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Effects of hyperbaric oxygen therapy on neuropathic pain via mitophagy in microglia

Guang Han[1], Kun Liu[1], Lu Li[1], Xingyue Li[1] and Ping Zhao[1]

Abstract

Purpose: Hyperbaric oxygen (HBO) therapy has been suggested to palliate neuropathic pain, but the mechanisms involved are not well understood. This study explored the involvement of microglial mitophagy via HBO relative to neuropathic pain therapy.

Materials and methods: A total of 80 male Sprague Dawley rats were randomly divided into two groups: a normal group ($n = 40$) and a mitophagy inhibitor group ($n = 40$) in which the mitophagy inhibitor cyclosporin A (CsA) was administrated prior to chronic constriction injury (CCI). Groups ($n = 10$ rats per group) consisted of the following: control (C), sham operation (S), sciatic nerve with chronic constriction injury (CCI), and a CCI plus HBO treatment (CCI + HBO). Pain-related behaviors were evaluated using mechanical withdraw tendency and thermal withdraw latency analysis. Mitochondrial membrane potential was measured, and Western blot was employed to assess expression of NIX and BNIP3. Immunofluorescence changes in neuron protein (NESTIN) and mitochondria inner or outer layer proteins (TIM23, TOM20) were examined.

Results: HBO significantly ameliorated pain-related behaviors, which were downregulated by mitophagy inhibitors ($P < 0.05$). Mitochondrial membrane potential indexes were decreased after HBO therapy, but were reversed in the mitophagy inhibitor group ($P < 0.05$). HBO upregulated NIX and BNIP3 expression, which did not occur in the CCI group ($P < 0.05$). However, expression was reduced when mitophagy inhibitors were administered. Immunofluorescence examination showed that mitophagy in microglia was induced by CCI, which was upregulated after HBO treatment. This phenomenon was not observed in the mitophagy inhibitor group.

Conclusions: HBO therapy palliated CCI-induced neuropathic pain in rats by upregulating microglial mitophagy. These results could serve as guidelines to improve neuropathic pain therapy using HBO to maximize therapeutic efficiency.

Keywords

Neuropathic pain, mitophagy, hyperbaric oxygen

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Background

Neuropathic pain is closely related to its pathophysiological state in the nervous system.[1,2] Research has discovered that neuropathic pain shares several similarities with other neurobiological processes, particularly with respect to the development and maintenance of pain.[3] In the past, neuropathic pain was mainly thought to be caused by spinal neuronal cell death, but this point of view has gradually become controversial.[4] In fact, apoptosis is not the only cellular transformative mode in the development of pain behavior.[5] During periods of pain, reactive oxygen species (ROS) are released, which can damage the nerve and induce neuropathic pain.[6,7] ROS are also important factors in autophagy.[8] Although research has shown that cellular autophagy can lead to neurodegeneration or neuronal dysfunction,[9] the molecular mechanisms of neuropathic pain are still unknown.

Autophagy is a lysosome-mediated intracellular catabolic process involved in the degradation of damaged

[1]Department of Anesthesiology, Shengjing Hospital, China Medical University, Shenyang, China

Corresponding author:
Ping Zhao, Department of Anesthesiology, Shengjing Hospital, China Medical University, Shenyang 110003, China.
Email: zhaopsjh@163.com

cells.[10] Mitophagy is a type of selective autophagy in which damaged mitochondria are removed to maintain proper cellular functions.[11] As a result, mitophagy clears excessive ROS inside the cell and blocks ROS sources, which are important in therapeutic management of neuropathic pain.[12] Recent studies have shown that this cellular activity is usually very low under normal conditions, but can be upregulated by stress stimuli such as hypoxia, nutrient starvation, energy depletion, and other pharmacological or viral stimulation.[13] After autophagosome formation is impaired, subsequent accumulation of damaged mitochondria interferes with cellular functions. Some authors have reported that neuropathic pain may promote LC3-II and p62 upregulation, while LC3-I and Beclin 1 levels were not dramatically increased.[14] This phenomenon illustrates that even though autophagy is a factor in neuropathic pain, it can be readily blocked for unknown reasons. Our previous study have verified LC3 and P62 associated with autophagy which may alleviate neuropathic pain.[15] Herein, we investigated whether degenerative mechanisms, particularly in the mitochondria, can modulate neuropathic pain following chronic constriction injury (CCI) in rats.

Hyperbaric oxygen (HBO) is widely used in clinics to treat neurological diseases, such as spinal cord injury and cerebral ischemia.[16,17] HBO is a noninvasive, easy procedure to implement, and patients are typically willing to undergo treatment. While HBO exerts neuroprotective effects via specific mechanisms, including inhibition of inflammation, reduction of hypoxia, and improvement of microcirculation,[18,19] the antinociceptive mechanisms of HBO are not well understood. A recently study suggests that the analgesic effect of HBO may involve cellular modulation.[20] We previously found that the antinociceptive effect of HBO treatment is associated with microglial autophagy.[15] The discovery of a new molecular mechanism related to HBO may be important in managing neuropathic pain.

