

The analgesic effect of early hyperbaric oxygen treatment in chronic constriction injury rats and its influence on nNOS and iNOS expression and inflammatory factor production

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Abstract

Objective: To observe the analgesic effect of early hyperbaric oxygen (HBO) treatment in chronic constriction injury (CCI) rats, and to analyze the influence of HBO on the expression of neuronal nitric oxide synthase and inducible nitric oxide synthase and on the levels of inflammatory factors.

Methods: Rats were assigned into three groups randomly: sham, CCI, and HBO groups. The CCI rat model was established, and HBO treatment at 2.5 ATA (60 min) was given one day after surgery, lasting for five consecutive days. The pain behaviors of the rats were observed at predetermined time points, and the activation of astrocytes at dorsal horns as well as the changes of the synaptic ultrastructures were observed. The expressions of inducible nitric oxide synthase and neuronal nitric oxide synthase were detected by Western blot, and the levels of tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) were detected by quantitative real-time PCR.

Results: Rats in the CCI group developed hyperalgesia when compared with the sham group. Mechanical withdrawal threshold decreased and thermal withdrawal latency shortened in CCI group. Also, astrocytes at the dorsal horn were activated, the synaptic structure was disordered, the expressions of inducible nitric oxide synthase and neuronal nitric oxide synthase were increased significantly, and the release of inflammatory factor (TNF- α and IL-1 β) was up-regulated. However, with early initiation of HBO treatment, rats in the HBO group showed significantly alleviated hyperalgesia, increased mechanical withdrawal threshold, and prolonged thermal withdrawal latency. HBO treatment inhibited astrocyte expression and maintained normal synaptic structure. The expressions of inducible nitric oxide synthase and neuronal nitric oxide synthase were decreased in the dorsal horn, and the release of inflammatory factor (TNF- α and IL-1 β) was reduced.

Conclusions: Early HBO treatment significantly improves hyperalgesia in rats with neuropathic pain. The decreased expressions of inducible nitric oxide synthase and neuronal nitric oxide synthase and reduced levels of inflammatory factors are important mechanisms by which early HBO helps to alleviate neuropathic pain.

Keywords

Hyperbaric oxygen, neuropathic pain, nitric oxide, astrocytes, TNF- α , IL-1 β , chronic constriction injury

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Background

Neuropathic pain (NP) is caused by injury or dysfunction of the nervous system. The hallmark symptoms of NP are hyperalgesia and allodynia, characterized by spontaneous, paroxysmal, and debilitating burning pain, usually with paresthesia.¹ NP is one of the most

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common types of chronic pain presentation in pain clinics. Central sensitization is an important mechanism by which acute pain turns into chronic pain,² and synaptic plasticity is regarded as the basis of this process.³ Nitric oxide (NO) participates in central pain modulation and NO synthase (NOS) is thought to regulate the synthesis of NO. Neuronal NOS (nNOS) and inducible NO synthase (iNOS) are protein variants of NOS and understood to play a role in central pain sensitization. They are expressed in the presence of inflammation.⁴ Further, astrocytes have a close structural relationship with synaptic gap junctions promoting neuronal signaling,⁵ and thereby regulate chronic NP through inflammatory reactions.⁶ Since NOS could activate spinal astrocytes, this further causes a series of biochemical and pathological responses in central pain sensitization.⁷ Thus, NOS might be involved in the delivery of nociceptive information at the spinal level, and thus participates in the pathophysiological process of synaptic plasticity as well as the evolution of chronic pain.

Hyperbaric oxygen (HBO) has been used in the clinical treatment extensively (central or peripheral nerve injury diseases). HBO has a strong neuroprotective effect: it can promote the recovery of injured nerves, reduce, or eliminate nerve edema,⁸ improve tissue oxygen supply,⁹ promote peripheral nerve regeneration,¹⁰ and inhibit inflammatory reactions.¹¹ The effect of HBO in treating chronic pain has been confirmed,¹² and recent studies also found that HBO could alleviate NP,^{13–15} but the study of its analgesic mechanism is still not clearly established. Previous studies found that HBO treatment once reduced hyperalgesia in rats with chronic compress injury (CCI) and HBO decreased the expression of spinal NOS (iNOS and nNOS).¹⁶ iNOS is expressed in activated microglia, and massive synthesis of NO could enhance pain generation by activating the astrocytes, which may further produce various cytokines, inflammatory factors, and neural active substances that release pain mediators to help generate and maintain NP.¹⁷

NP significantly affects the quality of life in patients and is a focus for research in many medical institutions. Investigating the mechanisms by which early HBO treatment reduces NP could provide a theoretical basis for the application of HBO in NP treatment, and thus it is of important clinical significance.

Materials and methods

Experimental animals

Clean degree of healthy male Sprague-Dawley rats (weighted 280.12 ± 5.37 g) were provided by China Medical University Shengjing Hospital' Animal Center. Rats were fed separately and given free access to water

and food in a room with 23°C to 25°C temperature, 40% to 60% humidity, and natural lighting. A total of 144 rats were numbered and randomly assigned into three groups (n = 48 in each group): a sham group, model group (CCI group), and HBO-treated group (HBO group). In addition, the three groups were further divided into four subgroups based on the days post-surgery (0, 3, 7, and 14 days). This study was conducted in accordance with the Guidelines for laboratory animal care and use by the Animal Welfare Act and the National Institutes of Health. The Ethics Committee of China Medical University Shengjing Hospital (2016PS063K) has approved animal experiments which are used in this study.

