Microglial activation induced by traumatic brain injury is suppressed by postinjury treatment with hyperbaric oxygen therapy

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ABSTRACT
Background: The mechanisms underlying the protective effects of hyperbaric oxygen (HBO) therapy on traumatic brain injury (TBI) are unclear. TBI initiates a neuro-inflammatory cascade characterized by activation of microglia and increased production of proinflammatory cytokines. In this study, we attempted to ascertain whether the occurrence of neuroinflammation exhibited during TBI can be reduced by HBO.

Methods: TBI was produced by the fluid percussion technique in rats. HBO (100% O2 at 2.0 absolute atmospheres) was then used at 1 h (HBO I) or 8 h (HBO II) after TBI. Neurobehavior was evaluated by the inclined plane test on the 72 h after TBI and then the rats were killed. The infarction area was evaluated by Triphenyltetrazolium chloride. Immunofluorescence staining was used to evaluate neuronal apoptosis (TUNEL + NeuN), microglial cell aggregation count (OX42 + DAPI), and tumor necrosis factor-alpha (TNF-α) expression in microglia cell (OX42 + TNF-α).

Results: The maximum grasp angle in the inclined plane test and cerebral infarction of the rats after TBI were significantly attenuated by HBO therapy regardless of whether the rats were treated with HBO 1 or 8 h after TBI compared with the controls. TBI-induced microglial activation, TNF-α expression, and neuronal apoptosis were also significantly reduced by HBO therapy.

Conclusions: Our results demonstrate that treatment of TBI during the acute phase of injury can attenuate microgliosis and proinflammatory cytokine TNF-α expression resulting in a neuroprotective effect. Even treating TBI with HBO after 8 h had a therapeutic effect.

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1. Introduction
Traumatic brain injury (TBI) is a leading cause of death and disability with limited treatment options. TBI survivors often suffer from severe cognitive ability and memory and neurologic deficits [1]. The morbidity and mortality caused by TBI are due to cell ischemia, hypoxia, inflammation, and apoptosis which occur after the TBI, ultimately leading to cell

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The anti-inflammatory effects of HBO have been shown to inhibit the endotoxin lipopolysaccharide-induced proinflammatory cytokines in monocytes and macrophages [11] and to prevent suppress pyrogenic fever by reducing the overproduction of circulating TNF-\(\alpha\) [12]. Other studies have shown that in rats with heat stroke, increased secretion of TNF-\(\alpha\) in the brain and plasma leading to inhibition of cardiac output could be attenuated by HBO treatment [13,14].

HBO at 1.5–3 absolute atmospheres (ATA) for 1–2 h is usually used in experimental studies in central nervous system (CNS) disorders and is considered to be quite safe. Hyperbaric oxygenation has been shown both experimentally and clinically to improve the outcome of cerebral trauma, especially when early treatment is performed within 6 h of TBI [15–17]. Also Sahni et al. [18] indicated that HBO therapy revealed some clinical advantages in chronic TBI cases. Recently, we have demonstrated the neuroprotective effects of HBO on attenuating inflammation and astrocyte activation by using 100% \(O_2\) at 2.0 ATA for 1 h/d for three consecutive days [19]. However, some authors do not support the use of HBO for TBI or stroke [20], and the value of HBO treatment in TBI remains controversial [1,21]. To date, the effect of HBO on TNF-\(\alpha\) expression in microglia if treated immediately or delayed after TBI has not been properly evaluated.

In this study, we hypothesized that HBO may have a therapeutic effect on TBI based on its anti-inflammatory effect on TBI-induced cell damage, apoptosis, and functional outcome impairment. To examine this hypothesis, experiments were conducted to assess the therapeutic effects of HBO on microglia activation, proinflammatory cytokine TNF-\(\alpha\) expression, and neuronal apoptosis in the area of the ischemic cortex. In addition, we also compared the motor deficits and cerebral infarction volume during TBI in rats with or without HBO therapy with the aim of elucidating whether HBO therapy attenuates TBI-induced cerebral injury by reducing proinflammatory cytokines and improving neurologic outcomes. Furthermore, we also compared the beneficial effects of HBO treatment at 1 and 8 h after TBI.

### 2. Materials and methods

#### 2.1. Experimental design

Table shows the summary of the overall experimental procedures. The end point for our study was 72 h after TBI because investigations tend to indicate that severe lateral fluid percussion causes motor and cognitive dysfunction, which persists from 72 h to 1 y after TBI [22].

#### 2.2. Animals

Adult male Sprague-Dawley rats weighing 300 ± 16 g were used in the experiments. They were kept under a 12/12-h light/dark cycle and allowed free access to food and water. All experimental procedures conformed to the NIH guidelines and were approved by the Chi Mei Medical Center Animal Care and Use Committee to minimize discomfort of the animals during surgery and the recovery period.