Materials and methods

Materials

The following materials were used in this study: ECL Western blotting kit (Solarbio, Beijing, China), horseradish peroxidase (HRP) conjugated rabbit anti-goat IgG, goat anti-rabbit IgG, goat anti-rat IgG (Pierce, USA), cyclosporin A (CsA; 32489, Sigma, USA), rabbit anti-rat NIX, BNIP3 IgG (D4R4B, CST, USA), mitochondrion membrane potential kit (MAK 147, Sigma, USA), mouse anti-rat Iba1 IgG (ab6142, Abcam, USA), mouse anti-rat TOMM20 IgG (ab56783, Abcam, USA), rabbit anti-rat TIMM23 IgG (11123-1-AP, Proteintech, China), a fluorescence microscope X81 (Olympus, Tokyo, Japan), and total protein extraction kit (Keygen Biotech, Nanjing, China).

Animals

Male Sprague Dawley (SD) rats (260 ± 20 g) were obtained from the Animal Care Center at Shengjing Hospital of China Medical University. The study protocol was approved by the Institutional Animal Care and Use Committee, Shengjing Hospital of China Medical University, China. A total of 80 rats were randomly separated into a normal control group without treatment ($n = 40$) and a mitophagy inhibitor group treated by intraperitoneal injection of CsA ($n = 40$). Each group included four subgroups ($n = 10$ rats per group) as follows: control group (C group) in which CCI and HBO were not performed; a sham operation group (S group) in which the sciatic nerve was exposed, but not ligated and HBO was not performed; sciatic nerve with CCI group (CCI group) in which the sciatic nerve was ligated, but HBO was not performed; and a CCI plus HBO treatment group (CCI + HBO group) in which HBO was initiated 6 h after CCI once a day for five days.

Experimental process

Rats in the mitophagy inhibitor group were intraperitoneally injected with CsA (10 mg/kg) one day before CCI, while rats in the normal group were intraperitoneally injected with saline. Baseline mechanical withdrawal thresholds (MWTs) and thermal withdrawal latencies (TWLs) were measured in all rats one day before CCI. CCI or sham operations were performed in the morning. Pain-related behaviors were observed one, two, three, four, five, six and seven days after CCI between 09:00 and 10:00 a.m. each day. Rats in the CCI + HBO group were first exposed to HBO 6 h after CCI once a day for the following five days. Rats were sacrificed, and the dorsal part of spinal cord was collected on the seventh day after CCI (Figure 1).

CCI model preparation

Rats were anesthetized by peritoneal injection of 1% sodium pentobarbital (40 mg/kg). CCI models were prepared using a common method in which a posterolateral incision on the right hind limb was made. We found the right sciatic nerve trunk and loosely ligated it with 4-0 silk thread to produce slight pressure on the epineurium until momentary muscle contraction was observed in the sciatic nerve distribution region. Incisions were closed to complete the CCI model. When regaining consciousness, rats were returned to their cages. In the S group, the sciatic nerve was exposed, but not ligated.

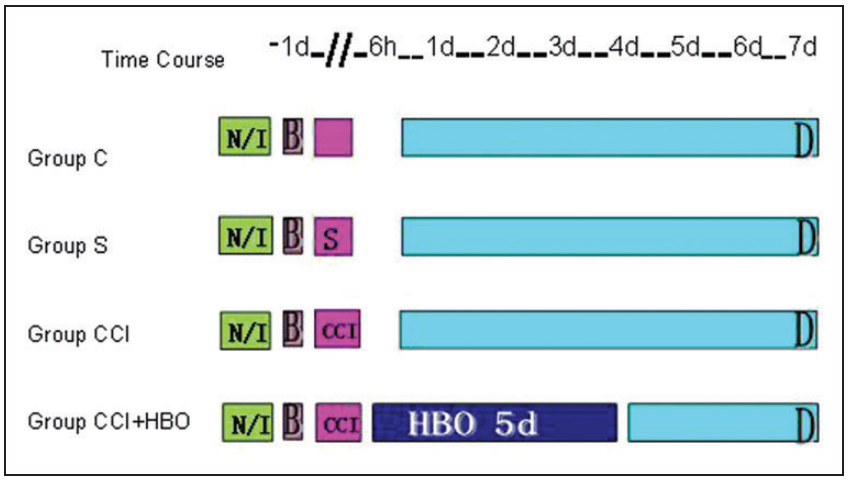

Figure 1. Experimental process. N: normal group; I: mitophagy inhibitor (CsA) group; B: baseline values; S: sham operation; CCI: chronic constrictive injury; d: day; D: death (sample taken); HBO: hyperbaric oxygenation.

