Effects of hyperbaric oxygen on pain-related behaviours and nitric oxide synthase expression in a rat model of neuropathic pain

Guang Han MD, Lu Li MD, Ling-xin Meng PhD

BACKGROUND: Neuropathic pain is complex, and a satisfactory therapeutic method of treatment has yet to be developed; therefore, finding a new and effective therapeutic method is an important issue in the field of neuropathic pain.

OBJECTIVE: To determine the effects of hyperbaric oxygen (HBO) on pain-related behaviours and nitric oxide synthase (NOS) expression in a rat model of neuropathic pain.

METHODS: Forty male Sprague Dawley rats were randomly divided into five groups (eight rats per group) including control, sham operation, sciatic nerve with chronic constriction injury (CCI), HBO pretreatment (pre-HBO) and HBO post-treatment (post-HBO) groups. Pain-related behaviours and NOS expression in the spinal cord were compared among the five groups.

RESULTS: Compared with the CCI group, the mechanical withdrawal threshold was significantly increased and thermal withdrawal latency was significantly extended in the pre-HBO and post-HBO groups (all P<0.05). After CCI, expression of spinal neuronal NOS and inducible NOS were significantly decreased in the pre-HBO and post-HBO groups compared with the CCI group (all P<0.05). Spinal eNOS expression changed very little.

DISCUSSION: HBO has been used as an effective and noninvasive method for the treatment of spinal cord injuries and high-altitude sickness, and in immunosuppression and stem-cell research; however, it has yet to be applied to the treatment of neuropathic pain. The present study indicated that HBO effectively increased mechanical withdrawal threshold and thermal withdrawal latency, demonstrating that HBO has therapeutic effects on neuropathic pain.

CONCLUSION: HBO inhibits pain in rats with CCI through the regulation of spinal NOS expression.

Key Words: Hyperbaric oxygen; Neuropathic pain; Nitric oxide synthase

Neuropathic pain is caused by central or peripheral nerve injury, and represents a heavy burden to both society and family. Neuropathic pain is a complex process involving multiple factors. The basis of neuropathic pain is central nervous system hyperalgesia. Peripheral nerve injury induces a variety of immune-inflammatory factors, such as interleukin (IL)-1β, tumour necrosis factor-alpha (TNF-α) and IL-6, resulting in hyperalgesia through the activation of various intracellular signalling molecules (1,2). A satisfactory therapeutic method to treat neuropathic pain has not been developed due to the complexity of the disease. Therefore, finding a new and effective therapeutic method is an important issue in the field of neuropathic pain. Hyperbaric oxygenation (HBO) can enhance antioxidant activity, accelerate the clearance of free radicals, increase blood oxygen content, improve microcirculation and repair injured nerve tissue. In recent years, HBO has been used in the treatment of fibromyalgia, complex regional pain syndrome, spasmodic headache and postradiotherapy pain (3,4). However, little research has been performed on the effects of HBO on neuropathic pain. The purpose of the present study was to observe the effects of HBO on pain-related behaviours and nitric oxide synthase (NOS) expression in a rat model of neuropathic pain, and to identify its efficacy and mechanism of action in the treatment of neuropathic pain.

METHODS

All study methods were approved by the Ethics Committee of the Shengjing Hospital of China Medical University, Shenyang, China.

Experimental animals
Forty male Sprague Dawley rats, weighing between 280 g and 350 g, were provided by the Laboratory Animal Center of the Shengjing Hospital of China Medical University, and were randomly divided into five groups (eight rats per group), including: a control group (group C) and HBO pretreatment groups (pre-HBO and post-HBO) and HBO post-treatment groups. Pain-related behaviours and nitric oxide synthase expression in the spinal cord were compared among the five groups.

RESULTS:
1. Compared with the CCI group, the mechanical withdrawal threshold was significantly increased and thermal withdrawal latency was significantly extended in the pre-HBO and post-HBO groups (all P<0.05). After CCI, expression of spinal neuronal NOS and inducible NOS were significantly decreased in the pre-HBO and post-HBO groups compared with the CCI group (all P<0.05). Spinal eNOS expression changed very little.

