

Hyperbaric oxygen therapy improves neurological function via the p38-MAPK/CCL2 signaling pathway following traumatic brain injury

Yingzi Jiang^{a,b,*}, Yuwen Chen^{a,b,*}, Chunling Huang^{a,b,*}, Anqi Xia^{a,b}, Guohua Wang^c and Su Liu^a

Objective The anti-inflammatory mechanisms of hyperbaric oxygenation (HBO) treatment on traumatic brain injury (TBI)-induced neuroinflammation remain unclear. The aim of this study was expected the effect of HBO on CCL2-related signaling pathway following severe TBI in rats.

Methods The severe TBI model in rats was induced by controlled cortical impact. TBI rats were treated with CCR2 antagonist, p38 inhibitor, or HBO. Modified neurological severity scores and Morris water maze were used to evaluate neurological and cognitive function. The expression levels of CCL2 and CCR2 were measured by ELISA and real-time fluorescence quantitative PCR. Phospho-p38 expression was analyzed by western blotting.

Results TBI-induced upregulation of CCL2, CCR2, and p38 in the injured cortex. Application of CCR2 antagonist improved neurological and cognitive function of TBI rats. Application of p38 inhibitor decreased expression of CCL2 and CCR2 in the injured of TBI rats, meanwhile improved neurological and cognitive function. HBO improved

neurological and cognitive function by decreasing the expressions of CCL2, CCR2, and phospho-p38.

Conclusions This study indicates that the p38-MAPK-CCL2 signaling pathway could mediate neuroinflammation and HBO therapy can modulate neuroinflammation by modulating the p38-MAPK-CCL2 signaling pathways following TBI. This study may provide theoretical evidence for HBO treatment in the treatment of TBI. *NeuroReport* 32: 1255–1262 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

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Keywords: hyperbaric oxygen, traumatic brain injury, p38, CCL2, CCR2

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Introduction

Traumatic brain injury (TBI) is one of the great challenges to public health worldwide that presents various neurological impairments ranging from mild alterations of neurological function to an unrelenting comatose state and death [1,2]. Severe TBI easily resulted in permanent impairment that including cognitive impairments, mood disorders, sensory and motor changes, speech and language dysfunctions, and persistent vegetative state [3–6]. Major research and clinical efforts have focused on therapeutic interventions to secondary injuries following TBI, because of secondary injuries may persist for weeks to months.

Hyperbaric oxygenation (HBO) denotes breathing of 100% oxygen at a pressure between one and three times that of atmospheric pressure. Previous experimental and

clinical studies had demonstrated the beneficial effects of HBO therapy following severe TBI on attenuation of secondary injury that significant improvement of Glasgow outcome scale score and reduction of overall mortality [6–9]. Our previous findings indicated that HBO significantly improved cognitive function and inhibited the proliferation of astrocyte [10]. HBO therapy has been recognized as an effective treatment for modulating neuroinflammatory responses secondary injuries induced by TBI [11–13]. Chemokines are important mediators of inflammation following TBI and modulate chemokine signaling, especially C–C motif ligand (CCL)2 (CCL2, also known as monocyte chemoattractant protein-1, MCP-1)/Chemokine C–C motif receptor 2 (CCR2), may be beneficial in TBI treatment [14]. We further found expression of chemokine CCL2 and its primary receptor CCR2 upregulated in the injured cortex after TBI, CCL2 protein was mainly co-localized with the astroglial marker glial fibrillary acidic protein, and then targeting the CCL2–CCR2 pathway may provide a novel therapeutic approach for the treatment of TBI [15,16]. This study was expected to

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explore the potential signaling pathway that modulates CCL2–CCR2 pathway following severe TBI in rats and the effect of HBO on the signaling pathway.

Materials and methods

Animals and surgery

Male Sprague-Dawley rats (230–260 g) were purchased from the Experimental Animal Center of Nantong University. The cortical controlled injury (CCI) was used to establish a severe TBI model as described previously [17,18]. Rats were accepted intraperitoneal anesthesia (10% chloral hydrate), fixed in a stereotactic frame, then subjected to right parietal craniotomy, and exposed to the underlying dura mater with a dental drill (3 mm posterior to bregma, 3 mm lateral to the midline, diameter of 6 mm). Coordinates of 3 mm posterior to bregma and 3 mm lateral to the midline were taken as the impact center. TBI was conducted using the TBI-0310 impactor device (Precision Systems and Instrumentation, St. Paul, Minnesota, USA). The impact parameters are as follows: speed 4 m/s, depth 3 mm, and impact time 150 ms. Sham operation rats underwent the same craniotomy but did not receive the impact. All experimental procedures were approved by the Experimental Animal Ethics Committee of Nantong University.