Establishing the CCI rat model

The CCI model was built in reference to the methods proposed by Bennett and Xie.¹⁸ In detail, the rats in three groups were anesthetized (10% chloral hydrate, 0.3 g/kg) by an abdominal injection. Then, an incision of 1 cm long was made at the posterolateral side of the left hind limb; the sciatic nerve was found behind the femur. Next, four 4-0 chromic ligatures spaced about 1 mm apart were loosely tied to compress the epineurium, and the muscles innervated by the sciatic nerve showed transient contractions. Afterwards, the skin was sutured and the rats were returned to their cages after waking up. For the sham group, the same steps were taken, but only sciatic nerve isolation was performed instead of ligation.

Application of hyperbaric oxygenation

In order to reduce the accumulation of steam or CO₂, fresh and dry soda lime was put at the bottom of the HBO chamber. Pure oxygen was used to ventilate the HBO chamber (DS400-IV, Weifang Huaxin Oxygen Industry Co., Ltd., Shandong, China) for 10 min. Rats of the HBO group were put into the HBO chamber on the first day after surgery, and the treatment lasted consecutively for five days, with an initial pressure increase at 0.125 ATA/min until 2.5 ATA, a maintenance at this level for 60 min, and a gradual pressure decrease for 20 min until normal pressure was achieved. The behaviors of the HBO rats in the chamber were observed carefully. As for the sham and the CCI groups, rats were also put into their chambers simulated with the same conditions except for the HBO treatment.

Observation and measurement of pain behaviors

After surgery, all rats were fed separately. Gait, autophagic behavior, hind limb bearing, body weight change, and pain behaviors at different time points were observed carefully.

The number of spontaneous paw withdrawals: The rats were put into a transparent glass box and allowed free walking. The number of spontaneous paw withdrawals of the left hind limb within 5 min was counted.

Measurement of mechanical withdrawal threshold (MWT): By using the Von Frey filaments (Stoeling, Chicago, IL) and the “up and down” method, MWT was measured. The test was performed between 10 am and 12 am every day. First, the rats were put on a test rack covered with a glass lid and were allowed adaptation for 15 min. After the rats stopped grooming or exploring, a scaled Von Frey filament was used to stimulate the paws for 3 s, with a force gradually increasing from 0.14 to 15 g until the filament bent. Each test was performed with an interval of 15 s. During the stimulation, the minimal force of Von Frey filaments causing rats to raise their paws for more than five times was regarded as MWT; “painless” was determined if the force was increased to 15 g and rats still did not show paw withdrawal. Paw withdrawals pulled out by body’s own movement was not recorded.

Measurement of thermal withdrawal latency (TWL): By using a thermal pain stimulator (Youer Equipment Scientific Co., Ltd., Shanghai, China), TWL was measured. The rats were put on the rack and allowed adaptation for 15 min. Then, the light was pointed to rats’ left hind paw and the timer was turned on. The timer was turned off once the rats raised their feet, and the test was performed three times, with an interval of 10 min for each time, and the mean value was used in statistical analysis. To avoid scalding, the upper limit of the withdrawal latency was set to 30 s. If rats still did not show paw withdrawal after 30 s, the stimulation was suspended automatically. The time record was 30 s.

Sampling

After testing of the pain threshold, the rats were anesthetized (10% chloral hydrate, 0.3 g/kg) and killed. On sterilized ice, the spinal cord segments (L4–L6) were sampled. The dorsal horn was quickly cut and put into liquid nitrogen. For each subgroup, six rats were sacrificed for sampling, respectively, on 0, 3, 7, and 14 days after surgery for Western blot and real-time PCR (RT-PCR).

Immunohistochemistry

At predetermined time points, the animals ($n = 3$ in each subgroup, five sections in each animal) were anesthetized (10% chloral hydrate, 0.3 g/kg) with an abdominal injection and perfused by 100 mL of saline to the ascending aorta, followed by perfusion of 4% paraformaldehyde (200 mL, pH 7.4). Then, spinal cord segments (L4–L6) were sampled and fixed in paraformaldehyde at 4°C

overnight, and added with 30% sucrose solution on the next day and placed at 4°C overnight until the tissues all sunk to the bottom of the tube. On the next day, a Leica microtome (Microm HM525; Thermo Fisher Scientific, Walldorf, Germany) was used to slice the frozen-section tissues into a thickness of 20 μm , and at room temperature, the sections were fully dried. Then, sections were washed with 0.01 M PBS (three times), 5 min each, treated by 3% H_2O_2 for 10 min in order to block the activity of endogenous peroxidase, washed again with PBS (three times), and incubated with 5% goat serum for 15 min to block unspecific sites. Then, the sections were added with 0.3% Triton-X-100 for 20 min, washed with 0.01 M PBS (three times), incubated with glial fibrillary acidic protein (GFAP) primary antibody (1:800, Wuhan Boster Biological Technology Ltd., Wuhan, China) in a wet box overnight (4°C), washed by PBS again, and incubated with goat-anti-rabbit IgG secondary antibody (ZSGB-BIO, ORIGENE, Beijing, China) in a wet box at room temperature (20 min). Following that, the sections were washed with PBS (three times) and developed using the Vectastain Elite ABC Kit (Vector Laboratories Co., Ltd., USA). After washing three times with PBS, the DAB solution (Sigma-Aldrich Co., Ltd., USA) was added to terminate the reaction and regulate development under a microscope. Sections were rinsed under water for 15 min, restained by hematoxylin and eosin for 1 to 2 min, dehydrated by serially diluted ethanol, transparent by xylene, and finally mounted in neutral resin. As for the negative control, PBS was used to substitute primary antibody. A confocal microscope (Nikon, Japan) was used to count the number of astrocytes in the dorsal horns; the Meta Morph software was used for image analysis and the results were presented as gray value.