HBO treatment

In the CCI + HBO group, rats were treated with HBO five times at a frequency of once per day following CCI. On the first day, HBO treatment was given 6 h after CCI. HBO conditions were described as follows. An HBO chamber was purified with greater than 90% pure oxygen. Pressure was increased at a rate of 0.0125 mPa/min to 0.25 mPa. Pure oxygen was allowed to fill the chamber for 10 min. Rats were exposed under high pressure for 60 min. Pressure was subsequently decreased for 30 min at a constant rate.

Samples

On the seventh day, rats were anesthetized by peritoneal injection of 1% sodium pentobarbital (40 mg/kg) and intubated from the left ventricle to the ascending aorta. Rats were perfused with a 0.9% saline solution until no red perfusate was effused. After 0.1 M phosphate buffer (pH 7.4) was perfused, the L4–L6 spinal cord segments were removed.

Pain-related behaviors

Pain-related behaviors were observed once a day for seven days after CCI between 09:00 and 10:00 a.m. MWT was tested using von Frey filaments. Rats were placed in a test box and allowed to acclimatize for 30 min. Each von Frey filament was applied to the plantar surface of the hind paw for 6 s to 8 s to observe the hind paw withdrawal response. TWL was assessed to quantitatively determine thermal sensitivity. Rats were placed on the glass surface of a thermal testing apparatus and allowed to acclimatize for 30 min before testing. A mobile radiant heat source located under the glass was focused onto the hind paw of each rat. The paw TWL was recorded by a timer three times, and the mean of these three trials was used. A cut-off time of 20 s was used to prevent potential tissue damage.

Mitochondrial membrane potential

The spinal cord tissue was separated and washed with PBS (pH = 7.4). Samples were minced on ice and filtered with two layers of wet lens cleaning paper. Cells were centrifuged at 4°C (500 r/min, 10 min). The supernatant was discarded and these steps were repeated until single cells were isolated for flow cytometry. Isolated spinal cord cells were counted to 1×10^6/ml under a microscope. After adjusting the number of cells, cells were centrifuged at 1000 r/min, 4°C for 5 min, and the supernatant was separated for Mitochondrial membrane potential (MMP) determination using an MMP kit (MAK 147, Sigma, USA) according to the manufacturer instructions.

NIX and BNIP3 expression

A total of 100 mg of spinal cord tissue in dorsal horn was homogenized and total protein was separated using a total protein extraction kit according to the instructions provided. Protein content was determined, and proteins were diluted with PBS to the same concentration. A total of 20 pg of protein was loaded for electrophoresis. After electrophoresis, gels were transferred to a cellulose membrane and immersed in confining liquid for 60 min. The membranes were incubated with anti-NIX and BNIP3 primary antibody (1:200) and anti-β-actin primary antibody (1:200) overnight. β-Actin served as an internal reference. Membranes were washed with PBS and incubated with secondary antibody (1:1) for 30 min, followed

by rinsing. Expression of NIX and BNIP3 was detected with ECL Western blotting kit using Quantity One software on a DocTM XR gel imaging system (Bio-Rad, USA).

Immunofluorescence

Spinal cord tissue was dissected on the seventh day after CCI. After separating the spinal cord into $0.5 \times 0.5 \times 0.2$ cm^3 and selecting the dorsal horn of the spinal cord. It was fixed with 2.5% glutaraldehyde. Before observing under the electron microscope, samples were placed in the refrigerator for 12 h at 4°C. Samples were washed with PBS buffer, fixed with osmic acid, dehydrated with acetone, and embedded with resin for subsequent experiments. Samples were cut into thin sheets and observed under fluorescence microscopy. To determine whether mitophagy was involved in neurons, the samples were subjected to immunofluorescence using mouse monoclonal anti-nestin antibody and Tomm20 or Timm23 antibody on an inverted fluorescence microscope X81 (Olympus, Tokyo, Japan).

Statistical analysis

Data analysis was performed using SPSS version 21.0 (IBM Corporation, USA). Data are expressed as the mean ± SD. Two-way analysis of variance (ANOVA) and Student-Newman-Keuls q tests were used to compare the indexes between groups. Differences were considered statistically significant at $P < 0.05$.

Results

MWT and TWL

Chronic constriction of the spinal nerve via ligation induced severe mechanical allodynia. Ligation of the spinal nerve produced early onset and long lasting mechanical hypersensitivity. Tactile hypersensitivity determined by von Frey filaments and heat stimulation developed one day after CCI and was maintained for up to seven days. Scores of MWT and TWL increased dramatically in the CCI + HBO group compared to the CCI group, which indicates that HBO can alleviate this hypersensitivity (Figure 2(a)) ($P < 0.05$). However, there was no effect following HBO when the mitophagy inhibitor CsA was added (Figure 2(b)) ($P > 0.05$).