Modified neurological severity scores

Neurological function of TBI rats and sham group rats was evaluated with the modified neurological severity method [19]. The evaluation aspects included motor (six points), sensory (two points), balance beam test (six points), lack of reflexes, and abnormal activity (four points). Normal score is 0 point and the maximal deficit score is 18 points. If the animals did not complete the required movements or lack of reflexes, one point was obtained. The higher the total score obtained, the more severe the nerve disfunction.

Morris water maze test

Escape latency to the platform and the number of platform crossings were recorded as described previously with the Morris water maze (MWM) test [16,20]. All groups of rats were received successive adaptive training before injury. Escape latency to the platform was measured on each of four daily trials and then averaged. If the animal did not find the platform within 120 s, the escape latency was recorded 120 s. Probe trials were conducted after the platform was removed to assess spatial memory that documented the number of crossings over the former platform. Two probe trials were conducted and the average was recorded for analysis.

Drugs and administration

Rats were randomly divided into five groups: sham, TBI, TBI + vehicle, TBI + low-dose, and TBI + high-dose group. The CCR2 antagonist (RS504393) (Tocris, Bristol, South West England, UK) or p38 inhibitor (SB203580) (Calbiochem, Merck, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO) and diluted

with PBS to a low dose (2.5 µg/10 µL) and a high dose (25 µg/10 µL) and then injected into the injured area, respectively, at 1 h following TBI as described previously [15]. Alternatively, the corresponding TBI vehicle group received 10 mL PBS+DMSO. All consecutive 3 days injections were performed while rats were induced anesthesia with 10% chloral hydrate. Drugs and solvent were injected at 2 mL/min (requiring about 5 min) and the needle was kept in place for 5 min by 10 mL Hamilton microliter syringes.

Hyperbaric oxygenation therapy

Rats were randomly divided into four groups: sham, sham + HBO, TBI, and TBI + HBO group. The rats of sham + HBO group and TBI + HBO group were subjected to continuous HBO therapy once a day for 10 days the first HBO therapy was given 6 h after TBI [14]. The rats were placed in a homemade cage and then transferred to a single hyperbaric chamber (Shanghai 701 Institute Yangyuan Medical HBO Chamber Factory, Shanghai, China). The therapeutic pressure of the oxygen chamber was increased to 0.2 MPa slowly for about 15 min, then maintained 0.2 MPa for about 60 min that oxygen concentration was kept above 95%, and finally slowly decreased to atmospheric pressure for about 15 min.

Tissue collection

Rats were anesthetized using 10% chloral hydrate via intraperitoneal injection, then perfused with PBS until the liver turned white by the left ventricle. After dissection on ice, the cerebral cortex around the injured area was collected, and then stored at –80°C after quick freezing with liquid nitrogen. The tissue was used for the following experiments.

Real-time fluorescence quantitative PCR

Total RNA was extracted from the cerebral cortex of the injured cortex using Trizol reagent (Invitrogen, Carlsbad, California, USA). According to the manufacturer's instructions (Takara, Shiga, Japan) total RNA (1 µg) was then reversed transcribed to cDNA. Real-time qPCR was performed in a Step One Plus real-time qPCR instrument using the primer sequences shown in Table 1. The RT-qPCR amplification program was as follows: pre-denaturation at 95°C for 3 min; 40 cycles of 95°C for 10 s and 60°C for 30 s; dissolution at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal

Table 1. Primer sequences

Genes	Primers	Sequences
GAPDH	Forward	TCCTACCCCAATGTATCCG
	Reverse	CCTTTAGTGGGCCCTCGG
CCL2	Forward	TGCTGCTACTCAITCACTGGC
	Reverse	CCTTATTGGGGTCAGCACAG
CCR2	Forward	TGCTACTCAGGAATCCTCCACAC
	Reverse	GGCCTGGTCTAAGTGCATGTCAAC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

control and gene expression level was analyzed using the $2^{-\Delta\Delta CT}$ method.

ELISA

A rat CCL2 ELISA kit from R&D Systems (MJE00; Minneapolis, Minnesota, USA), a rat CCR2 ELISA kit, CSB-EL004841RA from (CUSABIO TECHNOLOGY, Wuhan, Hubei, China). The tissue from the damaged area of the cerebral cortex was added to 1.5 mL EP tubes containing 250 μ L protein lysate and homogenized. The lysis reaction proceeded for 30 min, and the supernatant was collected by centrifugation. The total protein concentration was measured by bicinchoninic acid protein assay (Pierce, Rockford, Illinois, USA). The sample volume was 100 μ g per well, and ELISA was performed according to the manufacturer's instructions, respectively.