Electron microscope observation

The animals ($n = 3$ in each subgroup) were sacrificed for sampling at corresponding time points. The dorsal horns were cut into cubes with a size of $0.2 \times 0.2 \times 0.2$ cm, then fixed by 2.5% glutaraldehyde and washed by PBS, further fixed by 1% osmic acid and dehydrated in serially diluted acetone, embedded in EPON812 and sectioned to a thickness of 60 to 70 nm using a microtome, double-stained by uranyl acetate and lead citrate, and finally observed and photographed under a transmission electron microscope. Each rat spinal cord made 10 consecutive slices and identified layer II. Beginning from the inside of plate II then gradually moving to the outside, each copper mesh shot two photos. Each rat was taken 10 pictures ($n = 30$ in all). The ultrastructural injury visual score standards were as follows: “0” normal; “1” slight change; “2” mild change; “3” moderate change;

and “4” significant change. The observer was blinded to the experimental groups.

Western blot

Cryopreserved (-80°C) spinal tissues and cell lysate were homogenized on ice quickly. Then, the homogenates were centrifuged at 12,000 r/min and 4°C for 10 min and the supernatant was aspirated for protein quantification using the BAC method. Afterwards, protein samples were loaded at an amount of 5 μg , electrophoresed through a 10% SDS-PAGE gel and transferred to a PVDF membrane (Amersham Bio-sciences, Freiburg, Germany). The membrane was then blocked by 5% skimmed milk (dissolved in TBST) and incubated with primary antibodies diluted by TBST containing 5% skimmed milk at 4°C overnight. The primary antibodies included nNOS primary antibody (1: 500, Epitomics, Burlingame, CA), iNOS primary antibody (1: 500, Millipore Corp, Billerica, MA), and GAPDH primary antibody (internal reference, 1: 1000, Sigma Chemical Co, St. Louis, MO). On the next day, the membrane was washed by TBST and incubated with HRP-labeled secondary antibody (1: 2000, diluted by TBST containing 5% skimmed milk, ZSGB-BIO, ORIGENE, Beijing, China) at room temperature for 2 h. Afterwards, the membrane was developed using the HRP-ECL assay, and the light density of bands was analyzed using the Gel-Pro analysis system.

Real-time PCR

Total RNA extraction was performed according to the instructions provided with the Trizol reagent (Invitrogen, Carlsbad, CA). The concentration and quantity of RNA were measured using a spectrophotometer, and the RNA were reversely transcribed into cDNA. The primer sequences for interleukin-1 beta (IL-1 β) were as follows: upstream 5'-TGTGATGTTC CCATTAGAC-3' downstream 5'-AATACCACTTG TTGGCTTA-3'. Tumor necrosis factor-alpha (TNF- α): upstream 5'-CCACGCTCTTCTGTCTACTG-3'; downstream 5'-GCTACGGGCTTGTCCTC-3'. Beta-actin (β -actin): upstream: 5'-TGGCACCCAGCACAA TGAA-3'; and downstream: 5'-CTAAGTCATA GTCCGCCTAGAAGCA-3'. The Light Cycler RT-PCR Amplifier (Takara, Kyoto, Japan) was used to amplify IL-1 β , TNF- α , and β -actin, and the common amplification conditions were set as: 95°C 30 s, 95°C 10 s, 60°C 15 s, 72°C 10 s, 45 cycles, and melting curve analysis was performed after amplification.

Statistical analysis. Data analysis was performed using the SPSS 16.0 software. Metric data were first tested for normality. Data with a normal distribution, mean

\pm standard deviation, are presented. Data with nonparametrical distribution, the median and quarterback spacing, are presented. Data of MWT and TWL were analyzed using repeated measures analysis of variance (ANOVA). Equality of variance was tested using Mauchly's test of sphericity. Then, pairwise comparisons at predetermined time, multivariate ANOVA was used in the general linear model, followed by Scheffe post hoc tests. One-way ANOVA was used in data of RT-PCR and Western blot, followed by the Scheffe test for multiple comparisons. P values <0.05 were defined significant.

Results

Observation of pain behaviors

After CCI surgery, the rats showed claudication in the limbs that received the surgery, the paws could not be placed on the ground, the legs were bent, and rats tended to lick the limb with pain. Later on, the limbs with surgery showed continuous paw contracture and joint stiffness. After early HBO treatment with 2.5ATA, the paws could be placed on ground, the toes were separate, the injured limbs could bear weight, and claudication basically disappeared. From 3 days after surgery, decreased mechanical withdrawal threshold (P value <0.05) was observed in the CCI group, reaching to the bottom by 7 days, and still maintained at a relatively low level until 14 days after treatment (Figure 1(a)). Similar variations were also observed for the thermal withdrawal latency (Figure 1(b)), and the shortening of TWL was even prior to the decreasing of MWT. However, after early HBO treatment with 2.5ATA, rats in the HBO group had significantly increased MWT and prolonged TWL compared to the CCI group (P value <0.05).

Immunohistochemical staining

Immunohistochemistry showed that at 3 days after surgery, large amounts of GFAP-positive astrocytes could be seen in CCI rats' dorsal horns; the staining intensity reached the peak 7 days after surgery, and still maintained at a high level by 14 days after surgery. However, after HBO treatment, HBO rats showed significantly decreased number of activated astrocytes in the surgical side (Figure 2). The integral optical density of GFAP in the CCI rats' dorsal horns was significantly higher than that in HBO rats at all time points (P value <0.05).

