Effects of hyperbaric oxygen therapy on RAGE and MCP-1 expression in rats with spinal cord injury

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Abstract. The inflammatory response is an important source of secondary damage to neuronal tissue in the spinal cord following spinal cord injury (SCI). Hyperbaric oxygen (HBO) therapy reduces inflammation and promotes the restoration of locomotor function following SCI, however, the mechanisms underlying this effect remain to be determined. The aim of the current study was to investigate the mechanisms by which HBO therapy promotes recovery in a rat model of SCI by measuring expression levels of receptor for advanced glycation end products (RAGE) and monocyte chemoattractant protein-1 (MCP-1) in spinal cord tissue. Experimental animals (n=90) were divided into three groups: Sham-operated (SH), SCI (T-10 laminectomy) and SCI + HBO. Each group was further divided into five subgroups (n=6) that were examined at 12 h, and at 1, 3, 7 and 14 days post-injury. Recovery of locomotor function was evaluated using the Basso, Beattie and Bresnahan (BBB) scoring system. Neutrophil infiltration was analyzed using myeloperoxidase (MPO) activity assays. The expression of RAGE and MCP-1 was measured by immunohistochemistry, reverse transcription-quantitative polymerase chain reaction and western blotting. RAGE and MCP-1 expression and MPO activity were higher in the SCI groups than in the SH groups at each time point. HBO therapy reduced RAGE and MCP-1 expression and MPO activity compared with untreated, injured animals at early post-injury stages. In addition, HBO therapy improved BBB scores at post-operative day 7 and 14. HBO therapy was, therefore, demonstrated to relieve secondary inflammatory responses, potentially by inhibiting the expression of RAGE and MCP-1, resulting in significant recovery of locomotor function. The results of the present study may, therefore, be useful in improving the clinical application of HBO therapy for patients with SCI.

Introduction

The pathogenesis of spinal cord injury (SCI) involves two phases: A primary trauma, which is pivotal for initial tissue disruption, followed by a series of secondary cellular processes that accentuate tissue damage beyond the original injury site and that can lead to long-term functional spinal deficits and disabilities (1). Although considerable effort has been made to improve outcomes for patients with SCI, advances in therapy for this disease have been limited, and further efforts are necessary to improve the treatment of SCI. Previous reports have suggested that hyperbaric oxygen (HBO) therapy is beneficial for neurological recovery in SCI, and HBO has become an important therapeutic approach in the treatment of secondary SCI (2,3). However, the mechanism that underlies this effect is not well understood. The inflammatory response is an important source of secondary damage to neuronal tissue in the spinal cord following SCI (4). Receptor for advanced glycation end products (RAGE) binds diverse ligands, including the high mobility group box-1 (HMGB1) and S100 calcium binding protein families (5,6). Ligand binding to RAGE triggers a series of cellular signaling events, including the activation of nuclear factor-κB (NF-κB), which leads to pro-inflammatory cytokine production and causes inflammation (7). Abnormal upregulation and activation of RAGE is associated with diseases of the central nervous system (CNS), including traumatic brain injury, ischemic stroke and SCI (8-10). Monocyte chemoattractant protein-1 (MCP-1) is a member of the β-chemokine family that activates and recruits mononuclear phagocytes, T cells and B cells, and is induced in response to various CNS insults (11-13). However, previous studies have not addressed the effect of HBO on the expression of RAGE and MCP-1 following SCI. In the present study, HBO-induced changes in RAGE and MCP-1 expression levels were investigated in rats following SCI, and the effect of HBO therapy on SCI recovery was investigated.

Materials and methods

Animals. A total of 90 8-week-old adult Sprague-Dawley rats (Center of Experimental Animals of Capital Medical

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University, Beijing, China) weighing 220-250 g were maintained under environmentally controlled conditions and subject to a 12 h light/dark cycle, with food and water provided *ad libitum*. The animals were acclimated to the facility for 7 days prior to initiation of experimentation, then were divided into sham-operated (SH), SCI, and SCI + HBO groups using the randomization table method. Each group was further divided into five subgroups, each containing six animals, that were evaluated at 12 h, and 1, 3, 7 and 14 days post-injury. All procedures and handling techniques were in strict accordance with the Committee on the Ethics of Animal Experiments of Capital Medical University (Beijing, China).