2. DISCUSSION: HBO has been used as an effective and noninvasive method for the treatment of spinal cord injuries and high-altitude sickness, and in immunosuppression and stem-cell research; however, it has yet to be applied to the treatment of neuropathic pain. The present study indicated that HBO effectively increased mechanical withdrawal threshold and thermal withdrawal latency, demonstrating that HBO has therapeutic effects on neuropathic pain.

3. CONCLUSION: HBO inhibits pain in rats with CCI through the regulation of spinal NOS expression.

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Les effets de l’oxygène hyperbare sur les comportements liés à la douleur et à l’oxyde nitrique synthétisée dans un modèle de douleur neuropathique chez le rat

HISTORIQUE: La douleur neuropathique est complexe, et aucune méthode thérapeutique satisfaisante n’a encore été élaborée. La découverte d’une nouvelle méthode thérapeutique efficace constitue un enjeu important dans le domaine de la douleur neuropathique.

OBJECTIF: Déterminer les effets de l’oxygène hyperbare (OHB) sur les comportements liés à la douleur et l’expression de l’oxyde nitrique synthétisée (ONS) dans un modèle de douleur neuropathique chez le rat.

MÉTHODOLOGIE: Quarante rats Sprague Dawley mâles ont été répartis au hasard entre cinq groupes (huit rats par groupe), soit un groupe témoin, un groupe d’opération factice, un groupe de lésion de constriction chronique (LCC) du nerf sciatique, un prétraitement à l’OHB (pré-OHB) et un posttraitement à l’OHB (post-OHB). Dans les cinq groupes, les chercheurs ont comparé les comportements liés à la douleur et l’expression de l’ONS dans la moelle épinière.

RÉSULTATS: Par rapport au groupe de LCC, le seuil de retrait mécanique était considérablement plus élevé et la latence de retrait thermique, considérablement rallongée dans les groupes de pré-OHB et de post-OHB (tous P<0,05). Après la LCC, l’expression de l’ONS neuronale spinale et de l’ONS inductible augmentait. Cette expression était considérablement moins élevée dans les groupes pré-OHB et post-OHB que dans les groupes de LCC (tous P<0,05). L’expression de l’ONS spinale changeait très peu.

EXPOSÉ: L’OHB est une méthode efficace et non effractive pour traiter des lésions de la moelle épinière et du mal de l’altitude et pour effectuer des recherches sur l’immunosuppression et les cellules souches. Il n’a toutefois pas encore été utilisé pour le traitement de la douleur neuropathique. D’après la présente étude, l’OHB augmentait avec efficacité le seuil de retrait mécanique et la latence de retrait thermique, ce qui démontre les effets thérapeutiques de l’OHB sur la douleur neuropathique.

CONCLUSION: L’OHB inhibe la douleur chez des rats ayant une LCC grâce à la régulation de l’expression de l’ONS spinale.
a sham operation group (group S), in which the sciatic nerve was exposed but not ligated and HBO was not performed; a sciatic nerve with CCI group (CCI group), in which the sciatic nerve was ligated but HBO was not performed; a HBO pretreatment group (pre-HBO group), in which HBO was initiated 12 h before CCI; and a HBO post-treatment group (post-HBO group), in which HBO was initiated 12 h after CCI.

**Experimental process**

Baseline mechanical withdrawal thresholds (MWTs) and thermal withdrawal latencies (TWLs) were measured in all rats one day before CCI. The rats in the pre-HBO group were exposed to HBO in the evening, and the operation (CCI or sham) was performed in all rats, except the rats in group C, the following morning. The rats in the post-HBO group were exposed to HBO in the evening following the operation. Pain-related behaviours were observed one, two, three, seven, 14, 21 and 28 days after CCI, starting 12 h after the HBO treatment of the post-HBO group (Figure 1).