Electron microscope observation

The presynaptic components of the Sham group, such as the mitochondria and endoplasmic reticulum, were

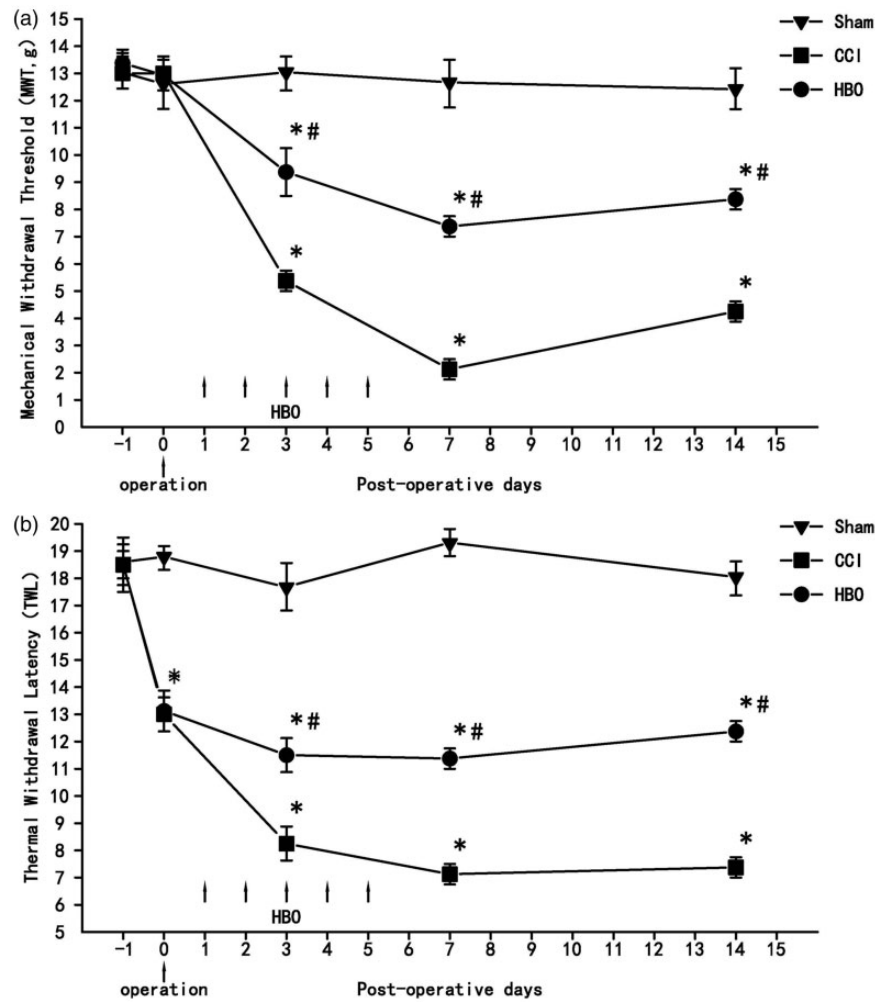


Figure 1. Early stage HBO treatment alleviates neuropathic pain. (a) Changes in MWT in three groups. (b) Changes in TWL in three groups. HBO was treated once daily (5 days after CCI), as indicated by arrows. * P value < 0.05 versus sham; # P value < 0.05 vs. CCI. MWT, mechanical withdrawal threshold; TWL, thermal withdrawal latency; CCI, chronic constriction injury.

almost normal or had very few vacuoles. The sham group showed clear synaptic structures, with obviously visible presynaptic and postsynaptic membranes, as well as the synaptic space. Many synaptic vesicles could be seen in the presynaptic membrane, and the distribution of postsynaptic density was even observed. However, in the CCI group, presynaptic components appeared with mitochondrial crests loss, endoplasmic reticulum swelling and expansion, and the formation of vacuoles. The synaptic structure was disordered, the presynaptic and postsynaptic membranes were mostly blurred or fused, the amount of postsynaptic density was increased, and the thickness was uneven. The ultrastructural injury visual score was highest on the seventh day. Compared with sham group, the ultrastructural injury visual score was significant (P value < 0.05). As for the HBO group, the losing of mitochondrial crests and swelling of the endoplasmic reticulum were reduced, compared with the CCI group. Though some of the membranes were

still fused, the synaptic space could be seen clearly. Some synapses were recovered to normal, with obviously visible presynaptic and postsynaptic membranes and synaptic spaces. Postsynaptic density was relatively even, and synaptic vesicles could be viewed in the presynaptic membrane (Figure 3). The ultrastructural injury visual score at each time point was significantly lower than CCI group (P value < 0.05).

Western blot

iNOS and nNOS expression in the dorsal horns of CCI and HBO rats were detected using Western blot. After CCI surgery, iNOS and nNOS expression both increased, reaching the peak at 7 days postsurgery, and declined slightly, but still higher than the sham group, at 14 days after surgery (Figures 4(a) and 5(a)). The variation patterns of iNOS and nNOS expression were consistent with the change of MWT and TWL. After HBO