Spinal cord injury. Traumatic spinal injury was induced using a Multicenter Animal SCI Study Impactor weight drop device (14). Briefly, the rats were anesthetized with 10% chloral hydrate (350 mg/kg) administered intraperitoneally. The fur above the vertebral column was cleared using clippers and cleaned with Betadine solution. A 20-mm midline incision was made in the thoracic region, and the vertebral column was exposed. A laminectomy was performed at the T-10 vertebra, exposing the dorsal cord surface with the dura intact. The vertebral column was stabilized with angled clamps on the T-8 and T-12 vertebrae. A 10-g weight was dropped from 25 mm onto the T-10 segment, resulting in a moderate spinal cord injury. The impact rod was removed immediately following the injury, and the muscles and the incision were closed in layers. Following surgery, animals were placed on a heating pad maintained at 37°C and monitored until recovery from anesthesia, then returned to the cages. A single dose of penicillin (0.8 mg/g) was administered daily by subcutaneous injection until hematuria ceased. Manual bladder expression was required daily until a reflex bladder was established. The sham-operated rats received the equivalent surgical procedure, but were not subjected to impact injury.

HBO therapy. Rats in the SCI + HBO group were placed in a hyperbaric chamber for 6 h post-surgery and exposed to 2.0 atmospheres absolute (ATA) of 100% oxygen for 60 min once daily. Following treatment, the chamber was flushed with 100% oxygen for 5 min, then the pressure was increased to 2.0 ATA for 10 min, followed by slow decompression over 15 min to normobaric air (21% oxygen). Rats in the SH and SCI groups were post-operatively treated with normobaric air at 1.0 ATA.

Assessment of locomotor function. Recovery of locomotor function was assessed using an open-field testing paradigm, the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale, which is based on a 21-point scale originally developed in spinal cord-injured rats (15). This scale assesses 10 distinct categories, ranging from limb movement to tail position, and involves detailed observations of joint movement, stepping and coordination. Uninjured animals exhibit a locomotor score of 21, whereas animals that exhibit complete hind limb paralysis are scored as 0. All animals were scored in an open field (120x120 cm) for 5 min at 12 h and at 1, 3, 7 and 14 days post-injury.

Tissue sample collection. Terminally anesthetized (10% chloral hydrate by intraperitoneal injection at a dose of 350 mg/kg) rats were transcardially perfused with cold saline and 4% buffered paraformaldehyde at the indicated time points post-surgery (12 h and 1, 3, 7 and 14 days). The spinal cord containing the injured center site was removed and divided into two segments: One was fixed with 10% formaldehyde solution at 4°C for 1 week for histological evaluation, and the remaining segment was preserved at -80°C for molecular analyses.

Myeloperoxidase (MPO) activity assay. MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in the spinal cord tissue as previously described (16). Each tissue sample was weighed, then homogenized in homogenate medium [0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7] and centrifuged at 20,000 x *g* for 30 min at 4°C. An aliquot of the supernatant was subsequently incubated with a solution of 1.6 mM tetramethyl-benzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured using a UV-2000 UV/Vis spectrophotometer (Unico Shanghai Instrument Co. Ltd., Shanghai, China) at 460 nm. MPO activity was defined as the quantity of enzyme required to degrade 1 mmol H_2O_2 per min at 37°C and was expressed as units of MPO/g wet tissue.

Immunohistochemical staining. Histopathological samples were fixed, embedded in paraffin, and cut into 5-μm slices. Following deparaffinizing and rehydrating, antigen retrieval was performed according to standard protocols (17). Following treatment with 3% H_2O_2 in methanol and normal non-immune goat serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 20 min at room temperature, the sections were incubated overnight at 4°C with primary antibodies against RAGE (1:1,000; catalog no. sc-365154; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or MCP-1 (1:2,000; catalog no. sc-28879; Santa Cruz Biotechnology, Inc.). The sections were then incubated with biotinylated-goat anti-rabbit immunoglobulin G (1:5,000; catalog no. K500710; Dako, Glostrup, Denmark) for 50 min at room temperature, then treated with streptavidin-peroxidase (Dako). Phosphate-buffered saline was used in place of the primary antibody in the negative control samples. Diaminobenzidine was used to visualize peroxidase activity, and sections were counterstained with hematoxylin. Five fields on each of the three slides per animal were randomly selected for visualization by light microscopy. Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (5 μg) was extracted from frozen spinal cord tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and an extraction kit (Takara Bio, Inc., Otsu, Japan), according to manufacturer protocols. First-strand cDNA synthesis from 2 μg RNA was performed using Moloney Murine Leukemia Virus reverse transcriptase (BIOER, Hangzhou, China), with a temperature protocol of 42°C incubation for 60 min, followed by 10 min incubation at 70°C. qPCR was performed using the BioEasy SYBR Green I Real Time PCR kit (BIOER) and a Line-Gene sequence detector (BIOER). The amplification reaction consisted of 45 cycles of

