Am J Transl Res 2017;9(5):2629-2638
www.ajtr.org /ISSN:1943-8141/AJTR0053438

Original Article

Hyperbaric oxygen treatment attenuates neuropathic pain by elevating autophagy flux via inhibiting mTOR pathway

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Received December 18, 2016; Accepted April 30, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: Peripheral neuropathic pain is a complex disease, and treated based on underlying diseases. Emerging evidences suggest that hyperbaric oxygen alleviates neuropathic pain. However, its cellular and molecular mechanism on pain relief is unknown. We hypothesize that hyperbaric oxygen alleviates neuropathic pain via activating autophagy flux and inhibiting mTOR pathway. Hyperbaric oxygen effectively inhibited nerve injury induced autophagy impairment and mTOR pathway activation in a rat spinal nerve ligation (SNL) model. Moreover, intrathecal injection of rapamycin, an autophagy inducer, enhanced hyperbaric oxygen effect by further decreasing mTOR activity. In contrast, chloroquine, an autophagy inhibitor, counteracted hyperbaric oxygen analgesic effect. These findings indicate that hyperbaric oxygen attenuated neuropathic pain by increasing spinal autophagic flux via inhibiting mTOR pathway. Our study provides pre-clinical evidences in expediting hyperbaric oxygen become a safe clinical treatment of neuropathic pain.

Keywords: Spinal nerve ligation, neuropathic pain, autophagy, hyperbaric oxygen, chloroquine, mTOR

Introduction

Neuropathic pain is a broader term that encompasses a series of different conditions resulting from injuries to peripheral or central nerve system [22]. It is characterized by spontaneous pain, hyperalgesia, and allodynia [46]. Traditional therapy is to treat underlying diseases with adverse effects. A new and effective chronic neuropathic pain treatment strategy is needed.

Autophagy is a process of adaptive and survival mechanism that protect cells from environmental changes and pathological stimuli [30]. Recent investigations showed that autophagy might play an important role in the process of neuropathic pain. It has been reported that basal autophagy is impaired in spinal nerve ligation (SNL) model [1, 16]. Autophagy is impaired in neurons, GABAergic interneurons, and astrocytes following peripheral nerve injury [57]. A deficiency of autophagic activity in Schwann cells is an early event of neuropathic pain chronification [34]. Moreover, rapamycin, an autophagy inducer, induces long-lasting analgesia, inhibits interleukin-1β secretion [10], improves nerve myelination [45], and prevents pain chronification. Accordingly, neuropathic pain was dramatic enhanced by autophagy inhibitors [2, 45].

The mammalian target of rapamycin (mTOR), which regulates the UNC-51-like kinase1/2 complex, is a key regulator in autophagic process [20]. mTOR pathway was activated in spinal cord [33] and spinal dorsal horn [16] in models of neuropathic pain and bone cancer model [23, 40, 41]. Activation of the mTOR complex promotes the phosphorylation of mTOR downstream effectors, which further lead to damage autophagy activity. Inhibition of mTOR kinase could be a possible pharmacological target in the management of chronic pain [14, 33]. Rapamycin can reduce mechanical allodynia, mechanical and temperature sensitivity [58], and suppress microglial activation after spinal cord injury [50]. Furthermore, recent evidence

suggests that mTOR pathway promotes peripheral pain plasticity [21, 43] via changes in translation control. mTOR pathway in cerebrospinal fluid-contacting nucleus (CSF-contacting nucleus) has been shown involved in neuropathic pain model [32].