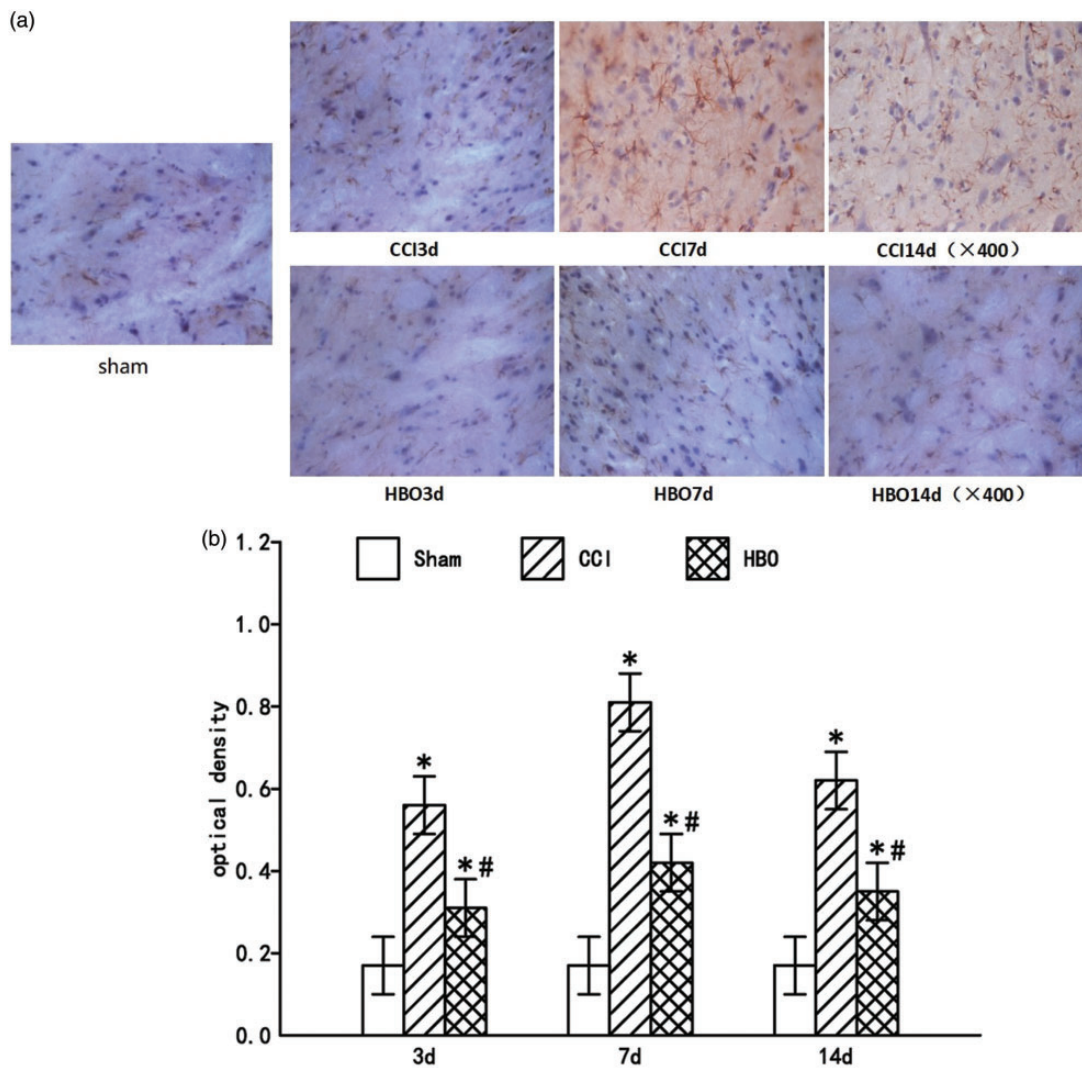


Figure 2. Immunohistochemical micrographs and image analysis showing astrocytes in the spinal dorsal horn in the sham, CCI (3, 7, and 14 days) and HBO (3, 7, and 14 days) groups. (a) Immunohistochemical micrographs; (b) Image analysis. GFAP, glial fibrillary acidic protein. Little GFAP positive astrocytes were viewed in sham operation group. Fewer GFAP positive astrocytes were viewed in the HBO group than CCI group. * P value <0.05 versus sham; # P value <0.05 versus CCI.

treatment, iNOS and nNOS expression was still significant (Figures 4(b) and 5(b)), but was decreased when compared with CCI group.

Quantitative RT-PCR

Inflammatory markers TNF- α and IL-1 β mRNA expression in the sham, CCI, and HBO groups were detected by RT-PCR. The results showed that the expression of TNF- α mRNA in the CCI group increased significantly since 3 days after surgery (P value <0.05), reached the peak at 7 days, and remained high at 14 days despite a slight decrease (P value <0.05) (Figure 6(a)). As for IL-1 β , it also showed an increasing trend (P value <0.05) similar to the expression of TNF- α (Figure 6(b)). Compared to the CCI group, the expression of both

inflammatory factors in the HBO group decreased: the difference was only significant at 7 days (P value <0.05) but insignificant at 3 days and 14 days (P value >0.05) for TNF- α , yet the difference was significant at all time points for IL-1 β (P value <0.05).

Discussion

NP is a universal chronic pain with multiple causes and complex pathophysiological mechanisms. The disease can cause severe symptoms and the lack of effective treatment has caught the attention of researchers to develop novel approaches for the management of this condition. An understanding of the mechanisms of NP and developing a multitargeted therapeutic approach is therefore of great clinical significance.