Western blot analysis

The tissue source and total protein concentration were the same as ELISA. Protein of 30 μ g of was loaded and separated on 10% SDS-PAGE gels (Beyotime, Beijing, China) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA) using a wet electroblotting system (Bio-Rad, California, USA). The membranes were blocked with 5% BSA for 2 h at room temperature. The antibodies used were shown as follows: p-p38 (9211, 1:1000; Cell Signaling, Boston, Massachusetts, USA), and GAPDH (MAB374,

1:10000; Millipore). Images were captured using the Odyssey Imaging System (LI-COR Bioscience, Lincoln, Nebraska, USA), and grayscale values were analyzed using Image J software (NIH, Bethesda, Maryland, USA).

Statistical analysis

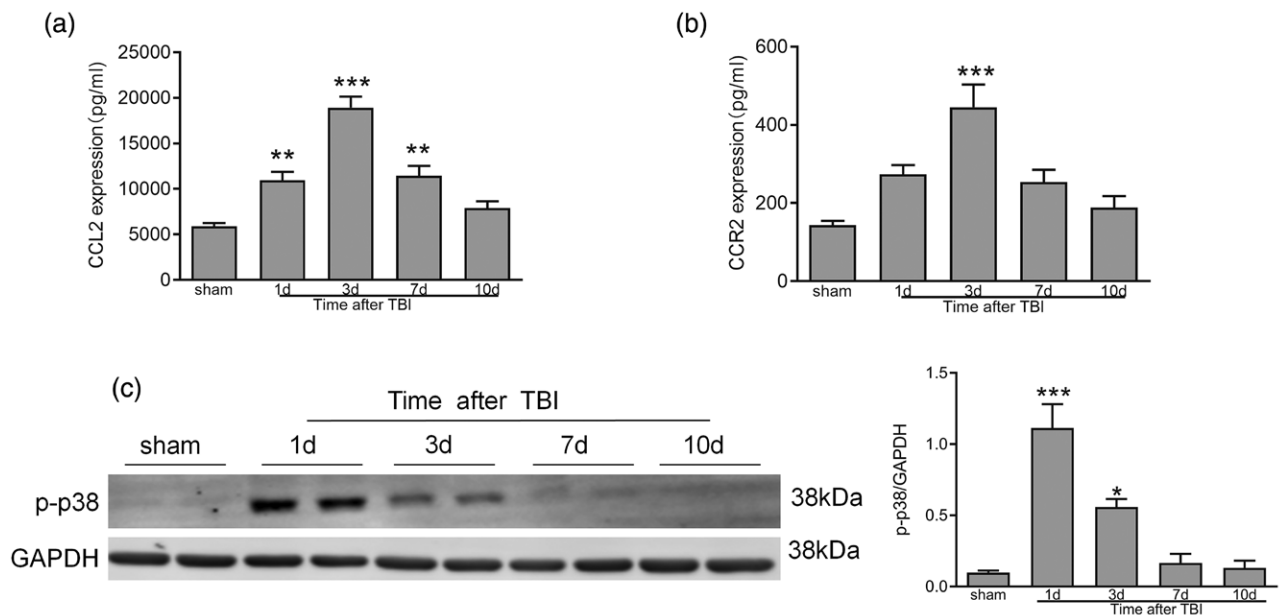
All data were expressed as mean \pm SEM. For western blotting, ImageJ was used to measure the grayscale values of specific bands. The relative expression level of p-p38 was standardized to the level of GAPDH. A two-way analysis of variance (ANOVA) was used to analyze the modified neurological severity scores (mNSSs) after HBO therapy with Bonferroni post-test to compare replicate means by row. Multi-group comparisons were performed using one-way ANOVA with post hoc Bonferroni correction. All data were analyzed using GraphPad Prism 8.0 (San Diego, California, USA).