MMP

To study whether mitophagy occurs, we examined the early events of mitophagy based on a decrease in the MMP. As shown in Figure 3, the mitochondrial membrane potential in the rat spinal cord in the CCI and CCI + HBO groups was reduced to 81.2% and 69.2%, respectively, compared with the C and S groups ($P < 0.05$). There were no differences between all groups when the inhibitor CsA was administered ($P > 0.05$) (Figure 3).

NIX and BNIP3 levels

In our previous study, we had verified HBO can upregulate autophagy by affecting the expression of LC3 and P62.[15] So now we explored possible mechanisms by which BNIP3 and NIX induce mitophagy in spinal nervous cells. We evaluated NIX and BNIP3 levels by Western blot in rats that underwent either CCI or sham surgeries. Expression of these markers was analyzed in the L4-L5 portion of the spinal cord seven days after injury. Compared with the C and S groups, NIX and BNIP3 expression was higher in the CCI and CCI + HBO groups. Samples from rats that underwent HBO showed higher NIX and BNIP3 levels than the CCI group ($P < 0.05$). However, HBO did not have an effect on expression after CsA was administered (Figure 4, $P > 0.05$).

Pathological changes in the spinal cord

To show the process of mitophagy, we observed the outer/inner membrane protein TOM20/TIM23 and neuron marker NESTIN by fluorescence microscopy, which has been shown to be an effective method of detecting mitophagy. Compared with the S group, there were a large number of red and green fluorescence signals merging into an orange signal in the CCI and CCI + HBO groups. Colorful fluorescence ratio in the CCI and CCI + HBO groups was greater than in the S group, especially in the CCI + HBO group (Figures 5 and 7, $P < 0.05$). This indicates that CCI induces mitophagy in the microglia and that HBO may upregulate this process. However, HBO did not have an effect when CsA was administered (Figures 6 and 7, $P > 0.05$).

Discussion

Chronic pain is an agonizing condition that can negatively affect quality of life and overall health. Current therapies include drugs, nerve blockers, and electrical stimulation, which provide limited relief. Thus, it is urgent to find more effective treatments. With the discovery of neurobiological and molecular mechanisms, we can attempt to study the effects of nerve injury on the autophagic process. Mitochondria are the primary source of ROS, and ROS can induce mutations in mtDNA that lead to protein deficiencies and restrict the ability to self-repair,[21] leaving cells more vulnerable to ROS attack. In addition, ROS can damage mitochondrial