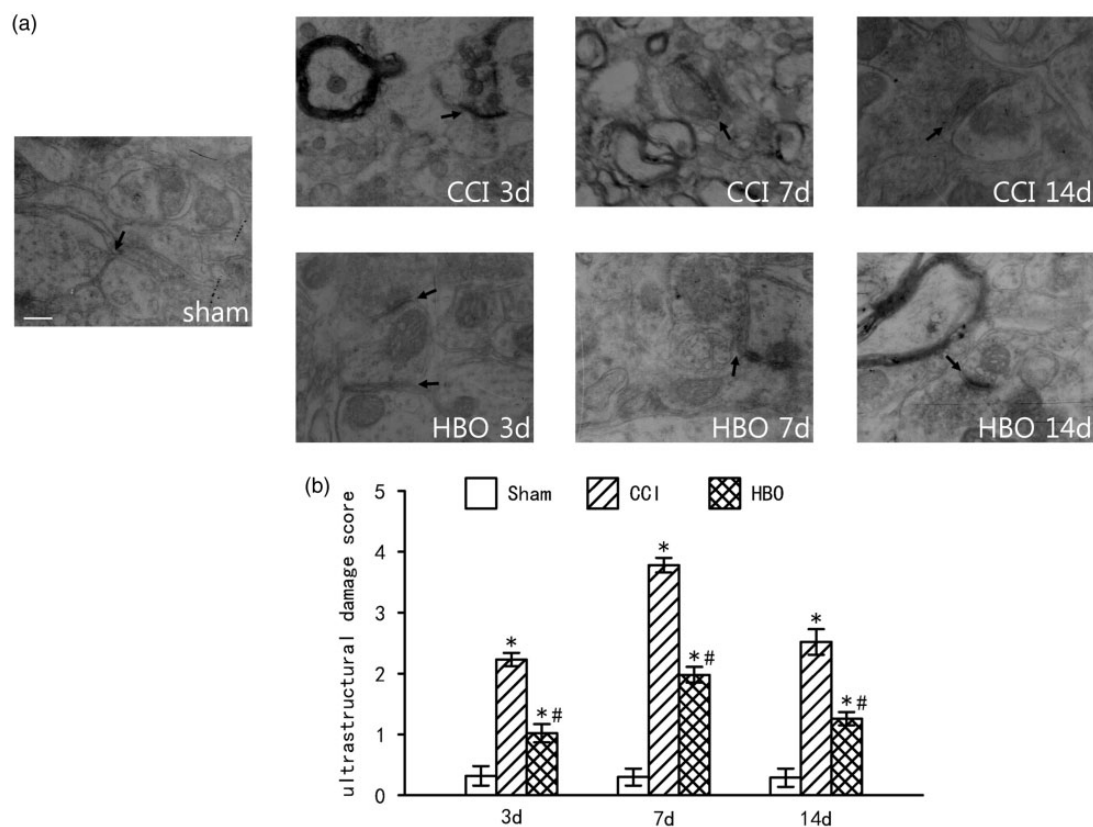


Figure 3. Ultrastructure of synapse in rat spinal cord in three groups (3, 7, and 14 days). (a) Electron micrographs; (b) Image analysis. Scale bars = 0.5 μ m, Magnification: 5000 \times .

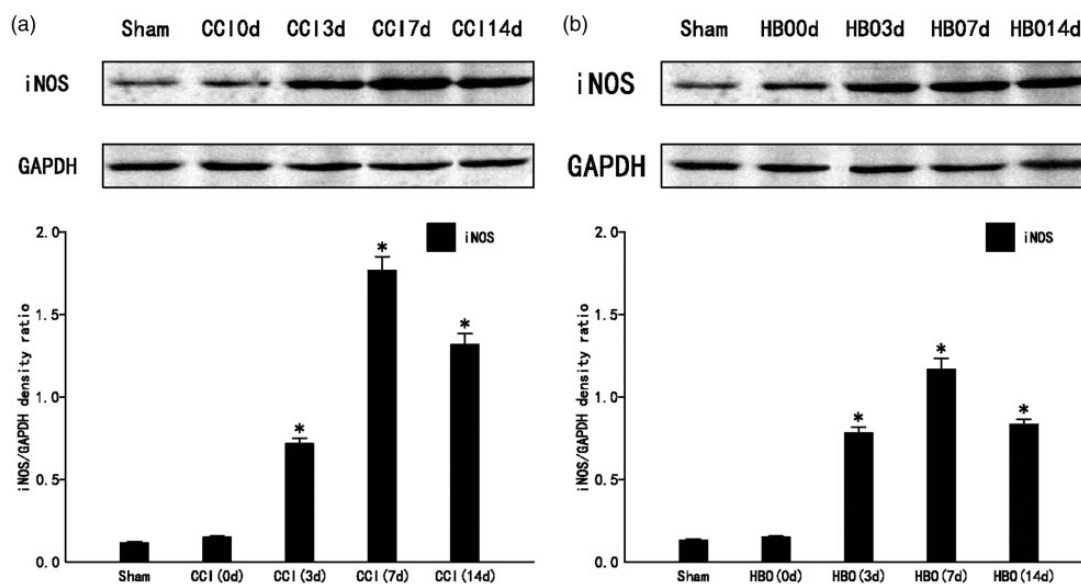


Figure 4. iNOS protein expression in the spinal dorsal horn in the sham, CCI (0, 3, 7, and 14 days), and HBO (0, 3, 7, and 14 days) groups. Western blot analysis of iNOS. (a) iNOS and GAPDH bands in sham and CCI (0, 3, 7, and 14 days) groups and statistical result of Western blot. (b) iNOS and GAPDH bands in sham and HBO (0, 3, 7, and 14 days) groups and statistical result of Western blot. Data are expressed as mean \pm S.E.M. **P* value < 0.05 versus sham.