Results

Traumatic brain injury-induced upregulation of CCL2, CCR2, and p-p38 expression in the injured cortex of traumatic brain injury rats

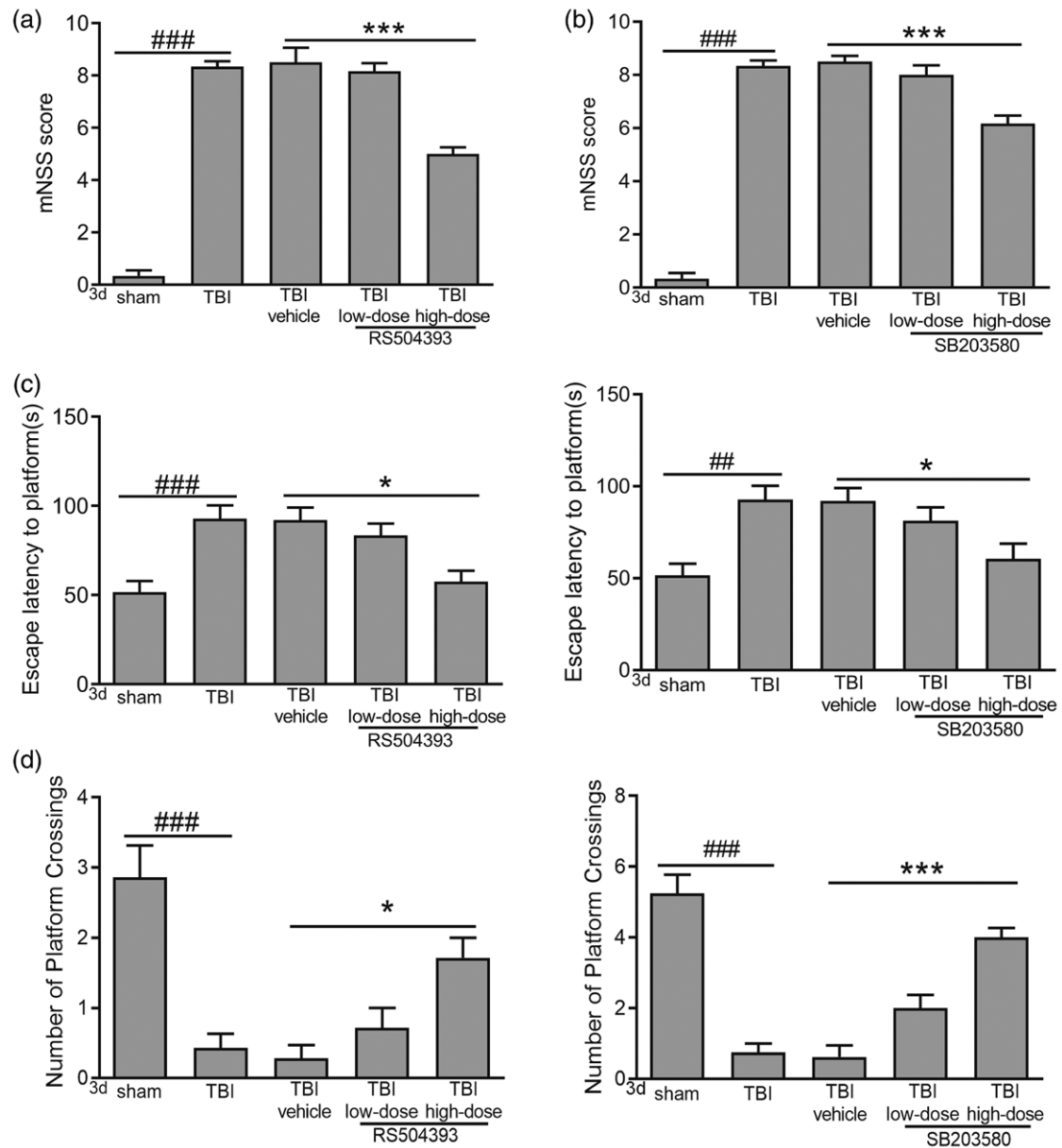
The expression level of CCL2 and CCR2 protein was measured by ELISA, and the expression of p-p38 in the injured cortex was assessed by western blot in the injured cortex at 1, 3, 7, and 10 days following TBI. As shown in Fig. 1a and b, the protein expression of CCL2 and CCR2 protein peaked on the third day and then showed

Fig. 1



TBI-induced upregulation of CCL2, CCR2, and p-p38 expression in the injured cortex of TBI rats. (a) Expression of CCL2 protein peaks on the third day after TBI, compared to sham group ($n=5$ /group). (b). Expression of CCR2 protein peaks on the third day after TBI, compared to sham group ($n=5$ /group). (c) The expression of p-p38 decreased after peaking on the first day after TBI ($n=3$ /group). Values are expressed as mean \pm SEM. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. sham group. TBI, traumatic brain injury.