**CCI model preparation**

After the rats had been anesthetized by peritoneal injection of 1% sodium pentobarbital (40 mg/kg), CCI models were prepared by a modification of the method described by Bennett and Xie (5). In detail, a posterosilateral incision on the right hindlimb was made and the sciatic nerve trunk was identified. The right sciatic nerve was loosely ligated with 4-0 silk thread to produce a slight pressure on the epineurium – namely, momentary muscle contraction in the sciatic nerve distribution region, followed by skin closure. After regaining consciousness, the rats were returned to cages. In group S, the sciatic nerve was exposed but was not ligated.

**HBO**

Rats in the pre-HBO or post-HBO groups were placed in HBO chambers 12 h before or after CCI, respectively. The chambers were infiltrated with pure oxygen for 10 min to allow the chamber oxygen concentration to exceed 90%, with the pressure at 0.25 MPa at a rate of 0.0125 MPa/min. The HBO treatment lasted 60 min under the condition of pure oxygen ventilation for 10 min. HBO was decreased to normal pressure within 30 min.

**Samples**

Rats were anesthetized by peritoneal injection of 1% sodium pentobarbital (40 mg/kg) and intubation from the left ventricle to the ascending aorta was performed, followed by perfusion with a 0.9% saline solution until no red perfusate effused. After 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde was perfused, the L4–L6 spinal cord segments were removed and fixed with 4% paraformaldehyde for future analyses.

**Observational items**

Pain-related behaviours were observed one, two, three, seven, 14, 21 and 28 days after CCI between 09:00 and 10:00 on each day (1). Rats’ gait, right hindlimb posture, autotomy and the bearing weight of the hindlimb were observed (2). MWT was tested using von Frey filaments, according to the method described by Dixon (6). The rat was placed in the test box and allowed to acclimatize for 30 min. Each von Frey filament was applied to the planter surface of the hind paw for 6 s to 8 s to observe the hind paw withdrawal response (3). TWL was assessed to quantitatively determine rats’ thermal sensitivity. Rats were placed on the glass surface of a thermal testing apparatus. The rats were allowed to acclimatize for 30 min before testing. A mobile radiant heat source located under the glass was focused onto the hindpaw of each rat. The paw TWL was recorded by a timer three times and the mean of these three trials was used in the present study. A cut-off time of 20 s was used to prevent potential tissue damage.

Fresh samples were fixed with 4% paraformaldehyde for 4 h, dehydrated according to standard protocols, embedded in paraffin and sectioned at a thickness of 40 μm. The slices were washed with phosphate-buffered saline (PBS) three times, and 3% H2O2 and 30% blocking serum were used to block endogenous peroxidase activity. NOS antibodies were added, respectively, and slices were incubated at 4°C for 48 h. The slices were washed with PBS three times, and biotinylated secondary antibody (goat anti-rabbit IgG) was added at 37°C for 1.5 h. The slices were washed with PBS three times, and horseradish peroxidase-labelled streptavidin was added at 37°C for 1.5 h. The slices were washed with PBS, visualized with diaminobenzidine, dehydrated in an alcohol gradient, were made transparent with xylene, and were sealed with gum followed by microscopic observation. Brown-coloured cells were considered to be positive. In negative control trials, primary or secondary antibodies were replaced with PBS and no positive neurons were present. In each spinal cord, a slice was selected from every five slices to count the number of nNOS-, eNOS- or iNOS-positive neurons. nNOS-positive neurons stained brown in the cytoplasm and were oval in shape, with a clear outline. A few nNOS-positive neurons were spindle- or cone-like in shape, and were of various sizes smaller than that of nNOS-positive glial cells. eNOS-positive neurons exhibited multiple neurites, which form a network around them.

Microscopy and image analysis were performed using the U-MCB Olympus system (Olympus Corporation, Japan). The analysis of slices was performed using the MetaMorph (Molecular Devices, USA)/Cool Snap (Roper Scientific, USA) +X/AX70 image analysis system. Four slices per group (post-HBO group), in which HBO was initiated 12 h after CCI.