#### 2.3. Traumatic brain injury

The animals received general anesthesia with a mixture containing ketamine (44 mg/kg, intramuscularly [i.m.]; Nankuang Pharmaceutical, Tainan, Taiwan), atropine (0.02633 mg/kg, i.m.; Sintong Chemical Ind. Co, Taipei, Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer, Leverkusen, Germany). They were placed in a stereotaxic frame and the scars were incised sagittally. The animals were then subjected to a lateral fluid percussion injury to induce TBI [23]. A moderate fluid percussion injury (FPI) (2.2 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a fluid percussion device (VCU Biomedical Engineering, Richmond, VA). This procedure was described in detail in our previous study [24,25]. Because a transient apnea might occur
after FPI, a standard respiratory care was performed by withdrawing the rat’s tongue out of mouth immediately after FPI for protecting the airway patent. If the experimental rat’s respiration recovering to the spontaneous and smooth pattern without oversecrretion, or cyanosis, and without systemic hypotension, animals would be recruited into the study.

2.4. Treatment intervention

The rats were randomly divided into four groups: sham operation with normobaric air (NBA: 21% O2 at 1 ATA), TBI + NBA, TBI + HBO I (1 h after TBI) with 100% O2 at 2.0 ATA for 60 min, and TBI + HBO II (8 h after TBI) with 100% O2 at 2.0 ATA for 60 min. NBA entailed continuous exposure to room air. The HBO group of animals was exposed in a HBO chamber (701 Space Research Institute, Beijing, China). The chamber was flushed with 100% oxygen at a rate of 5 L/min to avoid carbon dioxide accumulation. Decompression was done at 0.2 kg/cm²/min. The pressure chamber temperature was maintained between 22°C and 25°C. Rats were killed 72 h after TBI for neuropathology study.

2.5. Microglial activation and TNF-α expression assay

The activated microglia was evaluated at 72 h after TBI by detecting OX42 positive cells using an immunofluorescent assay [26]. The TNF-α expression in activated microglia were evaluated at 72 h after TBI by detecting OX42 + TNF-α positive cells using an immunofluorescent assay. The number of OX42 and TNF-α positive cells in the samples were measured in each slice and summed using computerized planimetry (PC-based Image Tools software, Media Cybernetics, Inc). Mouse monoclonal anti-OX42 antibody (ab78457; Abcam, Boston, MA) and polyclonal goat anti-TNF-α antibody (sc-1351; Santa Cruz Biotechnology Co, Santa Cruz, CA) were used in this study and detected with Alexa-Fluor, 568 anti-mouse (IgG) antibody (A11031; Life Technologies Co, Grand Island, NY), and DyLight 488 anti-goat (IgG) antibody (ab96931; Abcam), respectively.

2.6. Neuropathologic study

2.6.1. Neuronal apoptotic assay

Cellular identification of apoptotic cells was made by double staining with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling, and the neuronal nuclear marker Neu-N at 72 h after TBI [27]. The procedures have been described previously [24].

2.6.2. Cerebral infarction assay

Triphenyltetrazolium chloride staining was performed as described previously [24]. All animals were killed 72 h after TBI. The volume of infarction, as revealed by negative Triphenyltetrazolium chloride staining indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based Image Tools software). The volume of infarction was calculated as 2 mm (thickness of the slice) × [sum of the infarction area in all brain slices (mm²)].

2.7. Functional outcome evaluation

2.7.1. Maximum grasp angle

The inclined plane test was used to measure limb strength. The animals were placed, facing right and then left, perpendicular to the slope of a 20 × 20-cm ribbed rubber surface of an inclined plane starting at an angle of 40° [28]. The angle was increased or decreased in one-degree increments to determine the maximal grasp angle at which an animal could hold onto the plane. Measurements of the means of the left and right side maximal angles were made daily, but only the values 72 h after TBI were presented.

2.8. Statistical analysis

Immunoreactive cell count and lesion volumes were evaluated for Gaussian (normal) distribution and were expressed as means ± standard errors. The data were analyzed with one-way analysis of variance and, if P < 0.05, were analyzed using a Newman–Keuls post hoc test. Differences were considered significant when P < 0.05. All data were analyzed with SigmaPlot version 11.0 for Windows (Systat Software, San Jose, CA).

3. Results

3.1. HBO improved TBI-induced motor dysfunctions

Compared with the sham-operated group, the TBI groups had a significantly smaller maximal grasp angle (52 ± 3.71 versus 45.89 ± 5.22, P < 0.05). However, the TBI-induced maximal grasp angle dysfunctions were significantly ameliorated both in the onset of treatment at HBO I (P < 0.05) and HBO II (P < 0.05). However, maximal grasp angle showed no significant difference in the HBO I and HBO II groups; 51.65 ± 2.35 and 49.25 ± 2.24, respectively, P = 0.13 (Fig. 1).