Hyperbaric oxygen (HBO) treatment is effective of treating neuropathic pain in both preclinical and clinical studies [51]. HBO treatment can preserve muscle and neuronal ultrastructural integrity [38], reduce tumor necrosis factor-alpha production [31], decrease spinal apoptosis [19], and inhibits the activation of astrocytes [15]. In addition, HBO treatment synergistically alleviates neuropathic pain with drug treatment [42]. HBO alleviates neuropathic pain through upregulation of heme oxygenase-1 (HO-1) [11, 55], which in turn enhances autophagy [9]. Our previous study indicated that the expression of spinal neuronal nitric oxide synthase (NOS) and inducible NOS, were significantly decreased in HBO group when compared with the neuropathic pain group [17]. Furthermore, studies have demonstrated that HBO preconditioning could induce neuroprotection by elevating autophagic activity [53, 54].

The aim of this study is to address whether (1) HBO treatment could enhance spinal autophagy to attenuate SNL induced neuropathic pain; (2) pharmacological inhibition of autophagy reverse HBO analgesic effect; (3) mTOR signaling pathway responsible for HBO treatment following SNL; (4) HBO treatment induced spinal autophagy was mainly located in neurons or astrocytes.

Materials and methods

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Ethics Committee of China Medical University. All surgical procedures were performed under 10% chloralic hydras anesthesia, and all efforts were made to minimize animal suffering. Adult male Sprague-Dawley rats (200-300 g, Changsheng biological technology, China, n=48) were used in this study. The animals were housed individually in plastic boxes at 23~25°C with standard chow and water available ad libitum. Rats were randomly assigned into 7 groups: sham, SNL, SNL+HBO, vehicle (saline)+SNL+HBO, CQ+SNL+HBO, vehicle (DMSO)+SNL+HBO and RAPA+SNL+HBO.

Surgical procedures and treatment

Spinal nerve ligation (SNL) was performed according to the Kim & Chung model [26]. Under Chloral Hydrate (500 mg/kg) anesthesia, the rat was placed in a prone position and the left paraspinal muscles were separated from the spinous processes at the L4-S1 levels. The left lumbar L6 transverse process was carefully removed to identify the L4 and L5 spinal nerves. The left L5 spinal nerve was isolated and tightly ligated with a 4-0 silk thread. Complete hemostasis was confirmed and the skin was closed. The surgical procedure for the sham group was identical to the SNL group with no spinal nerve ligation.

Chloroquine intraperitoneal injection: Chloroquine (c6628, Sigma) was dissolved in saline before use (4 μg/ml). Rats (n=12) were random divided into two groups: chloroquine IP injection with hyperbaric oxygen (CQ+SNL+HBO), and saline IP injection with hyperbaric oxygen (vehicle+SNL+HBO). Chloroquine group received intraperitoneal administration of 8 μg/kg/day one dose per day from day 0 to day 5 after SNL surgery. Same volume of saline was given to saline with hyperbaric oxygen group.

Rapamycin (RAPA) intrathecal administration: a PE-5 intrathecal catheter was inserted into the subarachnoid space for drug delivery. Briefly, a laminectomy of the L5 vertebra was performed and the dura was cut. At the level of the L4/5 spinal cord, a polyethylene-5 catheter was inserted into the subarachnoid space. The location of the intrathecal catheter was confirmed by post-mortem examination after completion of the experiments. Rapamycin (CST, 9904S) was dissolved in DMSO and 10 μl (0.05 μg/μl), RAPA+SNL+HBO group was injected slowly once per day from day 0 to day 3 and received HBO treatment once a day for five consecutive days after SNL surgery. Same volume of DMSO was given to DMSO with HBO group.

Hyperbaric oxygen treatment

The cylindrical HBO treatment chamber (DS400-IV, Weifang Huaxin Oxygen Industry Co. Ltd., Shandong, China) was precoated with

soda lime on the bottom to minimize water vapor and CO_2 accumulation. Before pressurization, the chamber was flushed with pure oxygen for 10 min to displace the ambient air. After rats were placed in the chamber, the pressure was increased at a rate of 0.1 ATA/min to the desired pressure (2.0 ATA) and maintained for 60 min. The chamber then decompressed to normal room pressure at a rate of 0.1 ATA/min. All rats in SNL+HBO group received HBO treatment once a day for five consecutive days beginning on day 1 after SNL surgery. Rats in the sham group and SNL group were placed inside the chamber without HBO treatment.