**Statistical analysis**

Data analysis was performed using SPSS version 11.0 (IBM Corporation, USA). Data were expressed as mean ± SD. ANOVA and Student-Newman-Keuls q tests were used to compare MWT and TWL between groups. A paired t test was used to compare nNOS, eNOS and iNOS. Differences were considered to be statistically significant at P<0.05.

**RESULTS**

**General behaviours**

With the exception of the rats in groups C and S, the toes of the affected foot moved closer to one another with dorsiflexion, eversion and non-weight-bearing three days after CCI. Rats in the CCI group also exhibited limping, with the knee joint contacting the surface of the testing apparatus and the palm of the right foot occasionally suspended in midair. Fourteen days after CCI, muscle atrophy was apparent, and light stimulation to the sole of the affected foot caused the rats to place the affected limb into their mouth or swing the affected limb freely.
There were no significant differences in MWT among the groups before CCI. MWT was stable in group C, but was slightly decreased in group S without a statistically significant difference compared with group C (P>0.05). MWT was significantly lower in the pre-HBO, post-HBO and CCI groups compared with group S at each time point (P<0.05; Figure 2), and the reduction of MWT was highest in the CCI group. MWT was higher in the pre-HBO group compared with the CCI group at each time point (P<0.05), demonstrating that HBO has preventive effects on neuropathic pain. MWT was higher in the post-HBO group compared with the CCI group one day to 21 days after CCI (P<0.05), demonstrating that HBO had therapeutic effects on neuropathic pain. Based on the two latter findings, HBO pretreatment was more effective than HBO post-treatment.

Changes in TWL were essentially the same as those in MWT. There were no significant differences in TWL among the groups before CCI. TWL was stable in group C. TWL was significantly lower in the pre-HBO, post-HBO and CCI groups than in group S at each time point (P<0.05; Figure 2), and the reduction of TWL was the highest in the CCI group. TWL was higher in the post-HBO group compared with the CCI group one day to 21 days after CCI (P<0.05), demonstrating that HBO had therapeutic effects on neuropathic pain. Based on the two latter findings, HBO pretreatment was more effective than HBO post-treatment.

MWT
Twenty-eight days after CCI, the number of nNOS-positive neurons in the spinal cord was significantly increased in the CCI group and the pre-HBO and post-HBO groups compared with group S (P<0.05). The number of nNOS-positive neurons was significantly higher in the CCI group than in the pre-HBO and post-HBO groups (all P<0.05; Table 1 and Figure 3).

TABLE 1
Number of nNOS-positive cells in the spinal cord in each group 28 days after chronic constriction injury

<table>
<thead>
<tr>
<th>Group</th>
<th>nNOS-positive cells per 10⁴ cells</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.03±1.12</td>
</tr>
<tr>
<td>Sham operation</td>
<td>9.59±2.21</td>
</tr>
<tr>
<td>Chronic constriction injury</td>
<td>48.45±3.21*</td>
</tr>
<tr>
<td>Pre-HBO</td>
<td>28.86±3.53*</td>
</tr>
<tr>
<td>Post-HBO</td>
<td>32.84±2.68*</td>
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</tbody>
</table>

*P<0.05 when the pretreatment with hyperbaric oxygen (pre-HBO), post-treatment with hyperbaric oxygen (post-HBO) and chronic constriction injury groups are compared with the sham group; †P<0.05 when the pre-HBO and post-HBO groups are compared with the chronic constriction injury group. n=8 rats per group. nNOS Neuronal nitric oxide synthase

TABLE 2
Number of iNOS-positive cells in the spinal cord in each group 28 days after chronic constriction injury

