies with long, thin ramified processes at 7 days after hyperbaric oxygen (HBO) treatment, and significantly fewer GFAP-immunoreactive astrocytes were present in the HBO 2.0 and 2.5 groups than in the CCI group ($P<0.05$). Fewer GFAP-immunoreactive astrocytes were observed in the HBO 2.5 group than in the HBO 2.0 group ($P<0.05$). * $P<0.05$ vs. CCI group, # $P<0.05$ vs. HBO 2.5 group



increased cAMP response element-binding protein (CREB) expression and signaling activity, and bolstered regenerative

cell proliferation in the injured brain. They suggested that these effects of HBO treatment were mediated by the

reduction of ubiquitin-dependent CREB degradation due to the HBO-induced activation of protein phosphatase 1 γ .

Antinociceptive Mechanism of HBO Treatment in Rats with CCI

Astrocyte activation is generally believed to be closely correlated with neuropathic pain (Juif et al. 2012; Watkins et al. 2001). Several studies have demonstrated that astrocyte activation exacerbates neuropathic pain and induces hyperalgesia (central sensitization) via proinflammatory cytokine production (DeLeo and Yezierski 2001; von Hehn et al. 2012). Shi et al. (2012) compared glial activation in the spinal dorsal horn in human postmortem tissues among three HIV patient groups (HIV negative, HIV positive with and without chronic pain). They found that expression levels of microglial cells did not differ among groups, but that astrocytes were upregulated and proinflammatory cytokines and proteins in the mitogen-activated protein kinase signaling pathway were increased in patients with HIV and chronic pain. These findings suggest that astrocyte activation was involved in the maintenance phase of HIV-associated chronic neuropathic pain (Shi et al. 2012). Chen et al. (2012) found that connexin 43 expression by astrocyte deletion reduced neuropathic pain-induced gliosis in knockout mice. They suggested that connexin 43, a mediator of central nervous system inflammation, was critically linked to the development of neuropathic pain (Chen et al. 2012). As in previous studies, we noted that astrocytes were upregulated in the spinal dorsal horns of rats at 7 days after CCI. Thus, experimental and clinical evidence implies the important role of astrocytes in the development of neuropathic pain.

We found that the levels of inflammatory cytokines, namely IL-1 β and IL-10, in the lumbar spinal cord were altered markedly during chronic constriction injury of the sciatic nerve. During the four consecutive days after nerve injury, the daily hyperbaric oxygen treatment did not significantly decrease IL-1 β production, but did increase levels of the anti-inflammatory cytokine IL-10. However, 7 days of hyperbaric oxygen treatment suppressed IL-1 β expression while increasing IL-10 levels. These evidences imply that hyperbaric oxygen treatment may relieve neuropathic pain by suppressing expression of the proinflammatory cytokine IL-1 β level, while promoting expression of the anti-inflammatory cytokine IL-10.

In the central nervous system, activated astrocytes produce large amounts of inflammatory cytokines and participate in the regulation of various functions. In the spinal cords of rats with neuropathic pain, proinflammatory cytokines, such as IL-1 β and TNF- α , generated by astrocytes may promote central sensitization, reduce the pain threshold, and alter pain signal transduction pathways (Guo et al. 2007; Mika et al. 2009; Zhang et al. 2008). IL-10 is an important anti-inflammatory cytokine. IL-10 is known to suppress generation of IL-1 β and

TNF- α and inflammatory responses in central nervous system. Moreover, IL-10 in spinal cord plays a critical role in relieving neuropathic pain by regulating the production of pro- and anti-inflammatory cytokines by astrocytes (Kanaan et al. 1998; Moore et al. 2001). We found that the hyperbaric oxygen treatment could suppress the generation of proinflammatory cytokines and upregulate the expression of anti-inflammatory cytokines. These evidences further indicate that hyperbaric oxygen may suppress the inflammatory responses by inhibiting activated astrocytes and regulating their functions.

The results of the present study suggest that astrocyte inhibition in the spinal dorsal horn is one mechanism of neuropathic pain reduction by HBO treatment, and that it is at least partly responsible for the beneficial effect of this treatment. Furthermore, the pressure used in HBO treatment affects the degree of astrocyte inhibition. HBO may not have direct inhibited astrocytes in the present study. Activated astrocytes encourage the release of inflammatory cytokines, which in turn activate adjacent resting astrocytes (Chen et al. 2012; Hald et al. 2009). Thus, a positive feedback regulation mechanism between activated astrocytes and inflammatory cytokines may progressively amplify neuropathic pain. Several previous studies have confirmed the involvement of a distinct mechanism responsible for the antinociceptive effect of HBO treatment in the anti-inflammatory property (Eliav et al. 2009; Wilson et al. 2006). Gu et al. (2012) found that HBO treatment markedly suppressed nerve injury-inhibited, CCI-induced astrocyte activation, supporting the ability of this treatment to inhibit inflammation, as activated astrocytes can release proinflammatory cytokines. Thus, this positive feedback regulation may be interrupted by a series of HBO treatments.

The antinociceptive effect of HBO treatment may be due in part to protective factors other than astrocyte inhibition; this study could not exclude the effects of other mechanisms. Another limitation of our study was the small pressure gradient used in HBO treatment, which prevented us from clearly demonstrating whether the antinociceptive response phase was independent of HBO pressure.

Conclusion

In summary, this study demonstrated that HBO treatment plays dual roles in the aggravation and alleviation of neuropathic pain, although the aggravated pain effect (transient allodynia) was far less pronounced than the antinociceptive phase. Alternation of MDA and SOD levels may be a major mechanism underlying the transient allodynia induced by HBO treatment. Astrocyte inhibition and anti-inflammation may contribute to the antinociceptive effect of HBO treatment after nerve injury.

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Conflict of Interest There are no ethical/legal conflicts involved in the article.

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