Behavioral tests

The mechanical withdrawal threshold (MWT) test was carried out to assess the response of the hind paw to mechanical stimuli. Rats were placed in Plexiglas chamber, MWT test was performed by stimulating the plantar surface of the left hind paw using von Frey filaments (Stoelting Company, USA). Each von Frey filament was held for approximate 5 s. Each trial started with the application of a 0.6 g von Frey force following an up-and-down procedure. A positive response was defined as a quick withdrawal of the hind paw upon stimulation. When a positive response occurred, a filament with a lower force was applied. If a negative response occurred, a filament with a higher force was applied. This protocol was continued until the least force that caused withdrawal was identified. The cut off value was 15 g. MWT tests were performed on preoperative day 3 (baseline) and postoperative day 1, 3, 5, 7.

Western blotting

Rats were euthanized 7 days after surgery and the spinal cord segment (L4-L5) was rapidly removed, snap-frozen and stored at -80°C until further processing. For protein extraction, each spinal cord segment was homogenized in ice-cold RIPA lysis buffer (p0013B, Beyotime, China) in the presence of protease inhibitors (cod. P8349; Sigma-Aldrich, China) and incubated on ice for 30 min. Samples were then centrifuged at 14,000 rpm for 30 min at 4°C. Total protein content was determined in the supernatants by the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, China). For Western blot analysis, equal amounts of total proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and transferred onto PVDF membranes (Immobilon-P, IPFL 000 10, Millipore, USA). After blocking for 1 hour at room temperature in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% non-fat milk, the membranes were incubated overnight at 4°C with the primary antibody directed against the protein of interest. After several washes, an appropriate HRP-conjugated secondary antibody (ZB-2301, ZB2305, goat IgG; ZSGB-BIO, China) was applied for 1 hour at room temperature. Peroxidase activity was visualized using the ECL Western Blotting Detection kit (NC15079, SuperSignal west pico trial kit, Thermo, USA) and capture images (c300, Azure biosystems, USA). Signal intensity was measured using Image J software (NIH, Bethesda, MD, USA). For quantitative analysis, the Beclin 1, LC3-I, LC3-II, p62, p-mTOR, p-p70S6k, mTOR, p70S6k and signals of each sample were normalized to GAPDH. Changes in signal intensity were then expressed as fold increase of the ipsilateral versus the contralateral side for each individual animal.

The following primary antibodies and dilutions were used: anti-LC3 1:1,000 (2775s; CST), anti-p62/SQSTM1 1:1,000 (5114; CST), mTOR (2983, CST), p-mTOR (2971, CST), p70S6k (2708, CST), p-p70S6k (9234, CST), anti-GAPDH 1:10,000 (Vazyme; China), Beclin-1 antibody was obtained from Prof. Wang.

Immunofluorescence

Animals were deeply anesthetized and perfused transcardially through the ascending aorta with 0.9% NaCl solution, followed by cold 4% paraformaldehyde in 0.1 M PBS. L5 spinal cord segments were removed, post-fixed in the same fixative solution overnight, and cytoprotected with 30% sucrose in ddH_2O for 24 h at 4°C. The spinal cord was sectioned on cryostat at 20 μm thickness. Sections were incubated with primary antibody against LC3, followed by incubation with the FITC-conjugated secondary antibody (SA00003-2, Proteintech). For double immunostainings, sections were sequentially incubated also with anti-NeuN (neuronal marker; MAB377, Millipore) or anti-GFAP (astrocyte marker; MAB360, Millipore) antibody followed by incubation with TRITC-conjugated secondary antibody (SA00007-1, Proteintech). Cell nuclei