Fig. 2



The neurological and cognitive function of TBI rats was improved by CCR2 antagonist (RS504393) or p38 inhibitor (SB203580). (a) The mNSS score of TBI rats decreased after the application of high-dose CCR2 antagonists. (b) The mNSS score of TBI rats decreased after the application of high-dose p38 inhibitor. (c) The escape latency of TBI rats was reduced after the application of a high-dose CCR2 antagonist or p38 inhibitor. (d) The number of platform crossings of TBI rats increased after the application of high-dose CCR2 antagonist or p38 inhibitor. Values are expressed as mean \pm SEM ($n=8$ /group). *** $P<0.001$, * $P<0.05$ vs. TBI + vehicle group. ### $P<0.001$, ## $P<0.01$, vs. sham group. TBI, traumatic brain injury; mNSS, modified neurological severity score.

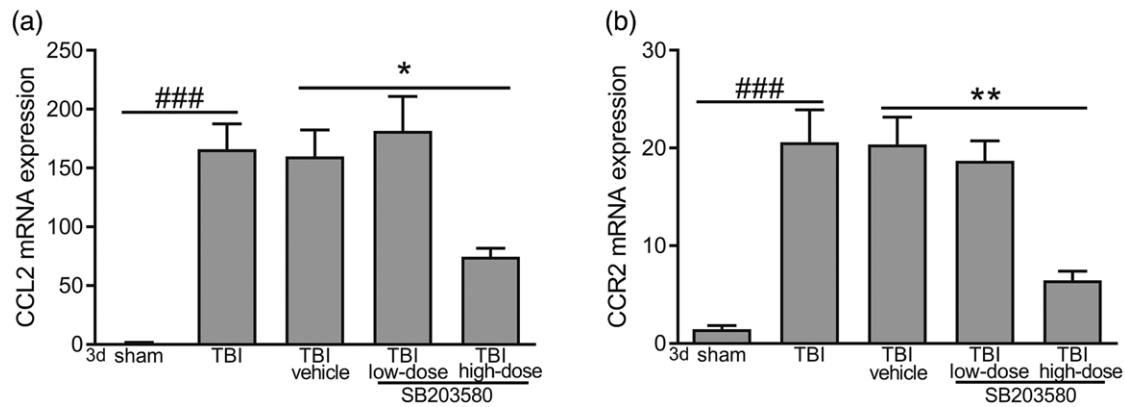
a decreasing trend after TBI. As shown in Fig. 1c, compared with the sham group, p-p38 peaked on the first day after TBI and then decreased.

CCR2 antagonist and p38 inhibitor improved neurological and cognitive function in traumatic brain injury rats

To evaluate the effect of CCL2–CCR2 and p38 on the neurological and cognitive function of TBI rats, mNSS

and MWM were tested after injection of CCR2 antagonist RS504393 or p38 inhibitor SB203580 in the injured cortex of TBI rats. As shown in Fig. 2a and b, the mNSS of rats after TBI were significantly higher than that of the sham group. Compared to TBI + vehicle group, the mNSS score of the TBI + high dose group decreased significantly at 3 days of TBI by the higher dose of RS504393 or p38 inhibitor SB203580, while the low dose had no

Fig. 3



p38 inhibitor (SB203580) downregulated CCL2 and CCR2 mRNA expression. CCL2 and CCR2 mRNA expression decreased significantly at 3 days after continuous injection of high doses of p38 inhibitor. Values are expressed as mean \pm SEM ($n=6$ /group). * $P<0.01$, ** $P<0.05$, vs. TBI + vehicle group; ### $P<0.001$, vs. sham group. TBI, traumatic brain injury.

effect. As shown in Fig. 2c and d, the escape latency of TBI rats was significantly prolonged and the number of platform crossings was reduced compared with the sham group. High-dose CCR2 antagonist RS504393 or p38 inhibitor SB203580 decreased the average escape latency and increased the number of platform crossings at 3 days following TBI.

p38 Inhibitors downregulated mRNA expression of CCL2 and CCR2 in traumatic brain injury rats

To verify whether p38 regulates the expression of CCL2/CCR2, the expression changes of CCL2/CCR2 were observed after the application of p38 inhibitor SB203580. As shown in Fig. 3, the mRNA expression of CCL2 and CCR2 decreased significantly after 3 days of continuous injection of high doses of p38 inhibitor compared with the TBI + vehicle group, indicating that p38 could regulate CCL2 and CCR2.