Fig. 1 – Means ± standard error of the mean values of maximal angle on an inclined table. The data were obtained 72 h after HBO treatment. #, P < 0.05, TBI compared with the HBO I and HBO II group (n = 6 in each group).
3.2. **HBO attenuated infarction volume after TBI**

The TBI-induced infarction volume was significantly decreased by HBO in the onset of treatment at HBO I (86.34 ± 5.26 mm³ versus 38.42 ± 4.46 mm³, *P < 0.05, Fig. 1) or HBO II (86.34 ± 5.26 mm³ versus 48.4 ± 3.2 mm³, *P < 0.05, Fig. 1). However, compared treatment with HBO I to HBO II after TBI showed no significant difference in TBI-induced infarction volume (38.42 ± 4.46 mm³ versus 48.4 ± 3.2 mm³, P = 0.25, Fig. 2).

3.3. **HBO attenuated neuronal apoptosis after TBI**

The TBI-induced neuronal apoptosis were significantly decreased by HBO in the onset of treatment at HBO I or HBO II (*P < 0.05, *P < 0.05, Fig. 3). However, the total number of TBI-induced neuronal apoptosis in perilesioned cortex showed no significant difference between the HBO I and HBO II groups; 8 ± 1.4 versus 14 ± 3.1, respectively P = 0.08, Fig. 3).

3.4. **HBO attenuated microglia activation and local TNF-α expression**

We first evaluated microglial activation and tested the possibility that HBO might suppress TBI-induced brain microgliosis. Microgliosis (increased expression of OX42) acquired an amoeboid morphology with retracted, thickened processes, and enlarged soma. OX42-DAPI double staining showed that there were significantly more microglia in the perilesioned cortex of the TBI rats compared with the sham rats. HBO I and HBO II significantly reduced the TBI-induced
activated microglia ($P < 0.05$, *$P < 0.05$, Fig. 4) expression in the cortex 72 h post-TBI. However, no significant difference in TBI-induced activated microglia expression between HBO I and HBO II groups; 18 ± 1.9 and 18 ± 2.2, respectively, $P = 0.17$).

Then, we evaluated the TNF-$\alpha$ expression in activated microglia and tested the possibility that HBO might suppress TBI-induced brain inflammation. As expected, OX42 $\cap$ TNF-$\alpha$ double staining showed that the amount of positive TNF-$\alpha$ in the activated microglia in the perilesioned cortex of the TBI rats was significantly higher than in the sham rats. It also showed that TBI-induced positive TNF-$\alpha$ in the activated microglia was significantly lower in the HBO I and HBO II rats compared with the TBI rats ($P < 0.05$, *$P < 0.05$, Fig. 5). However, the total number of TBI-induced TNF-$\alpha$ in the activated microglia showed no significant difference between the HBO I and HBO II groups; 14 ± 0.9 and 18 ± 1.1, respectively, $P = 0.06$).

4. Discussion

4.1. Novelty of the present study

To the best of our knowledge, this is the first study to present the neuroprotective effects of HBO on TNF-$\alpha$ expression on activated microglia in traumatic CNS injury. It also demonstrated that the therapeutic window of HBO therapy could be expanded to 8 h after TBI, which may be explored in the...
clinical setting. These data will hopefully serve as a foundation for future studies on the HBO therapy in TBI.

4.2. HBO treatment attenuated microglial activation and TNF-α expression in activated microglia in the cortex

It has been suggested that activated microglia can be detected by Iba1 or C3b-OX42 reactions. C3b complement receptor (CR3) is a marker for microglia. OX42 antibody, as demonstrated in our study, binds strongly to CR3, especially when the activated microglia has intense expressions of CR3 [29]. The significant presence of activated microglia was demonstrated 3 d after TBI by using OX42 antibody detection was consistent with the previous study [8,30].

In humans with TBI, microglial activation has been also reported as early as 3 d after injury [31]. In the present study, we found that OX42-DAPI double staining was significantly present in the perilesioned cortex of TBI rats compared with sham rats 3 d after TBI. These observations are similar to those of prior reports [30,31]. Furthermore, we also found that activation of microglia after TBI could be prevented by HBO.

On injury, activated microglia is an early and prominent source of TNF-α in the CNS [32]. TNF-α is a prototypical proinflammatory cytokine that can signal apoptotic cell death [33]. Thus, in CNS insults, microglia-induced TNF-α production can critically influence subsequent events. In the present study, we found that TNF-α expression in the activated microglia after TBI could be attenuated by HBO.