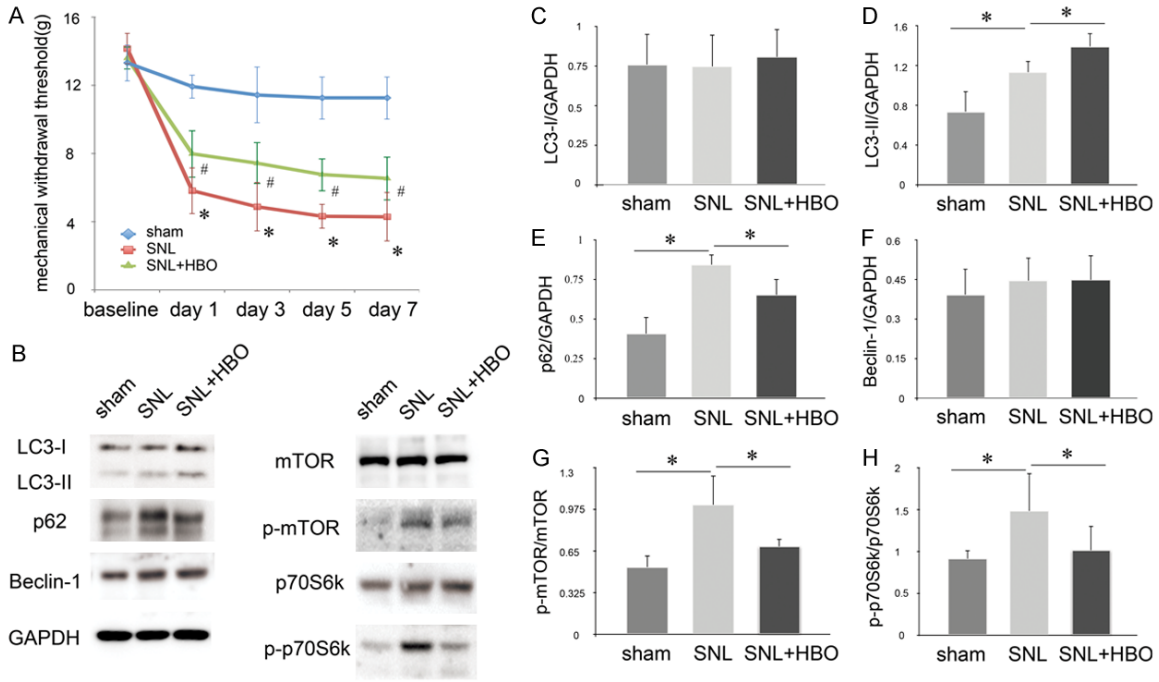

Figure 1. (A) The mechanical withdrawal threshold (MWT) tests were carried at 2 days before operation (baseline), Day 1, 3, 5, 7 post operation. Hyperbaric oxygen treatment significantly increases the level of MWT. (B) The immunoblotting show the level of LC3-I, LC3-II, p62, Beclin 1 and mTOR, p70S6k, p-mTOR, p-p70S6k in the spinal cord in sham group, SNL group and SNL+HBO group were shown. The SNL model was characterized by increase LC3-II, p62, mTOR and p70S6k vs. sham group. SNL+HBO group shows higher LC3-II and lower p62 level and mTOR pathway is inactived vs. SNL group (C-H). Data was mean ± SEM of six rats for each group, *P<0.05.

were counterstained with DAPI. Immunofluorescence images were taken under a Nikon C1Si confocal microscope.

Statistical analysis

All statistical analysis was carried out with IBM SPSS Statistics 22 software. Data were analyzed by Student's t-test or one-way or two-way ANOVA followed by the Tukey post hoc test. The results were expressed as the mean ± SEM. *p* values <0.05 were considered significant.

Results

Hyperbaric oxygen produced anti-nociceptive effect and induced autophagy in spinal cord

Ligation of the spinal nerve L5 produced an early onset and long lasting mechanical hypersensitivity. SNL rats received hyperbaric oxygen treatment for 5 days significantly reduced severity and duration of mechanical allodynia (**Figure 1A**) as measured by Von Frey filaments.