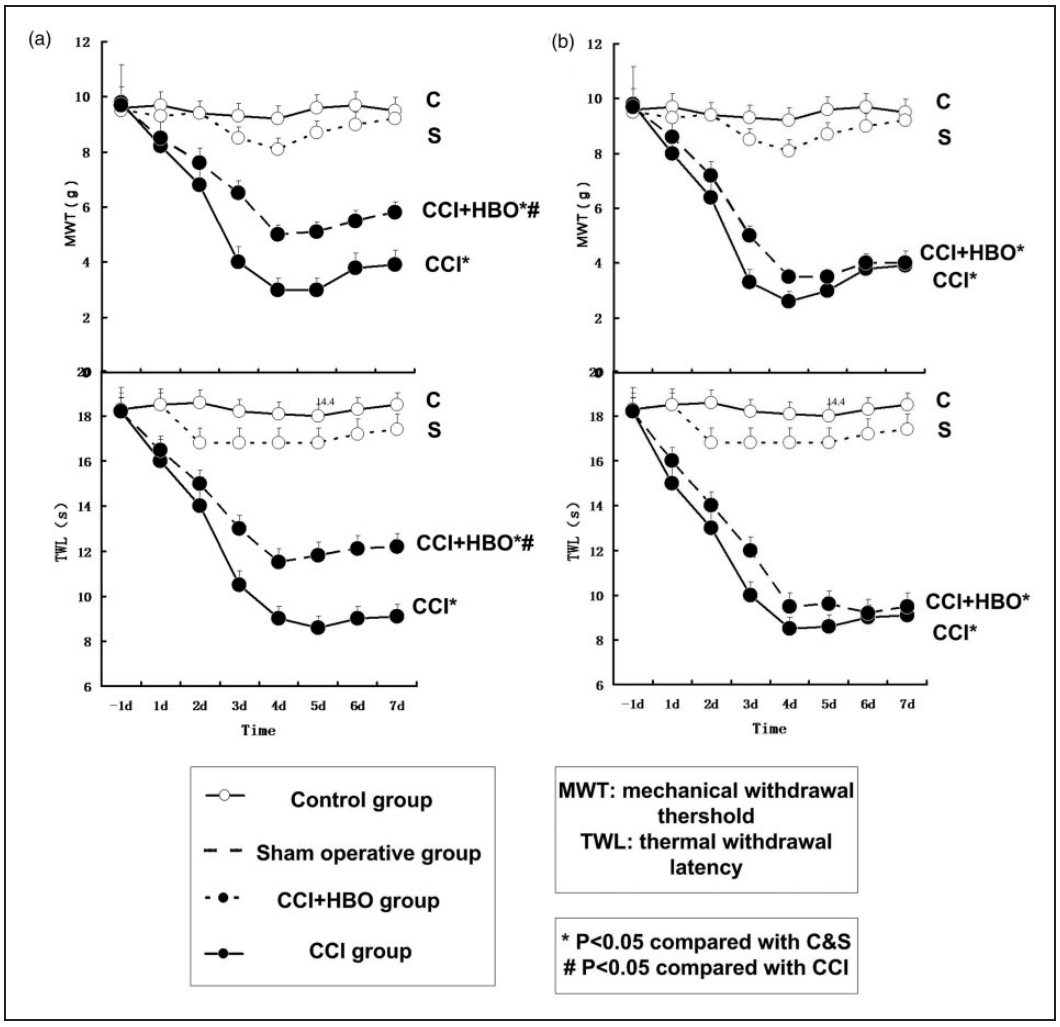

Figure 2. Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in each group ($n = 8$ rats per group). CCI: chronic constriction injury; HBO: hyperbaric oxygenation; S: sham operation; d: Day; w: week. (a) Normal and (b) adding mitophagy inhibitor CsA.

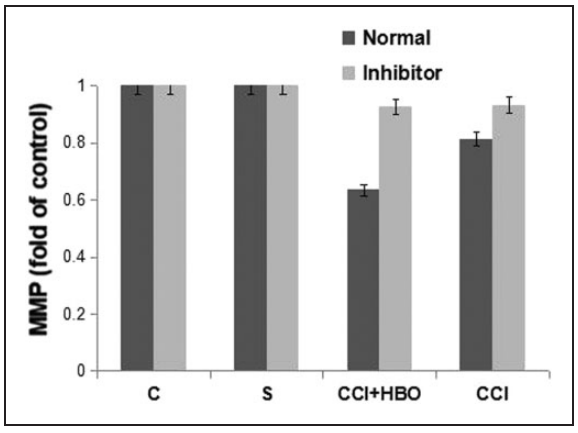

Figure 3. Mitochondrial membrane potential of the rat spinal cord. The MMP in CCI and CCI + HBO group reduced to 81.2% and 69.2% separately, compared with C group and S group ($P < 0.05$).While there are no difference in all groups when inhibitor CsA administration ($P > 0.05$).

proteins and lipids by inducing oxidative stress.[22] These damaged mitochondria have the potential to further induce more ROS via defective electron transport chains.[23] Therefore, damaged, aging, and excess mitochondria are risk factors in cells. Mitophagy may manifest as follows: (a) mitochondria aggregated at the periphery of the nucleus; (b) degeneration of mitochondria and appearance of a vacuole bilayer membrane structure; (c) mitophagy formation by the double membrane structure package mitochondria; (d) mitophagy is a process which fuse with lysosomes to form mitophagy-lysosomes; and (e) mitochondria that are unable to degrade.[24] These phenomena can directly reflect damage to the mitochondria as well as mitophagy. Appropriate clearance of mitochondria is important for maintaining homeostasis in cells, which is accomplished by mitophagy.

Mitophagy can also result in mitochondrial vesicle acidification and mtDNA degradation. Mitochondrial depolarization directly affects changes in mitochondrial