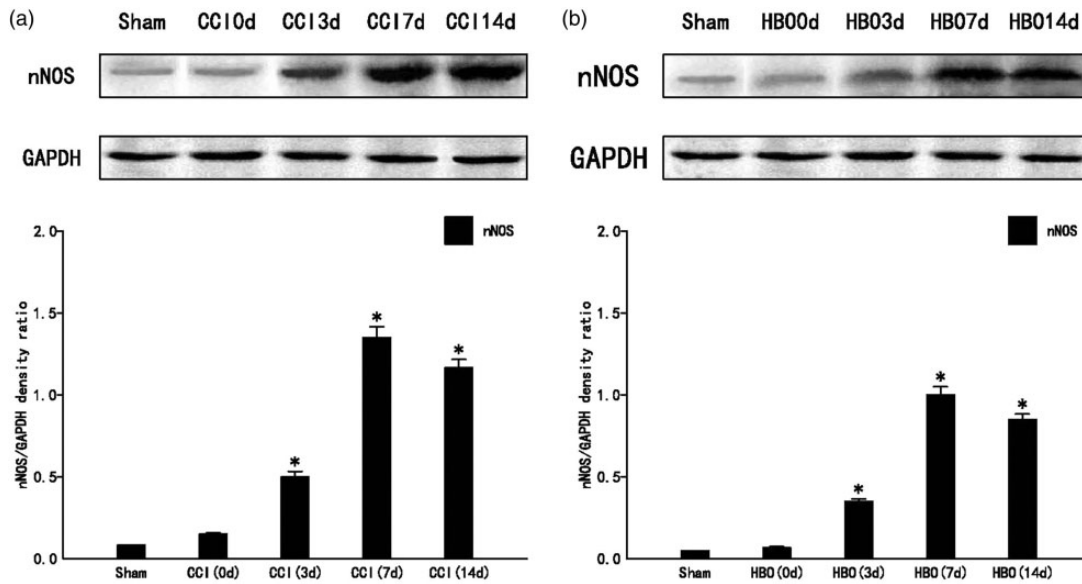


Figure 5. nNOS protein expression in the spinal dorsal horn in the sham, CCI (0, 3, 7, and 14 days), and HBO (0, 3, 7, and 14 days) groups. Western blot analysis of nNOS. (a) nNOS and GAPDH bands in sham and CCI (0, 3, 7, and 14 days) groups and statistical result of Western blot. (b) nNOS and GAPDH bands in sham and HBO (0, 3, 7, and 14 days) groups and statistical result of Western blot. Data are expressed as mean \pm S.E.M. **P* value <0.05 versus sham.

HBO has been widely used in clinics due to its economical, convenient, and reusable properties, and it has also been preliminarily applied in pain treatment.¹⁹ The CCI model is commonly used in pain studies. This model has a high repeatability, and the early shortening of thermal latency as well as the decrease of mechanical pain threshold is similar to the clinical features of NP in terms of peripheral and central pain sensitization. In this study, rats receiving early HBO treatment (2.5ATA) showed separate toes, their paws could be placed on ground, limbs could bear weight, gait recovered to normal, spontaneous pain was reduced significantly, the MWT was increased, and the TWL was prolonged. These results showed that early HBO treatment at 2.5ATA could have the potential to attenuate NP effectively.

Previous studies on pain have mainly focused on neurons, but recently, glial cells receive more attention, which have the widest distribution and the largest number in the nervous system. Glial cells, particularly astrocytes, play important roles in the generation and transmission of pain signals.²⁰ Astrocytes could produce neurotrophic factors (such as brain-derived neurotrophic factor), cytokines (glutamic acid, substance P, IL-1, IL-6, nerve growth factor, etc.), and neurotransmitters, thereby participating in the transduction and regulation of pain signals.²¹ As a specific marker for astrocytes, GFAP is often used for the detection of astrocytes. During the development of NP, activated astrocytes could release increased amount of GFAP, which might be associated with hyperalgesia and

allodynia,²² and also the maintenance of chronic pain. In this study, the activation of astrocytes occurred in the early stage of CCI, reached the peak until 7 days, and maintained to 14 days after surgery. This was consistent with the generation of pain, indicating that the formation of hyperalgesia is closely related to the activation of astrocytes. However, early HBO treatment inhibited astrocytes activation and blocked transduction of pain signals, thus producing an analgesic effect.

NP is also associated with anatomical remodeling. Astrocytes could regulate the maturation of neurons and synapses, and thus participate in the modulation of neural plasticity,²³ possibly by forming a trimer with microglia, presynaptic neuron, and postsynaptic neurons.²⁴ HBO could maintain cell membrane stability, decrease membrane damage, and alleviate local swelling. Also, HBO might reduce abnormal synaptic connections between the A β endings and the dorsal horn lamina II neurons, and thus prevent abnormal neural activation, maintain transmission and integration of sensory information, and attenuate hyperalgesia. Therefore, by changing cell ultrastructure, HBO could reduce the formation of abnormal synapses, decrease neuronal damage, and further alleviate hyperalgesia.

The mechanism of NOS in NP is somehow complicated. For one, it is believed that NO is synthesized by activated NOS that could penetrate cell membrane and diffuse to the presynaptic membrane, which further promotes the release of glutamate and aggravates hyperalgesia;²⁵ on the other hand, NO and cyclic guanosine monophosphate (cGMP) could activate target factors