LC3 is the most widely monitored autophagy-related protein. The nonlipidated and lipidated forms are referred to as LC3-I and LC3-II respectively [5, 28]. LC3-II is autophagosomal membrane-associated form, which indicates autophagosome formation. Spinal cord segments were harvested for Western blotting on POD7. There was no significant difference in LC3-1 expression among Sham, SNL and SNL+HBO groups (**Figure 1C**). However, LC3-II level was significantly increased in SNL group in comparison to the sham group. SNL+HBO group further increased LC3-II expression as compared to SNL (**Figure 1D**).

Increases in the level of autophagosomes or LC3-II can reflect either the induction of autophagy or inability to clean autophagosome or amphisome [5]. To confirm whether the autophagy is induced with HBO, we further analyzed expression of SQSTM1/p62 protein, which should be accumulated when autophagy is impaired. As expected, SNL group showed a higher p62 level than sham group, HBO treatment reversed this effect (**Figure 1E**).

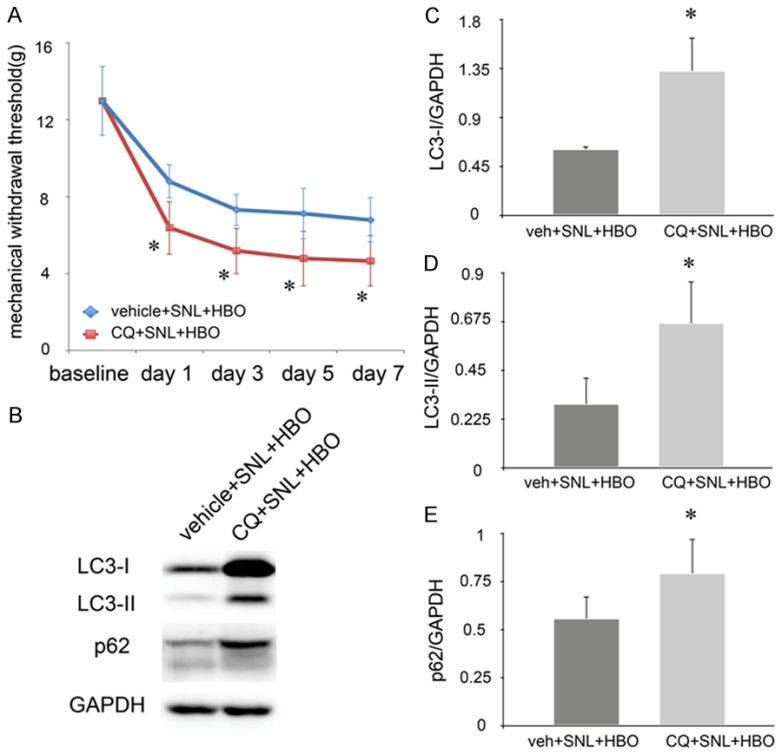

Figure 2. A. Significant reductions were observed in MWT from day 1 in CQ+SNL+HBO group in comparision to vehicle+SNL+HBO group. B. Representative Western blots of LC3-II and p62 from CQ+SNL+HBO group and vehicle+SNL+HBO group were shown. C-E. LC3-II and p62 protein levels were significantly increased in the CQ+SNL+HBO group compared to vehicle+SNL+HBO group. Values were expressed as mean ± SEM. n=6, *P<0.05.

Beclin-1/Atg6 is a key protein in the induction of autophagy and is essential for autophagosome formation [18]. A slight increase of Beclin-1 was observed in SNL and SNL+HBO groups as compared to sham group (**Figure 1F**) but the difference did not reach statistical significance.

mTOR and mTOR pathway downstream target protein p70S6K were evaluated by western blotting. Both p-mTOR and p-p70S6K were increased in SNL as compared to sham group, and HOB treatment further decreased the activity of these proteins (**Figure 1G**, **1H**).