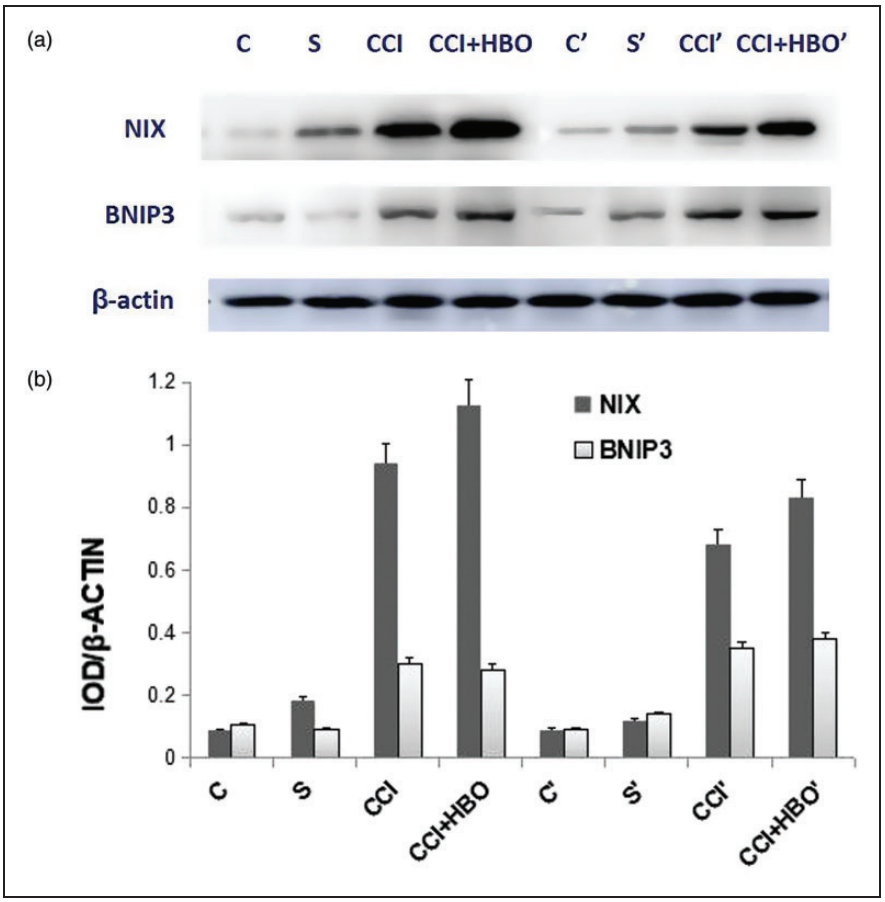

Figure 4. Qualitative (a) and quantitative (b) Western blot of NIX and BNIP3 after treatment with sham peration, CCI + HBO, and CCI. C', S', CCI', and CCI + HBO' represented the corresponding groups after mitophagy inhibitor CsA administration. Weakest blot was present in the C and S group and blots became gradually stronger after CCI and especially CCI + HBO therapy ($P < 0.05$). The blots presented in the CCI' and CCI + HBO' group became similar with C' and S' group ($P > 0.05$).

membrane.[25] Polarization is an important indicator of mitophagy. Many studies have reported that Parkinson's disease (PD) is a progressive neurodegenerative disease that is related to accumulating impaired mitochondria.[26] To date, there have been no reports on neuropathic pain. Therefore, we measured the function of mitochondria and explored related mechanisms.

CsA is an amino acid polypeptide with immune inhibitory properties. It is also a specific inhibitor of mitochondrial permeability transition pores.[27] The mitochondrial permeability transition pore is a nonspecific pore in the inner and outer membranes of mitochondria and is a common pathway for cell injury or death. It is also one of the end effectors of hypoxia/refusion and other types of damage.[28] CsA can inhibit cell apoptosis or necrosis by blocking the mitochondria permeability transition pore channel. As an immune inhibitor, CsA also can act with cyclophilin D to inhibit opening of the mitochondrial permeability transition pores.[29] As a result, CsA inhibits mitochondrial depolarization and mitophagy, leading to a marked decrease in the number of mitophagy bodies. We used CsA to verify that HBO can act through mitophagy given that specific indices, such as MMP and mitochondrial inner or outer protein levels, changed during the experimental period. We can deduce that HBO has an effect through mitochondrial permeability transitive pores or on the mitochondrial membrane.

Our previous study showed that hypersensitivity to mechanical and thermal stimulation developed on the third day following CCI and lasted up to 28 days.[30] However, the present study only observed changes for seven days after CCI. We found that gradual downregulation of MWT and TWL was detectable in the CCI model seven days after injury. Although mitophagy is a continuous phenomenon, we found that the seventh day of pain processing was the optimal time point for determining mitophagy based on earlier experiments (data not