Hyperbaric oxygenation treatment improved the neurological and cognitive function of rats following traumatic brain injury

The effect of HBO therapy on the neurological and cognitive function of TBI rats was observed by mNSS and MWM. After consecutive 10 days HBO treatment, the mNSS score decreased significantly on days 3, 7, and 10 (Fig. 4a). The escape latency in the locomotor navigation test was significantly shorter in the TBI + HBO group than in the TBI group on days 3, 7, and 10 (Fig. 4b). In the spatial exploration trial, the number of platform crossings increased significantly with HBO treatment than that of the TBI group on days 3, 7, and 10 (Fig. 4c). These results showed that HBO treatment could improve the neurological and cognitive function of rats following TBI.

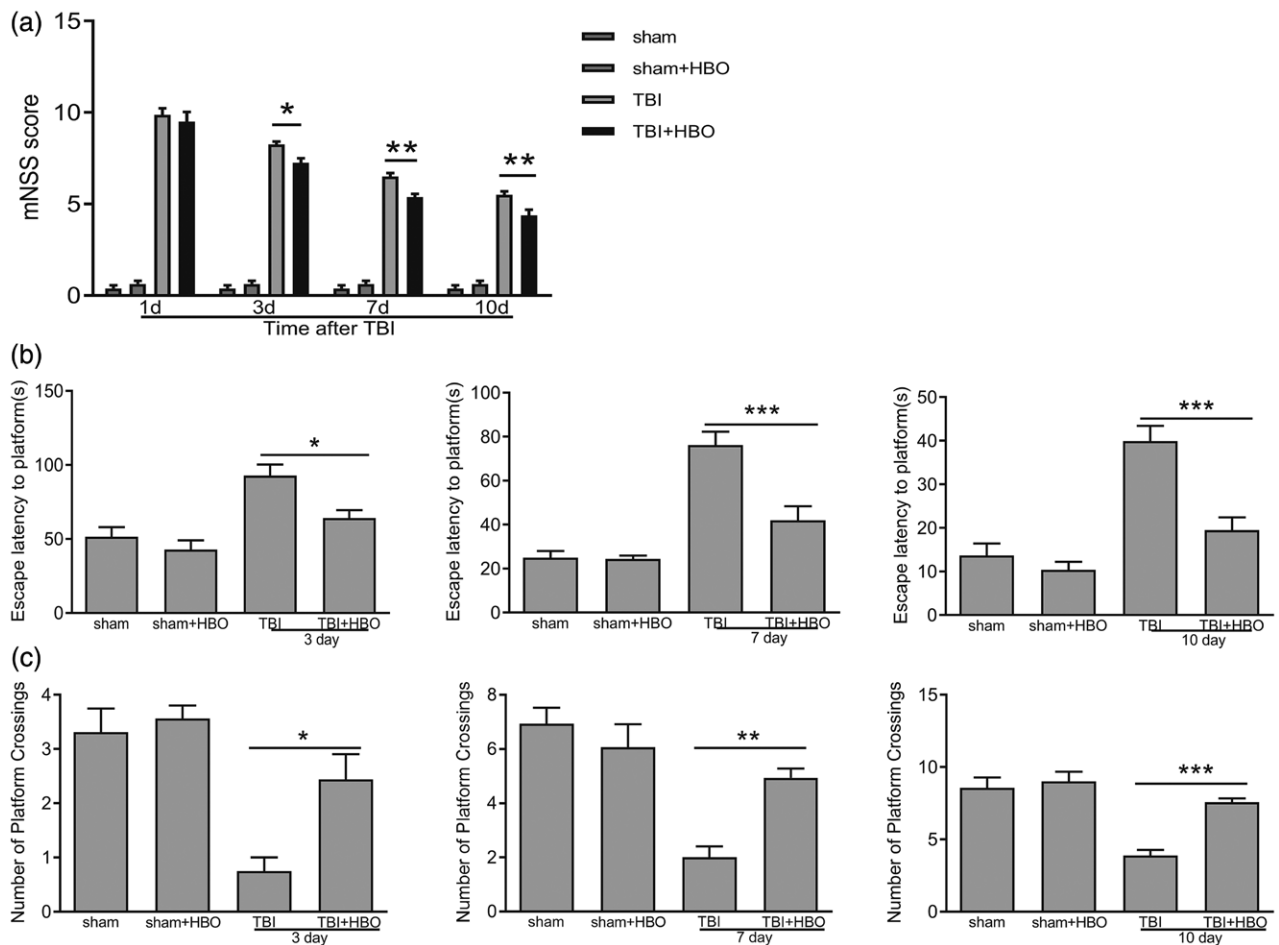
Hyperbaric oxygenation treatment downregulated CCL2 and CCR2 mRNA expression by p-p38 signaling in the injured cortex after traumatic brain injury in rats