Blockage of autophagy activity diminished HBO's analgesic effects

To further confirm that HBO alleviates pain through activation of autophagy, rats were injected with Chloroquine (CQ), an autophagy blocker. A significant reduction of MWT was observed in CQ+SNL+HBO group in comparison to vehicle+SNL+HBO group (**Figure 2A**) on day 1.

LC3-II formation and p62 expression were markedly increased in CQ+SNL+HBO group in comparison to vehicle+SNL+HBO group (**Figure 2B-E**), which suggested that CQ inhibited the autophagic activation by HBO treatment.

Rapamycin enhance HBO treatment effect via inhibiting mTOR pathway

The above results suggest that HBO treatment inhibits mTOR pathway and increases autophagy in spinal cord. To further test this hypothesis, we examined the effects of intrathecally administrating rapamycin on SNL-induced mechanical pain (**Figure 3A**) and the expression of mTOR and p70S6K phosphorylation level (**Figure 3B**). A significant increase of MWT was observed in RAPA+SNL+HBO group in comparision to vehicle+SNL+HBO group (**Figure 3A**) from POD3. The activity of mTOR and p70S6K was decreased in RAPA+SNL+HBO group, compared with vehicle+SNL+HBO group (**Figure 3C**, **3D**) as detected by western blotting.

LC3 is mainly expressed in neurons following hyperbaric oxygen treatment

To further characterize the cellular localization of spinal autophagy following HBO treatment, immunofluorescent staining was performed using antibodies against LC3, neuronal marker NeuN, and astrocytic marker GFAP. LC3 was mainly expressed in neurons as LC3 was colocalized with NeuN in spinal cord sections in SNL+HBO group (**Figure 4**).

Discussion

Neuropathic pain is characterized by pain hypersensitivity that is mediated by both

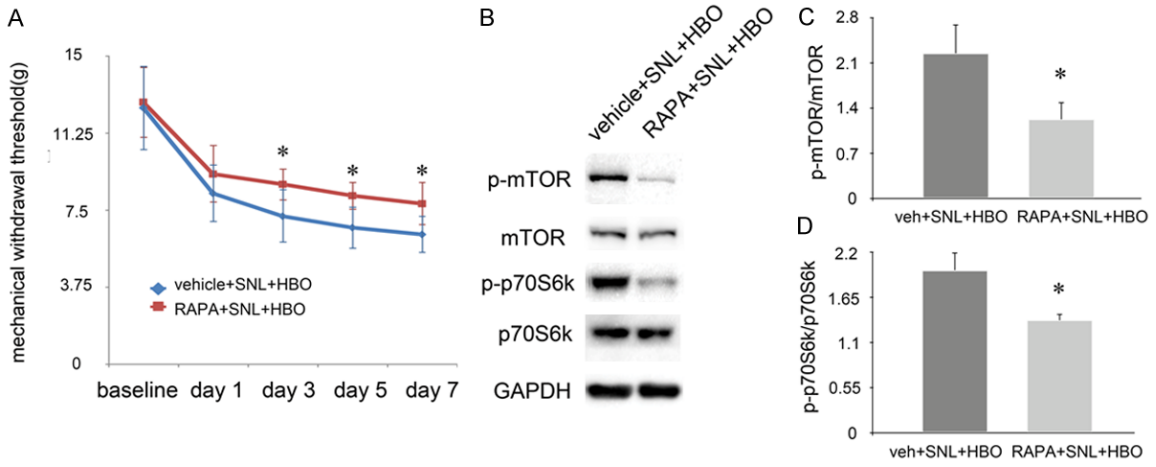

Figure 3. MWT (A) from day 3 show a higher level in RAPA+SNL+HBO group compared with vehicle+SNL+HBO group. (B) Evaluation the effect of rapamycin in SNL+HBO rats: Western blot analysis of spinal cord from RAPA+SNL+HBO group and vehicle+SNL+HBO group rats. (C, D) p-mTOR and p-p70S6k were significantly decreased in the RAPA+SNL+HBO group compared to the vehicle+SNL+HBO group. Signals of each band were normalized to the respective GAPDH. Data were expressed as mean ± SEM. n=6, *P<0.05.