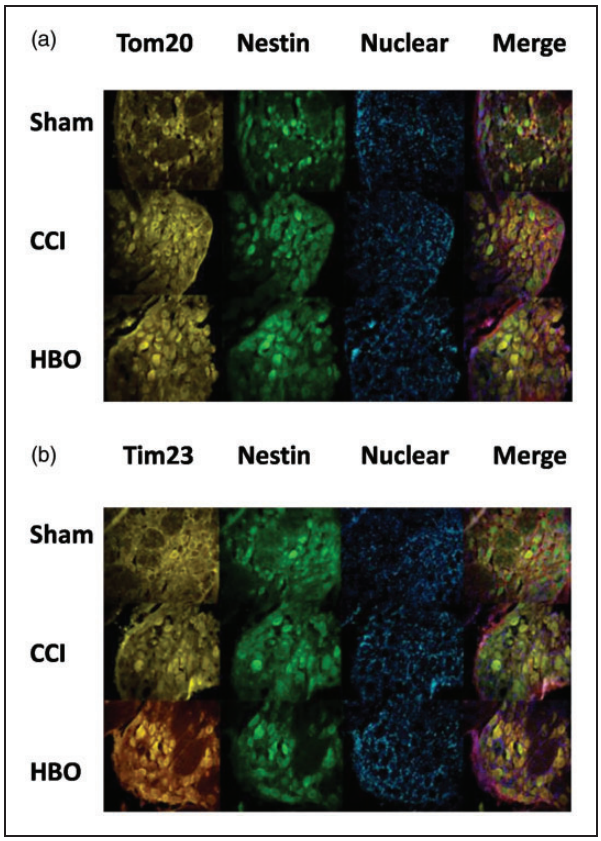

Figure 5. Immunostaining of each group with TOMM20, TIMM23, and NESTIN. Compared with the S group, there were a large number of red and green fluorescence signals merging into an orange signal in the CCI and CCI + HBO groups. Colorful fluorescence ratio in the CCI and CCI + HBO groups was greater than in the S group, especially in the CCI + HBO group.

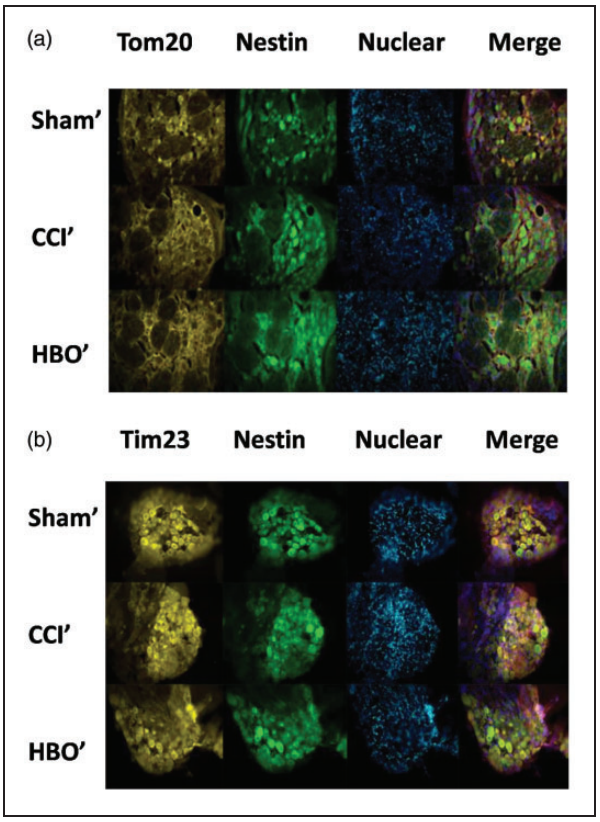

Figure 6. Immunostaining of each group with TOMM20, TIMM23, and NESTIN after mitophagy inhibitor CsA administration. C', S', CCI', and CCI + HBO' represented the corresponding groups after mitophagy inhibitor CsA administration. Compared with the S' group, there were a large number of red and green fluorescence signals merging into an orange signal in the CCI and CCI + HBO groups. Colorful fluorescence ratio was similar in these three groups.

shown), and day 7 was selected to evaluate MMP, NIX, and BNIP3. MWT and TWL in the CCI and CCI + HBO groups were gradually reduced, but HBO induced upregulation, which indicates that HBO can effectively alleviate neuropathic pain. This effect was not significant when the mitophagy inhibitor was administered. Therefore, HBO may influence neuropathic pain through mitophagy. Mitophagy is also likely involved in molecular antinociceptive mechanisms of HBO.

LC3 and P62 are both key proteins involved in autophagy. With the presence of Atg4, LC3 precursor was processed into soluble LC3-I which can change into lipid soluble LC3-II-PE under the action of phosphatidylethanolamine (PE), Atg3, and Atg7.[31] LC3-II participates the extension of autophagy lysosome membrane until autolysosome formation.[32] P62 that locates in the cytoplasm combines with the ubiquitin protein. Then P62 protein combines with LC3-II protein to build a complex formation. And it is degradated in lysosome ultimately.[33] In autophagy process, P62 will continue to be consumed. In our previous study, compared with the CCI group, the expression of LC3-II in HBO group was higher in dorsal horn part of spinal cord. While the expression of P62 was low in HBO group, which indicated that the autophagy activity of HBO was higher without P62 accumulation. Although the CCI group is not as high as the HBO group, it also induces autophagy, which may be a stress response after spinal cord injury.