95°C for 20 sec, 60°C for 25 sec and 72°C for 30 sec. PCR products were detected by the incorporation of SYBR Green during the reaction and verified by generating an amplification curve and by gel electrophoresis. The primer sequences for RAGE, MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are provided in Table I. Amplification products were quantified relative to the level of GAPDH, using the $2^{-\Delta\Delta Cq}$ method (18).

Western blot analysis. Frozen spinal cord tissue was homogenized in ice-cold isolation buffer containing 250 mmol/l sucrose, 10 mmol/l triethanolamine, 1 mg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 15,000 x *g* for 10 min at 4°C, and the protein concentration in the supernatant was measured using a bicinchoninic acid protein quantitation kit (Beijing Sunbio Biotech Co., Ltd., Beijing, China). Total protein (50 μg) in each sample was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% skimmed milk powder for 2 h at room temperature, then incubated overnight at 4°C with primary antibodies against RAGE (1:1,000; catalog no. sc-365154; Santa Cruz Biotechnology, Inc.), MCP-1 (1:2,000; catalog no. sc-28879; Santa Cruz Biotechnology, Inc.), or actin (1:2,000; catalog no. sc-1616R; Santa Cruz Biotechnology, Inc.) as indicated. Membranes were washed, then incubated with horseradish peroxidase-conjugated secondary antibodies (catalog nos. sc-2004 and sc-2005; Santa Cruz Biotechnology, Inc.) diluted to 1:10,000 for 2 h at 37°C, and immunoreactive protein was visualized using an enhanced chemiluminescence western blotting detection system (BestBio Inc., Shanghai, China). The film was scanned (Konica Minolta Medical Imaging, Inc., Wayne, NJ, USA), and protein expression was quantified from 2 blots using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA) and LabWorks (UVP, Inc., Upland, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of 3 replicates. One-way analysis of variance was used to compare the mean responses between the treatments, followed by least significant difference or Student-Newman-Keuls *post hoc* tests. P<0.05 was considered to indicate a statistically significant difference. Analyses were performed using SPSS (v15.0; SPSS Inc., Chicago, IL, USA).

Results

HBO promotes recovery of locomotor function following SCI. To evaluate the extent of locomotor function, BBB locomotor scores were calculated for control and experimental animals. As demonstrated in Fig. 1, the mean BBB scores of the SCI and SCI + HBO groups were significantly lower than those of the SH group at every time point (P<0.01). BBB scores in rats in the SCI and SCI + HBO groups improved from day 3, though the rats in the SCI + HBO group improved more rapidly; statistical analysis indicated a significantly greater increase in the BBB score in the SCI + HBO group compared with the SCI group on days 7 and 14 (P=0.006 and P=0.001, respectively; Fig. 1), indicating that HBO therapy promotes locomotor function in rats with SCI.

Table I. Sequences of primers for reverse transcription-quantitative polymerase chain reaction.