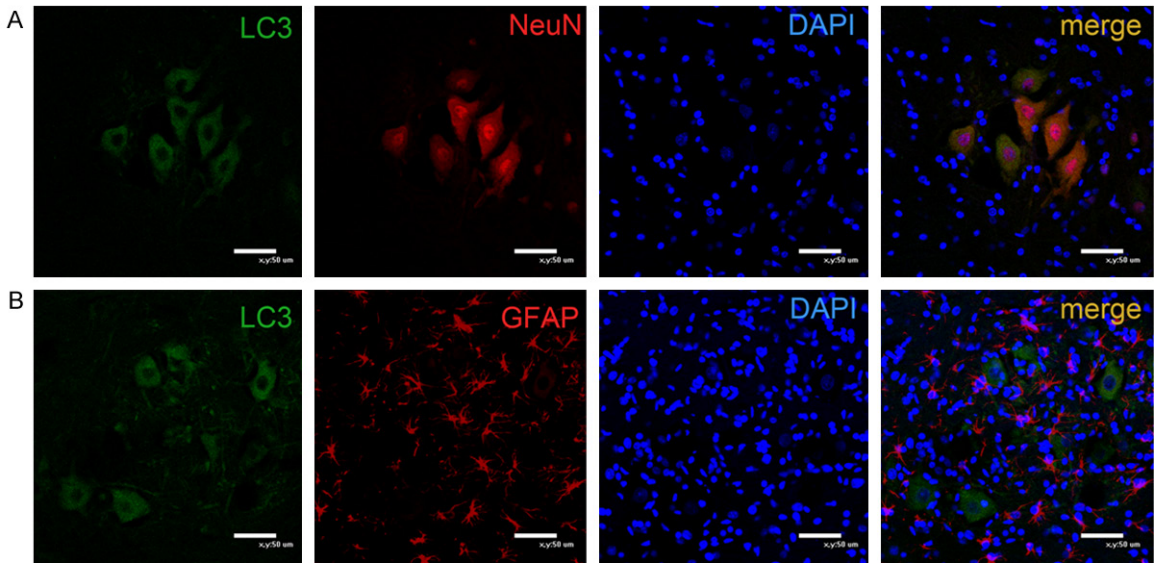

Figure 4. Double immunostaining showed that LC3 (green) were present almost in neuronal cell exclusively, as shown by NeuN (red)-positive (A) cell bodies, not within GFAP (red)-positive structure (B). Each image is from L5 spinal cord sections of SNL+HBO group. Scale bars =50 μm. SNL+HBO group rats L5 were shown above, n=6.

peripheral and central neuronal synaptic plasticity [33]. It is a significant health problem worldwide. Treatments of neuropathic pain remain a challenge due to the complex changes in the functions of receptors, enzymes, and voltage-dependent ion channels in sensory neurons [29]. Neighboring astrocytes and microglia act as powerful modulators of pain [6].

Autophagy responds to environmental cues through regulatory factors that signal to the autophagic machinery, which consists of products of the autophagy-related genes (atg) [4]. Beclin 1 autophagy signaling complex includes subunits ATG14L, VPS34 and p150/VPS15 [37]. Stimulation of Beclin1-interacting complex generates phosphatidylinositol-3-phosphate (PI3P), which promotes autophagosomal mem-

brane nucleation [13, 39]. ATG14L-Beclin 1-VPS34 complex may reflect the central pathway for pre-autophagosomes initiation [4]. LC3 was the first mammalian protein discovered to be specifically associated with autophagosomal membranes. The conversion of a cytosolic truncated form of LC3 (LC3-I) to autophagosomal membrane-associated and phosphatidylethanolamine-conjugated form (LC3-II) indicates autophagosome formation. It is worth to be noted that impaired autophagosome-lysosome fusion may result in increasing LC3 level [4]. A method for detecting the autophagic flux is measuring p62 (SQSTM1/sequestosome 1) degradation [3, 24, 36].