<table>
<thead>
<tr>
<th>Group</th>
<th>iNOS-positive cells per 10⁴ cells</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.58±1.21</td>
</tr>
<tr>
<td>Sham operation</td>
<td>11.52±3.27</td>
</tr>
<tr>
<td>Chronic constriction injury</td>
<td>42.45±4.23*</td>
</tr>
<tr>
<td>Pre-HBO</td>
<td>28.62±4.15*</td>
</tr>
<tr>
<td>Post-HBO</td>
<td>30.84±3.82*</td>
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</tbody>
</table>

*P<0.05 when the pretreatment with hyperbaric oxygen (pre-HBO), post-treatment with hyperbaric oxygen (post-HBO) and chronic constriction injury groups are compared with group S; †P<0.05 when the pre-HBO and post-HBO groups are compared with the chronic constriction injury group. n=8 rats per group. iNOS Inducible nitric oxide synthase

nNOS
Twenty-eight days after CCI, the number of nNOS-positive neurons in the spinal cord was significantly increased in the CCI group and the pre-HBO and post-HBO groups compared with group S (P<0.05). The number of nNOS-positive neurons was significantly higher in the CCI group than in the pre-HBO and post-HBO groups (all P<0.05; Table 1 and Figure 3).

TABLE 3
Number of eNOS-positive cells in the spinal cord in each group 28 days after chronic constriction injury

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<th>eNOS-positive cells per 10⁴ cells</th>
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<tr>
<td>Control</td>
<td></td>
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<tr>
<td>Sham operation</td>
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<tr>
<td>Chronic constriction injury</td>
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<tr>
<td>Pre-HBO</td>
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<td>Post-HBO</td>
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DISCUSSION
Neuropathic pain is caused by central or peripheral nerve injury, and characterized by spontaneous pain, hyperalgesia and allodynia. Neuropathic pain induces central nervous system changes that contribute to hyperalgesia (7-9). Peripheral nerve injury induces a variety
The present study was to observe the effects of HBO on NOS (nNOS, nNOS, iNOS and eNOS). In neuropathic pain, NOS expression and with different physiological actions of many NOS subtypes including those conflicting findings remains unclear, and may be associated with neurogenic pain. Indeed, the increased iNOS expression in the spinal cord can promote hyperalgesia. It has been determined that, in a rat model of neuropathic pain (20). nNOS and eNOS are collectively referred to as constitutive NOS, and are regulated by Ca\textsuperscript{2+} and present in normal tissue, while iNOS is not regulated by Ca\textsuperscript{2+}. In neuropathic pain, iNOS levels are elevated in the spinal cord, and after transcription and translation, iNOS plays an important role in NO synthesis. Therefore, in peripheral nerve injury, the increased iNOS expression in the spinal cord can promote hyperalgesia. It has been determined that, in a rat model of neuropathic pain, the expression of iNOS, eNOS and iNOS in the spinal dorsal horn has not been investigated. The purpose of the present study was to investigate the effects of HBO on the levels of nNOS, eNOS and iNOS expression in the spinal dorsal horn in a neuropathic pain rat model. HBO has preventive and therapeutic effects on brain injury caused by hypoxia, ischemia or carbon monoxide poisoning by regulating NO levels (17). HBO also can activate endogenous opioid peptides that produce analgesic effects (16). These studies suggest that HBO can not only improve oxygen supply and correct hypoxia, but also regulate the immune system to produce therapeutic effects. Therefore, we believe that HBO regulates NOS expression to relieve pain.

HBO can enhance antioxidant activity, relieve nerve tissue edema, accelerate the clearance of free radicals, increase blood oxygen content, produce triphosphadine, promote the regeneration of capillary vessels and improve microcirculation (18). The mechanism of the neuroprotective effects may be that HBO effectively increases plasma oxygen tension and improves tissue oxygen capacity; relieves nerve tissue hypoxia and promotes aerobic metabolism and the regeneration of capillary vessels; and inhibits inflammatory reactions, preventing leukocyte aggregation, and decreasing the secretion of TNF-α, IL-1 and IL-6 (16,19).