Target gene	Sequence (5'-3')
RAGE	F-AGAAACCGGTGATGAAGGACAA
	R-TCGTTTTCGCCACAGGATGG
MCP-1	F-TCTGGGCCTGTTGTTCACAGT
	R-TGCTGCTGGTGATTCTCTTGTAGT
GAPDH	F-GCAAGTTCAACGGCACAG
	R-CGCCAGTAGACTCCACGAC

RAGE, receptor for advanced glycation end products; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
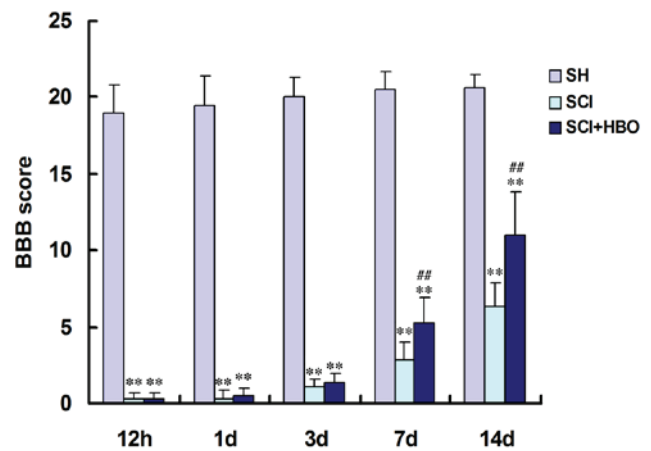

Figure 1. Locomotor function recovery assessment via BBB scores for hind limb motor function in the SH, SCI and SCI + HBO groups (n=6 for each group) at 12 h, and 1, 3, 7 and 14 days post-injury. Values are expressed as the mean ± standard deviation. **P<0.01 vs. SH group. ##P<0.01 vs. SCI group. BBB, Basso, Beattie and Bresnahan; SH, sham-operated; SCI, spinal cord injury; HBO, hyperbaric oxygen.

HBO reduces neutrophil infiltration following SCI. The effect of HBO therapy on neutrophil infiltration was assessed via MPO activity assays at multiple time points following SCI. The MPO activity was significantly increased in the SCI and SCI + HBO groups compared with the SH group at each post-operative time point (Fig. 2). However, the MPO activity of the SCI + HBO group was significantly lower than the SCI group at all time points from day 1 (Fig. 2), suggesting that HBO therapy reduces neutrophil infiltration following SCI.

HBO suppresses the expression of RAGE and MCP-1 following SCI. Immunohistochemistry was then used to detect RAGE and MCP-1 expression and localization. As demonstrated in Fig. 3A, RAGE and MCP-1 were predominantly expressed in the grey matter. RAGE- and MCP-1-positive cells were detectable in the SH group at all time points, however, the proportion of RAGE- and MCP-1-positive cells was significantly higher following SCI, with or without HBO therapy, with the exception of MCP-1 on day 14 (Fig. 3B). However, the proportion of RAGE- and MCP-1-positive cells was
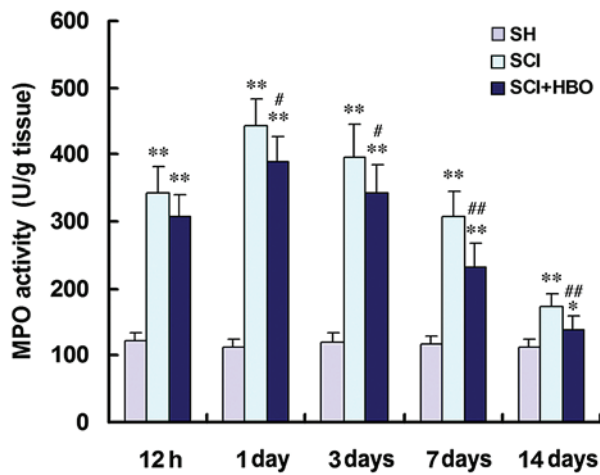
Figure 2. MPO activity in SCI and HBO-treated rats. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. SH group. #P<0.05, ##P<0.01 vs. SCI group. MPO, myeloperoxidase; SH, sham-operated; SCI, spinal cord injury; HBO, hyperbaric oxygen.

significantly lower in the SCI + HBO group compared with the SCI group at all time points from day 1, with the exception of MCP-1 on day 14 (Fig. 3B).