Our data showed that LC3-II accumulation was accompanied with a significant elevation of p62 levels in SNL model indicating a blockade of autophagic flux in SNL model [1, 2, 16, 57]. Hyperbaric oxygen treatment has positive effects on relief of neuropathic pain in rat models and clinical patients. SNL promoted the expression of LC3-II, the autophagosome-associated LC3 form. However, LC3-I and Beclin 1 expression were not changed. In contrast, p62 was strongly up-regulated, indicating a block of autophagosomes turnover in this experimental neuropathic pain model. HBO treatment induces the formation of autophagosomes following SNL and degradation of p62, indicating spinal autophagy is elevated after HBO treatment. However, the expression of p62 can also be modulated by other factors [28]. Presentation of p62 might not be sufficient to estimate the autophagic flux.

Lysosomal degradation can be inhibited by administrating inhibitiors, which can neutralize the lysosomal pH such as bafilomycin A_1, NH_4Cl and chloroquine [25, 44]. Bafilomycin A1 can cause autophagosomes fusion block and neutralize the pH as well, but the inhibition of fusion may the result of the block in ATP2A activity [35]. Moreover, chloroquine can block autophagosomes fusion with lysosomes in comparasion with NH_4Cl. To confirm the upregulation of spinal autophagy following HBO treatment, chloroquine was intrathecally administrated as an autophagy degradation inhibitor. Immunoblotting results showed that LC3-I, LC3-II level were significantly increased, and p62 level was parallel with LC3-II. These data, for the first time, show that HBO treatment relieves neuropathic pain by enhancing spinal autophagic flux. Interestingly, Beclin-1 expression had no differences among sham, SNL and SNL+HBO groups.

Molecular mechanisms underlying neuropathic pain are complex, and neurons and microglia cells play different roles in neuropathic pain. To further characterize the cellular localization of autophagy, LC3-NeuN and LC3-GFAP co-localization were investigated by immunofluorescence in SNL+HBO group. Our data showed that LC3 was mainly expressed in NeuN positive cells, not in GFAP, indicating HBO treatment induced spinal autophagy activation in neurons not in astrocytes. HBO treatment could attenuate neuropathic pain by inhibiting astrocyte activating [59], while the exact mechanisms through which autophagy in neurons may be linked to astrocyte activation in neuropathic pain following HBO treatment need to be further elucidated.

mTOR can negatively regulate autophagy [20, 33]. mTOR pathway is an important molecular mechanism of pain onset and maintenance. Previous studies have reported that mTOR pathway is activated in neuropathic pain not only in spinal level, such as spinal neurons [58] and glial cells [7, 50], but also in rostral ventromedial medulla (RVM) [52] and cerebrospinal fluid-contacting nucleus [32]. Using Rapamycin, we showed that HBO treatment reduces mTOR phosphorylation and up-regulate autophagy in SNL model. Moreover, inhibition of mTOR signaling pathway promotes autophagy is a novel perspective for management of neuropathic pain. This study provides a new molecular understanding of hyperbaric oxygen treatment in neuropathic pain relieving.

Acknowledgements

We thank Prof. Liu Yang for expert technical advice, Prof. Jin Li, Wang Lili for skillful advice. Supported by National Natural Science Foundation of China grant: No. 81600971.

Disclosure of conflict of interest

None.

Authors' contribution

Zhao Ping and Liu Yongda designed and conceived the experiments. Liu Yongda performed the experiments. Liu Yongda, Wang Zhibin analyzed experimental data. Reagents, materials

and analysis tools are contributed by Zhao Ping, Liu Yongda, Han Guang.

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