To verify whether HBO treatment regulates the expression of CCL2 and CCR2 gene expression, their mRNAs were measured detected by RT-qPCR after 10 days of continuous HBO therapy. In Fig. 5a and b, the results showed that the mRNA expression of CCL2 and CCR2 with HBO therapy were significantly decreased compared at days 1, 3, and 7 with the TBI group, indicating that HBO treatment could downregulate the expression of CCL2 and CCR2. To verify whether HBO therapy regulates CCL2/CCR2 expression via p38 signaling, we tested the expression level of p-p38 protein after 3 days of continuous HBO treatment by western blot. In Fig. 5c, the results show that the level of p-p38 decreased significantly in the TBI + HBO group compared with the TBI group, suggesting that HBO treatment modulated expression of CCL2 and CCR2 by downregulating the expression of p-p38.

Discussion

Chemokine CCL2 is known to recruit monocytes and macrophages to promote inflammation. In this study, we found that the expressions of the chemokine CCL2 and its major receptor CCR2 increased and peaked on the third day in the injured cortex after TBI, this same as previously [16,21]. Meanwhile, neurological and cognitive functions of TBI rats improved significantly with CCR2 antagonists. Deficiency of the CCR2 receptor improved neurocognitive functional recovery and neuronal survival in a CCI mouse model of TBI [22]. Our previous study had demonstrated CCL2-CCR2 signaling might initiate a deleterious inflammatory response contributing to secondary neurodegeneration and cognitive dysfunction after

Fig. 4

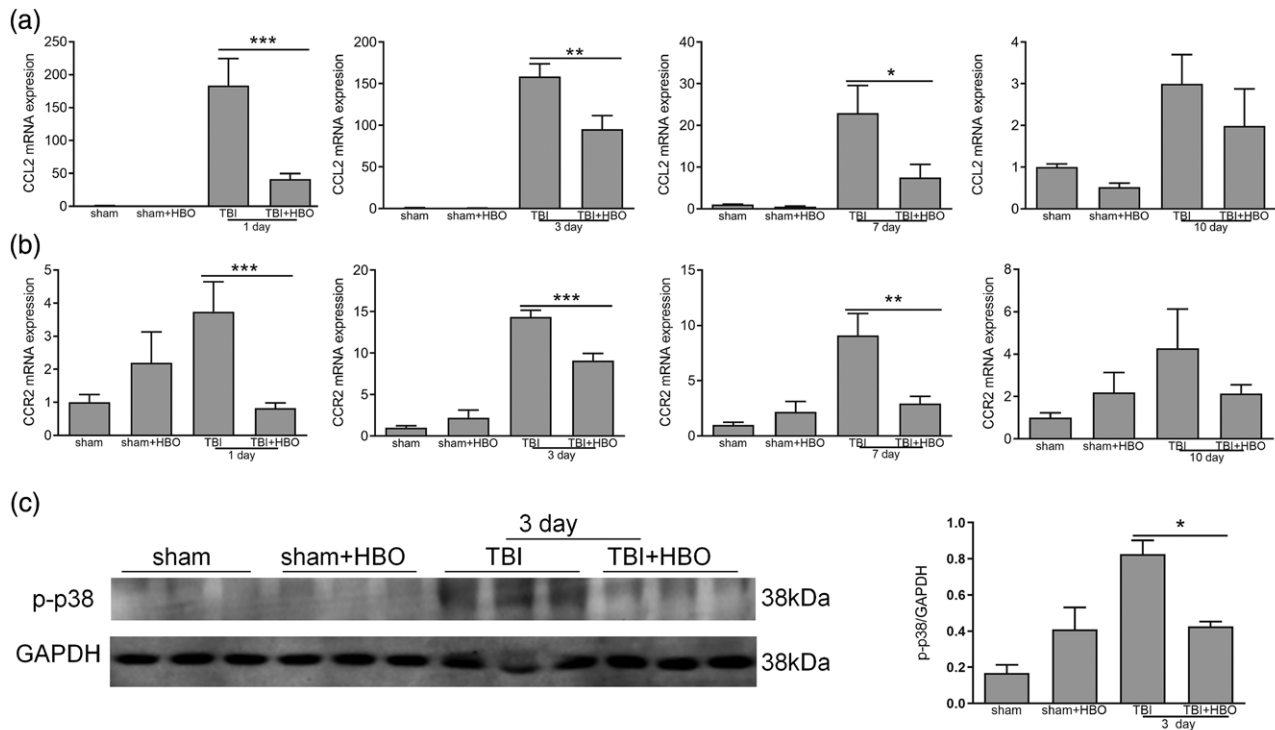


The neurological and cognitive function of TBI rats was improved after HBO therapy. (a) mNSS scores were lower after HBO treatment than TBI group at 3, 7, and 10 days. (b) In the locomotor navigation test, the escape latency shortened significantly with HBO treatment on days 3, 7, and 10. (c) In the exploration test, the number of platform crossings was higher after HBO treatment than TBI group on 3, 7, and 10 days. Values are expressed as mean \pm SEM ($n=8$ /group). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. TBI group. HBO, hyperbaric oxygen; mNSS, modified neurological severity score; TBI, traumatic brain injury.