RAGE and MCP-1 mRNA and protein expression levels were then assessed by RT-qPCR (Fig. 4) and western blot (Fig. 5), respectively. RAGE and MCP-1 mRNA and protein expression levels were consistently low in the SH group (Figs. 4 and 5, respectively). Following SCI, RAGE and MCP-1 mRNA and protein expression levels were significantly increased at 12 h compared with the SH group, reaching their maximum level at the 1 day time point (Figs. 4 and 5, respectively). RAGE and MCP-1 mRNA and protein levels were significantly higher in the SCI group compared with the SH group at 12 h, and days 1, 3 and 7 post-operatively (all P<0.01; Figs. 4 and 5, respectively). On day 14, the expression of RAGE mRNA (P<0.0001; Fig. 4), RAGE protein (P<0.0001; Fig. 5) and MCP-1 protein (P=0.021; Fig. 5) was increased in the SCI group compared with the SH group. However, the expression levels of RAGE and MCP-1 mRNA (Fig. 4) and protein (Fig. 5) were significantly lower in the SCI + HBO group on days 3 and 7 post-surgery compared with the SCI group. RAGE mRNA expression levels were significantly reduced in the SCI + HBO group compared with the SCI group from day 3 (P<0.05; Fig. 4) as were protein expression levels from day 1 (P<0.05; Fig. 5B). MCP-1 mRNA expression levels were significantly reduced in the SCI + HBO group compared with the SCI group on days 1, 3 and 7 (P<0.05; Fig. 4) as were protein expression levels on days 1, 3, 7 and 14 (P<0.05; Fig. 5). These data suggest that HBO therapy suppresses the expression of RAGE and MCP-1 following SCI.

Discussion

Numerous studies have demonstrated that mechanical injury following SCI results in secondary injuries, including inflammatory responses, hemorrhage, ischemia, excessive free radical generation, vascular dysregulation, and immune cell infiltration (19,20). Specifically, inflammation following SCI is important for the regulation of remyelination and in neuronal

and glial cell death (21,22). Therefore, the inhibition of the inflammatory response is an important factor in neuroprotection and for promoting the recovery of locomotor function.

HBO therapy is a clinical therapy that involves administering 100% oxygen at a pressure higher than atmospheric pressure at sea level for a prescribed amount of time (23). HBO therapy is a well established treatment for acute and chronic SCI (3,24-26). Exposure to HBO decreases the expression levels of superoxide dismutase, glutathione peroxidase and nitric oxide synthase, relieves secondary inflammatory responses, inhibits apoptosis following injury and promotes the regeneration of nerve tissue (3,24-26). Previous studies have demonstrated that HBO relieves secondary inflammatory responses by decreasing the expression of NF-κB, HMGB1, Toll-like receptor 2, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) following SCI (27,28). In addition, Chen *et al* (29) reported that exposure to HBO attenuates inflammation, as indicated by reduced expression of MCP-1 and other inflammatory cytokines in a traumatic brain injury model. However, the effect of HBO therapy on the expression of RAGE and MCP-1 following SCI remains unclear. Following SCI, the inflammatory response becomes intense and is most destructive within the initial few days following injury (30). Thus, in the present study, the anti-inflammatory and neuroprotective effects of HBO were measured during the early phase of SCI (12 h-14 days). Application of HBO following SCI was revealed to inhibit the infiltration of neutrophils, suppress the expression of inflammatory cytokines (including RAGE and MCP-1), and promote the recovery of locomotor function. To the best of our knowledge, this is the first study to examine the effect of HBO therapy on RAGE and MCP-1 expression following SCI.

Neutrophils are critical cellular components of the inflammatory response and the first inflammatory cell type to reach damaged tissue (31). Activated neutrophils promote tissue repair by inducing the phagocytosis of necrotic tissue in the wound area, while simultaneously promoting the release of elastase via a respiratory burst and producing large amounts of reactive oxygen species (ROS) (32). These ROS induce two effects: i) They attack the polyunsaturated fatty acids on the cell membrane, causing lipid peroxidation and disrupting the cell osmotic balance; and ii) they activate complement systems, resulting in a positive feedback loop in which neutrophils are further activated to produce more ROS (22,32). These downstream effects expand the inflammatory response and exacerbate secondary injury (31,32). The results of the present study reveal that HBO therapy significantly inhibits the activity of neutrophils following SCI, which reduces secondary injury and provides a neuroprotective effect.