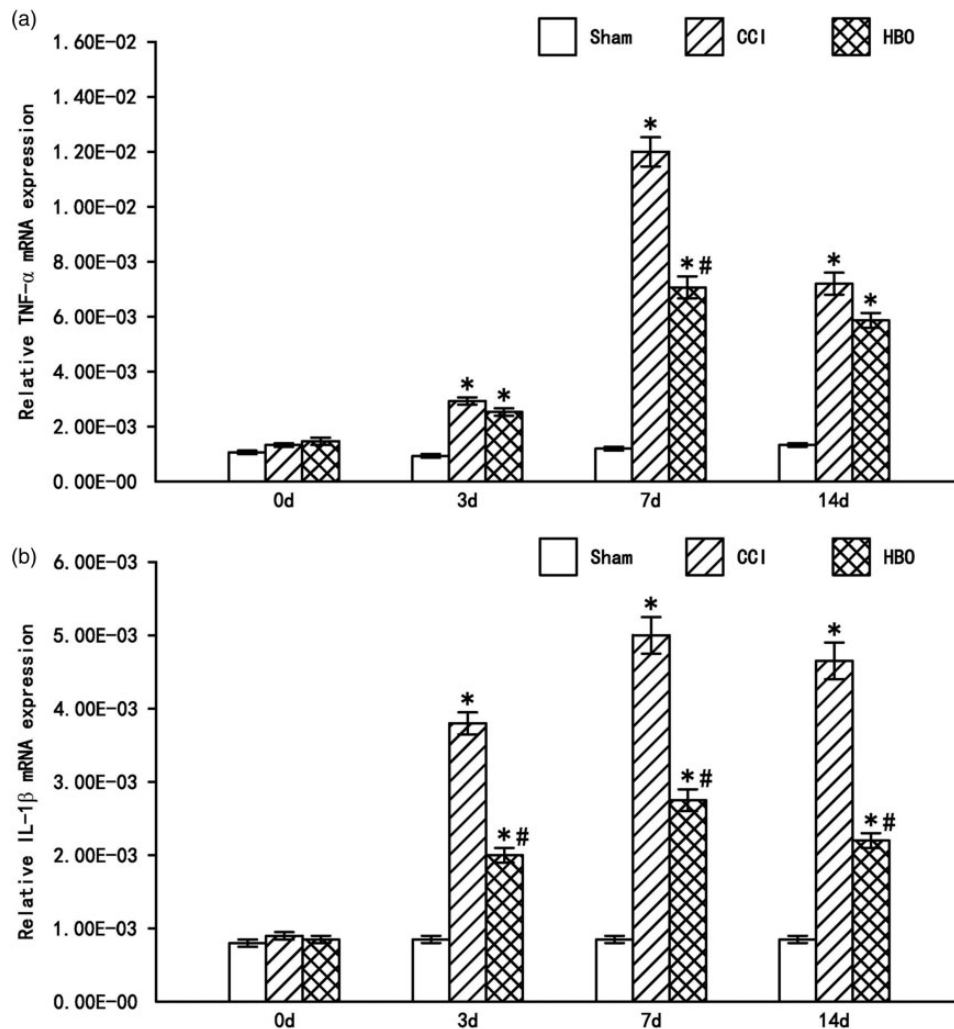


Figure 6. HBO attenuates TNF- α and IL-1 β expression in the spinal dorsal horn. TNF- α and IL-1 β analysis using real-time RT-PCR. (a) TNF- α mRNA relative expression (TNF- α / β -actin); (b) IL-1 β mRNA relative expression (IL-1 β / β -actin). Data are expressed as mean \pm S.E.M. * P value <0.05 versus sham; # P value <0.05 versus CCI.

and exert an analgesic effect by activating the NO-cGMP-protein kinase G signaling pathway, which opens the ATP-dependent potassium channels and induces an anti-injury effect.²⁶ NP increased protein expression of iNOS and nNOS in the dorsal horn, whereas HBO treatment decreased their expression, and this might further inhibit the activation of astrocytes and exert repairing and protective effects on damaged neural tissues.

The root cause of NP is nerve damage, and the following inflammatory reactions are believed to be critical to NP.²⁷ Since the mast cells, macrophages, T cells, and other inflammatory cells are activated; the produce of inflammatory mediators (histamine, IL-1 β , TNF- α , and nerve growth factors) would aggravate nerve damage and cause hyperalgesia. When peripheral nerve damage is experienced, the immediate rise of TNF- α would trigger a cascade of inflammation reactions,²⁸ and the

expression of IL-1 β mRNA and protein also showed significant increase in the spinal tissues. However, intrathecal application of TNF- α and IL-1 β antagonists could inhibit neuropathic hyperalgesia in a dose-dependent manner, indicating that NP might be associated with TNF- α and IL-1 β activation.^{29,30} After NP, glial cells are activated, and the release of various inflammatory cytokines (TNF- α , IL-1 β , IL-6, etc.) and pain transmitters (excitatory amino acid, NO, prostaglandin, etc.) will be increased.³¹ HBO could suppress inflammation and reduce pain, by inhibiting the function of T lymphocytes and macrophages and down-regulating the release of inflammatory cytokines,³² thus to alleviate inflammation under pathological conditions. Also, HBO could inhibit the activation of glial cells,^{33,34} which further decreases the release of cytokines, causing a more significant drop in the level of IL-1 β . Additionally, by regulating the level of NOS, HBO could further inhibit

the activation of glial cells and reduce the production of cytokines, NO, and inflammatory factors (TNF- α and IL-1 β), thus to achieve the goal of attenuating NP.

Conclusion

After HBO processing, iNOS and nNOS expression was decreased at spinal level, the activation of astrocytes was inhibited and normal synaptic ultrastructure was maintained, the release of inflammatory factors (TNF- α , IL-1 β) was decreased, and hyperalgesia was thereby improved. This study suggests that decreased expression of iNOS and nNOS and reduced level of inflammatory factors are important mechanisms by which early HBO treatment helps to alleviate NP.

Authors' Contributions

YYD and PY participated in the whole design of this study. YYD drafted the manuscript and carried out the molecular genetic studies and western blot. TH and ZKH prepared the animal model. YQZ carried out the behavioral test. HXL carried out the Immunohistochemistry. PY and GZ helped to draft the manuscript and statistical analysis. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act. Animal experiments used in this study have been approved by the Ethics Committee of China Medical University Shengjing Hospital (2016PS063K).

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