cortical injury [16]. Modulation of CCL2–CCR2 inflammatory pathways may be a broadly effective treatment strategy on TBI [23]. Inhibiting TBI-induced upregulation of CCL2 via modulating the AKT/NF- κ B p65 signaling pathway played a neuroprotective role in rat TBI [24]. We further found that the expression of p-p38 increased after TBI. It was reported that targeting p38 mitogen-activated protein kinase (MAPK) signaling pathway attenuated TBI-induced neuroinflammation and apoptosis that demonstrated play a neuroprotective role in preventing secondary damage post-TBI [25–28]. Our study demonstrated P38 inhibitor decreased the expression of CCL2/CCR2, and meanwhile, improved neurological and cognitive functions suggested that p38-MAPK partly preventing secondary damage post-TBI via CCL2/CCR2 inflammatory pathway. Thus, the p38-MAPK–CCL2/CCR2 inflammatory pathway probably has an important role in neuroinflammation following TBI.

Some studies had reported that the potential mechanisms underlying the anti-inflammatory effects of HBO treatment on TBI-induced neuroinflammation in rats. HBO treatment reduced TBI-induced microglial activation, attenuated inflammation significantly by reduced expression of IL-1 β , IL-6, macrophage inflammatory protein-2, CCL2, matrix metalloproteinase-9, and tumor necrosis factor- α expression, but IL-10 played an important role in the neuroprotection of HBO therapy against TBI [29–32]. Hyperbaric oxygen therapy significantly alleviated secondary brain injury by inhibiting the activation of the TLR4/NF- κ B signaling pathway after TBI of rats [33]. HBO therapy had been demonstrated to relieve secondary inflammatory responses of spinal cord injury by inhibiting the expression of CCL2, resulting in significant recovery of locomotor function [34]. In this study, HBO treatment improved the neurological and cognitive function of rats following TBI, and downregulated

Fig. 5



CCL2 and CCR2 mRNA expression and p-p38 expression were downregulated after HBO treatment. (a) mRNA expression of CCL2 was downregulated after 1, 3, 7 days of HBO treatment. CCL2 mRNA expression was downregulated after 10 days of HBO treatment, but the difference was not statistically significant ($n=6/\text{group}$). (b) mRNA expression of CCR2 was downregulated after 1, 3, 7 days of HBO treatment. CCR2 mRNA expression was downregulated after 10 days of HBO treatment, but the difference was not statistically significant ($n=6/\text{group}$). (c) The expression of p-p38 significantly decreased after continuous HBO treatment for 3 days ($n=3/\text{group}$). Values are expressed as mean \pm SEM. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. TBI group. HBO, hyperbaric oxygen; TBI, traumatic brain injury.

expressions of p-p38, CCL2 and CCR2 after TBI in rats. Taken together, these indicated that HBO treatment probably modulated neuroinflammation by targeting the p38-MAPK-CCL2/CCR2 signaling pathways following TBI. Our previous study found that HBO treatment inhibited neuroinflammation via regulation of NF- κ B, JNK, and ERK signaling pathways but not including p38 in LPS induced in primary astrocytes [35]. Possible reasons for the discrepant result may be explained by the different microenvironments between *in vivo* and *in vitro*. In addition, the pathogenic factor for the pro-neuroinflammation between *in vivo* and *in vitro* was diverse.

In conclusion, our study indicates that the p38-MAPK-CCL2 signaling pathway could mediate neuroinflammation after TBI and HBO therapy can modulate neuroinflammation by targeting the p38-MAPK-CCL2 signaling pathways following TBI. This study may provide theoretical evidence for HBO treatment in the treatment of TBI.

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The protocol of this study was approved by the Experimental Animal Center of Nantong University (permission number: 20191106-003).

All data used during the current study are available from the corresponding author on reasonable request.

Y.J., Y.C., C.H., A.X., G.W., and S.L. performed the experiments. S.L. conceived and designed the study. A.X. and G.W. analyzed the data. All authors read and approved the final article.

Conflicts of interest

There are no conflicts of interest.

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