RAGE, a transmembrane protein and member of the immunoglobulin superfamily, is expressed in endothelial cells, neurons, macrophages and monocytes (33). RAGE is involved in various inflammatory mechanisms and participates in numerous diseases, including CNS disorders, by binding to diverse ligands. In particular, several studies have reported that RAGE was upregulated following SCI in rats and mice (10,34). In confirmatory studies using RAGE-deficient animals, RAGE was demonstrated to be involved in various pathophysiological processes of SCI (35). In the present study, RAGE expression was demonstrated to be significantly increased from 12 h
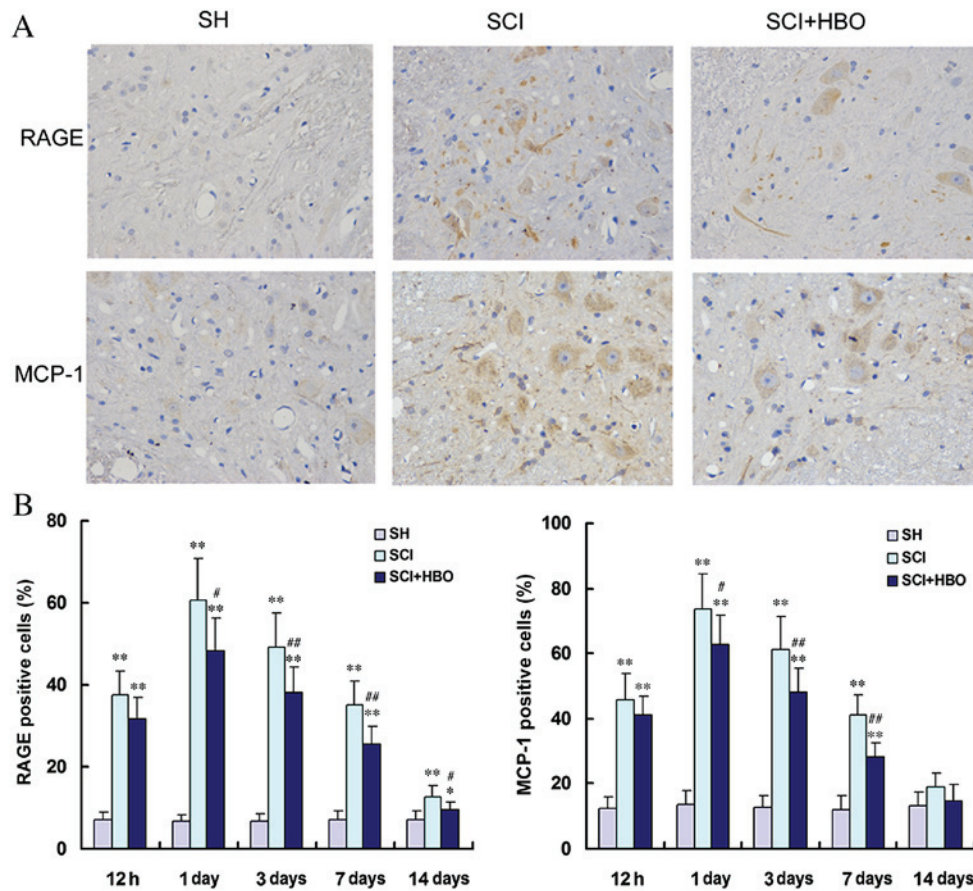
Figure 3. Expression of RAGE and MCP-1 in the control and experimental groups. (A) Representative photomicrographs of RAGE and MCP-1 immunohisto-chemistry in the spinal cord at 1 day post-surgery (original magnification, x400). (B) RAGE- and MCP-1-positive cells were quantified at x400 magnification. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. SH group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. SCI group. SH, sham-operated; SCI, spinal cord injury; HBO, hyperbaric oxygen; RAGE, receptor for advanced glycation end products; MCP-1, monocyte chemoattractant protein-1.
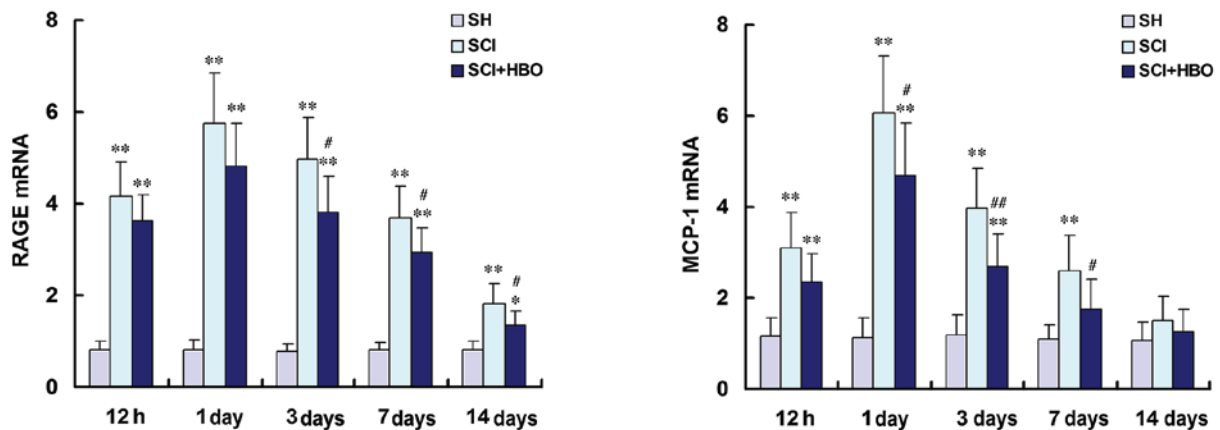