HBO has been used as an effective and noninvasive method for the treatment of spinal cord injuries and high-altitude sickness, and in immunosuppression and stem-cell research; however, it has yet to be applied to the treatment of neuropathic pain. The present study indicated that HBO effectively increased MWT and TWL, demonstrating that HBO has therapeutic effects on neuropathic pain. Our study provides a basis for HBO application in the treatment of neuropathic pain.

TABLE 3
Number of eNOS-positive cells in the spinal cord in each group 28 days after chronic constriction injury

<table>
<thead>
<tr>
<th>Group</th>
<th>eNOS-positive cells per 10(^4) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.87±2.53</td>
</tr>
<tr>
<td>Sham operation</td>
<td>18.23±2.41</td>
</tr>
<tr>
<td>Chronic constriction</td>
<td>22.34±2.88</td>
</tr>
<tr>
<td>Pre-H HBO</td>
<td>21.86±3.09</td>
</tr>
<tr>
<td>Post-H HBO</td>
<td>21.43±3.28</td>
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</table>

No statistically significant difference was observed when the pretreatment with hyperbaric oxygen (pre-HBO), post-treatment with hyperbaric oxygen (post-HBO) and chronic constriction injury groups were compared with the sham operation group.

of immune-inflammatory factors, such as substance P, IL-1β, TNF-α, prostaglandin and IL-6, resulting in hyperalgesia through activation of intracellular signalling molecules (10). These signals are regulated by positive feedback that is influenced by several mediators. However, the exact mechanisms governing the pathogenesis of neuropathic pain remain unclear and effective therapeutic methods have yet to be developed. Peripheral neuropathic pain is the most common and severe, and treatment with opiates often provides little relief. Therefore, neuropathic pain continues to be an important issue in the field of pain management. What causes changes in NOS expression in neuropathic pain? In neuropathic pain caused by peripheral nerve injury, NOS in the injured nerve and corresponding spinal cord plays an important role in pain regulation. Some studies have shown that pain afferent nerve endings release excessive amounts of glutamate that activates N-methyl-D-aspartate receptors and NOS, which increases neuron excitability. The increased production of nitric oxide (NO) by NOS, in turn, further enhances the release of glutamate and leads to pain. (11-13). Other studies have indicated that NOS and NO mediate the analgesic effects of morphine and clonidine, and the inhibition of NOS activity can reduce their analgesic effects (9,14-16). The cause of these conflicting findings remains unclear, and may be associated with different physiological actions of many NOS subtypes including nNOS, iNOS and eNOS. In neuropathic pain, NOS expression and activity in the spinal dorsal horn remain unclear. The purpose of the present study was to observe the effects of HBO on NOS (nNOS, iNOS and eNOS) expression in the spinal dorsal horn in a neuropathic pain rat model. HBO has preventive and therapeutic effects on brain injury caused by hypoxia, ischemia or carbon monoxide poisoning by regulating NO levels (17). HBO also can activate endogenous opioid peptides that produce analgesic effects (16). These studies suggest that HBO can not only improve oxygen supply and correct hypoxia, but also regulate the immune system to produce therapeutic effects. Therefore, we believe that HBO regulates NOS expression to relieve pain.

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Neuropathic pain in a rat model

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pain rat model. In the present study, HBO effectively decreased the levels of spinal nNOS and iNOS expression, but failed to alter the level of eNOS; therefore, HBO likely relieved hyperalgesia through regulating the expression of spinal nNOS and iNOS.

HBO increases MWT and TWL in a neuropathic pain rat model, demonstrating HBO has preventive and therapeutic effects on neuropathic pain. The expression of NOS-positive neurons in the spinal dorsal horn is increased in rats with CCI, suggesting that NOS-positive neurons in the spinal dorsal horn play an important role in the central mechanism of chronic neuropathic pain. HBO likely relieves pain through decreasing NOS expression. However, in the present study, HBO was administered only once; therefore, the optimal frequency of HBO treatment remains to be explored.

REFERENCES