BNIP3 and NIX are proteins related to the BH3-only family, which induce both cell death and autophagy. They are also two key proteins for mitophagy function that play a role in the initiation step and formation of autophagosomes.[34] NIX is a target of mitochondria for elimination and BNIP3 regulates mitophagy in response to hypoxia. NIX and BNIP3 were markedly increased with accumulation of autophagosomes in the L4-L5 spinal cord segment on the injured side in rats that underwent CCI, but this effect was absent in the sham group. NIX recruits autophagy components that do not rely on mitochondrial depolarization, thus inducing

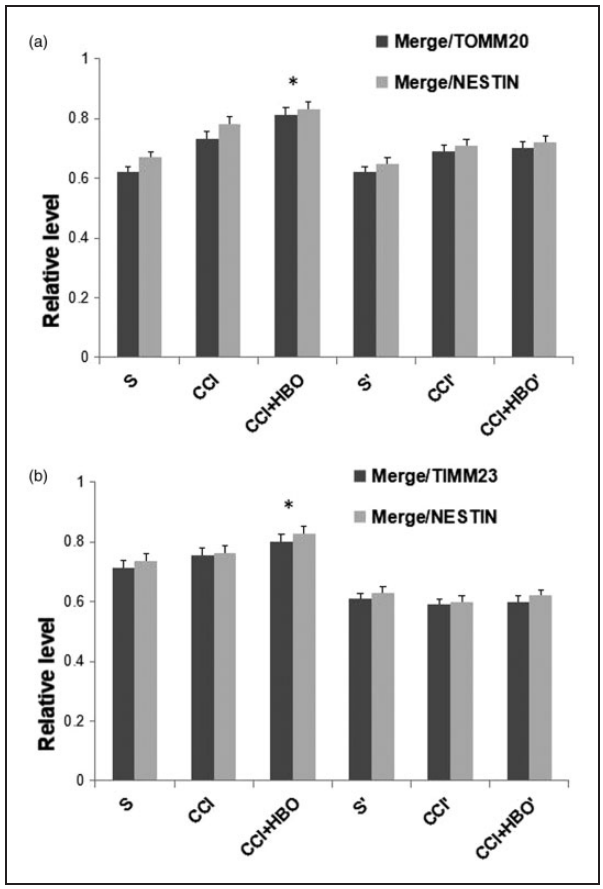

Figure 7. The quantification and statistical analysis on the immunuostaining. Normal groups (a) and inhibitor groups (b) immunostaining quantification shows relative fluorescence ratio in the CCI and CCI + HBO groups was greater than in the S group, especially in the CCI + HBO group ($P < 0.05$). However, HBO did not have an effect when CsA was administered ($P > 0.05$).

mitochondrial autophagy.[35] In many cases, BNIP3 and NIX expression induces autophagy possibly due to specific protein characteristics.[36] However, depolarization of the mitochondrial membrane is not necessarily indicative of mitophagy induction. It may represent activity at the mitochondrial membrane or a functional change in the mitochondria. We found a slightly higher expression of NIX and BNIP3, resulting in reduced accumulation of dysfunctional macromolecules or organelles. Progression of mitophagy disruption has been shown to correlate with neurological dysfunction.[37] Moreover, the autophagic machinery is required for remodeling of neuronal dendrites and axons and for maintaining CNS plasticity.[38] In neurodegenerative diseases, impairment of this last function has been suggested as a cause for progressive anatomical and functional alterations of the CNS when massive cell loss is still undetectable.[39] The molecular and cellular processes responsible for long-lasting abnormal sensory activation underlying chronic pain are poorly understood. However, it is now clear that structural and functional changes in neurons result in alterations in sensory processing following nerve injury and contribute to the development and maintenance of neuropathic pain.[40] To our knowledge, this is the first work reporting an impairment of spinal autophagy in a model of neuropathic pain and provides a platform for future studies regarding the role of this degradative pathway in pain processing.

In summary, our data demonstrate that mitophagy may participate in pain processes. There are several limitations to this study. First, the time point seven days after CCI was optimal for mitophagy, which was discovered through initial experiments. While formation of mitophagy is a dynamic process, we only selected a single time point, which may have certain limitations. To further clarify the dynamic changes in the mitophagy process, we should observe multiple time points. Second, using both autophagosome and lysosome markers to co-localize and monitor this dynamic process may provide more persuasive results. Finally, the mitochondrial membrane potential is not completely representative of mitochondrial function. ATP levels, respiratory function, and intracellular calcium ion levels should be monitored in future experiments. More in-depth study to explore important neuroprotective mechanisms of HBO on mitophagy will be necessary.

Authors' contributions

GH designed the experiments, wrote and interpreted the manuscript; KL performed immunofluorescence observation and participated in data analysis; LL performed the behavioral study and mitochondrial membrane potential detection; XYL performed the Western blot experiments; PZ supervised experiments and revised the manuscript. 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.

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