Figure 4. Expression levels of RAGE and MCP-1 mRNA in the spinal cord were determined by reverse transcription-quantitative polymerase chain reaction at the indicated time points. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. SH group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. SCI group. RAGE, receptor for advanced glycation end products; SH, sham-operated; SCI, spinal cord injury; HBO, hyperbaric oxygen; MCP-1, monocyte chemoattractant protein-1.

following SCI and persisted for 14 days. RAGE expression was further observed to be significantly decreased in animals with SCI that were treated with HBO therapy compared with untreated animals with SCI. HBO administration following SCI was also demonstrated to reduce neutrophil infiltration and increase the BBB scores of injured rats. Therefore, it is

surmised that decreased expression of RAGE as a result of HBO therapy may contribute to a reduced inflammatory response and an improvement in the restoration of locomotor function. Following binding to ligands including HMGB1 and S100β, RAGE triggers the activation of NF-κB, induces the production of pro-inflammatory cytokines, including IL-1β,
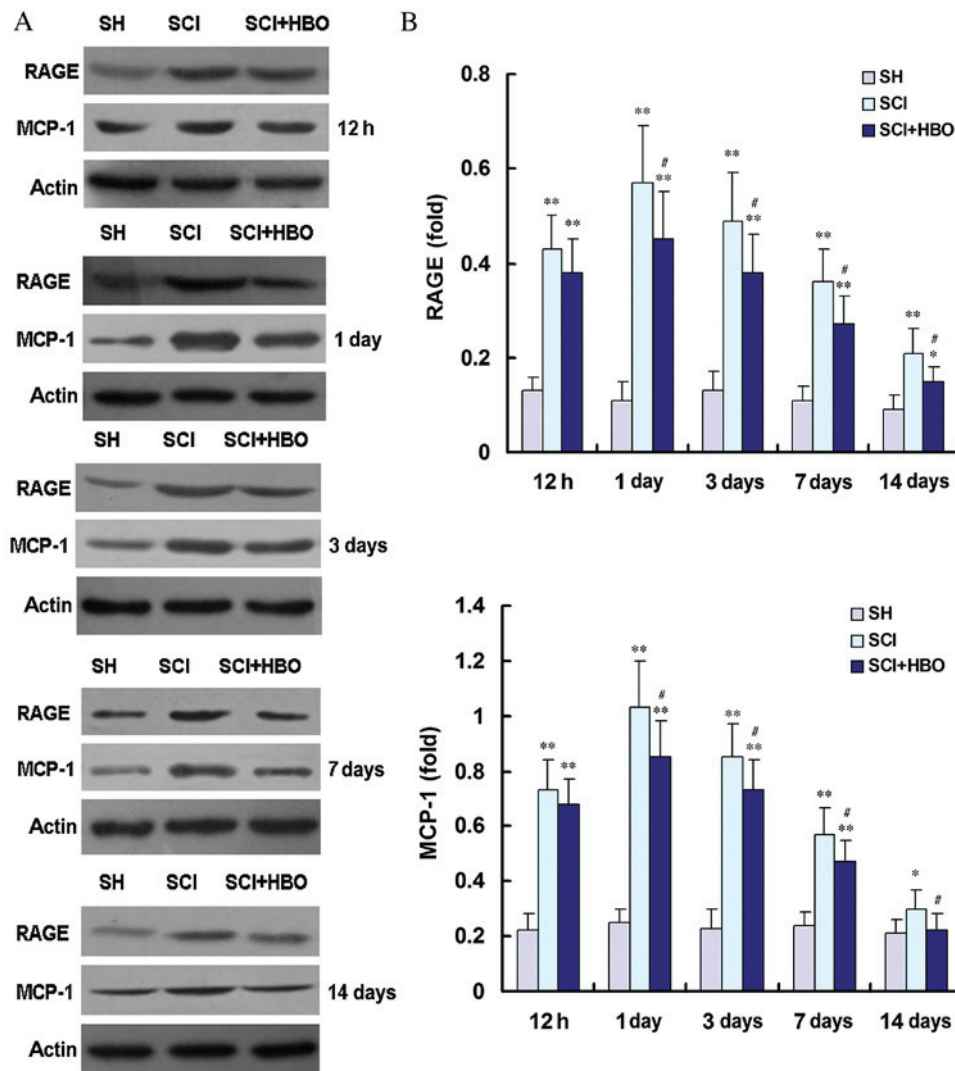
Figure 5. RAGE and MCP-1 protein expression levels in the spinal cord. (A) Representative immunoblots of RAGE and MCP-1 protein expression. (B) Quantitative analysis of RAGE and MCP-1 protein expression levels, relative to actin. Values are expressed as the mean ± standard deviation. $^{*}P<0.05$, $^{**}P<0.01$ vs. SH group. $^{#}P<0.05$ vs. SCI group. RAGE, receptor for advanced glycation end products; MCP-1, monocyte chemoattractant protein-1; SH, sham-operated; SCI, spinal cord injury; HBO, hyperbaric oxygen.

IL-6 and TNF-α, and causes inflammation (36). The expression of RAGE is also controlled by NF-κB transcription factor (7). Therefore, RAGE is upregulated in environments that are rich in RAGE ligands (37). Previous studies by this group have demonstrated that HBO therapy decreases the expression of NF-κB and HMGB1 following SCI (27,28). These results may, therefore, partially explain why HBO therapy dramatically relieves RAGE expression following SCI.

MCP-1 is a potent chemoattractant with a significant role in recruiting lymphocytes and monocytes into inflammatory sites. In the CNS, MCP-1 is produced by a variety of cells, including reactive astrocytes, neurons, activated microglia and endothelial cells (38). Several recent studies have reported upregulation of MCP-1 expression following SCI in rats (39,40). Inhibition of MCP-1 also attenuates leukocyte infiltration and tissue destruction in SCI (41). In the present study, the expression of MCP-1 was significantly increased following SCI, beginning at 12 h, peaking at day 1, and gradually declining to control levels by day 14; these findings are consistent with those of previous studies (42). Furthermore, HBO therapy

significantly decreased the expression of MCP-1 in animals with SCI that were treated with HBO therapy compared with untreated animals in the SCI group. HBO therapy-mediated attenuation of neutrophil infiltration was also observed. Therefore, is it proposed that HBO therapy inhibits the expression of MCP-1 and that this change in MCP-1 is important in reducing the inflammatory response following SCI. This method may, therefore, be beneficial in promoting the recovery of locomotor function in rats with SCI. The question of how HBO therapy influences MCP-1 expression following SCI requires further investigation.

In conclusion, exposure to HBO following SCI has been demonstrated to reduce secondary inflammatory responses by decreasing the expression of RAGE and MCP-1, resulting in a significant restoration of locomotor function. These results are preliminary, however, they provide an important insight into the molecular mechanism by which HBO therapy promotes locomotor recovery in SCI rats, and may be useful for improving the clinical application of HBO in human patients with SCI. Further study will be required to elucidate the

mechanism by which HBO therapy influences the expression of RAGE and MCP-1 following SCI.

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