Fig. 4 – Means ± standard error of the mean values of the number of OX42 positive cells in the ischemic cortex. The data were obtained 72 h after sham-operated, TBI, or TBI + HBO treatment (n = 6 in each group). * * P < 0.05 TBI compared with HBO I and HBO II. Top panels depict representative OX42 + DAPI staining for one rat in the different groups.
However, the dual role of activated microglia, beneficial and detrimental for neuroprotection and functional recovery, should be kept in mind [34]. Based on our findings, the attenuated inflammation by HBO was associated with improved neuronal survival adjacent to the impact site and by an improved performance on measures of maximum grasping angle carried out 72 h after injury. Thus, we supposed that early treatment with HBO, as in the present study, might inhibit the detrimental effects associated with neuroinflammation while attenuating the inflammatory pathways that lead to neuroprotection and functional recovery. These results suggested suppression of microglial activation in acute stage by HBO could be a promising treatment strategy for TBI.

It has been demonstrated that both neutrophil and astrocyte are important factors to cause neuronal insults by releasing proinflammatory cytokine [5,15]. Lin et al. [19] showed HBO significantly inhibited astrogliosis after TBI in acute stage. In our study, we further found HBO significantly attenuated microgliosis and TNF-\(\alpha\) expression, which associated with neuronal improvement after TBI.

4.3. HBO effects on neuropathologic and functional outcomes

Apoptotic cell death is one of the secondary injury mechanisms after TBI [35]. In TBI, apoptosis has been shown to

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**Fig. 5** — Means ± standard error of the mean values of the number of OX42 + TNF-\(\alpha\) positive cells in the ischemic cortex. The data were obtained 72 h after sham-operated, TBI, or TBI + HBO treatment (\(n = 6\) in each group). # \(P < 0.05\), TBI compared with HBO I and HBO II. Top panels depict representative OX42 + TNF-\(\alpha\) staining for one rat in the different groups.
commonly occur in the perilesioned area as a result of secondary brain insults in animal and human studies [19,36,37]. Consistent with previous studies, we found that the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling-positive cells seen in the cerebral cortex surrounding the primary injury site were significantly increased 3 d after TBI but were reduced with HBO treatment. This supports the involvement of apoptosis in secondary brain injuries after TBI in our model. Furthermore, this result also suggests that the protective effects of HBO may be at least partly due to reductions in apoptotic activity of brain tissue after TBI.

In a recent study, 3 and 6 h after TBI in rats, a single treatment with HBO yielded a significant reduction in brain damage. However, by 12 h after TBI, the efficacy of HBO II treatment was considerably attenuated [17]. Based on our results, we considered that the therapeutic window of HBO therapy could be expanded to 8 h after TBI. Thus, we suggest that treatment with HBO 8 h after TBI may be a promising treatment strategy for traumatic brain injury in clinical application.

However, whether the decreased numbers of apoptotic cells following HBO is a direct ant apoptotic effect or secondary consequence due to HBO anti-inflammatory effects is needed to be determined in the future.

4.4. Pressure and duration considerations when HBO treatment

To avoid the risk of oxygen toxicity and to improve the efficacy of HBO therapy, at present, the levels of pressure and length of time for HBO treatment after TBI are suggested less than 3 ATA for 1–2 h, for example, 1.5 ATA for 60 min [16], 2.0 ATA for 1 h/d for three consecutive days [19], 2.8 ATA during two consecutive sessions of 45 min [15], and 3.0 ATA for 60 min or 3 ATA hourly for 3 or 5 d [17]. In the present report, 2.0 ATA for 60 min was applied after TBI. Overall, the results were encouraging although we did not evaluate the side effect of HBO such as lipid peroxidation injury.

4.5. Other HBO effects beyond anti-inflammation

HBO has been shown both experimentally and clinically to improve the outcomes of cerebral trauma [38,39]. Its therapeutic effects are based on the ability of a hyperoxic environment to reduce cerebral blood flow by vasoconstriction-reduced intracranial pressure and increase oxygenation. Furthermore, the increased blood oxygen levels seen during HBO treatment are released passively and can penetrate into ischemic areas more deeply than under normobaric conditions [40,41]. Beside the anti-inflammation effects, HBO therapy could reduce secondary brain damage by improving oxidative metabolism [42], stimulating both neurogenesis and angiogenesis [19], stimulating hypoxia-inducing factor-1 accumulation [43], and reducing apoptosis-related proteins such as Bcl2 family [44], Bcl2, and Caspase-3 messenger RNA expression [17] in the perilesioned area of the rat model of cerebral contusion. Therefore, HBO may be a very useful therapy for TBI patients because of its multiple and effective effects.

5. Conclusion

There are two important findings in the present study that may add to the current knowledge on HBO. First, besides attenuating neuronal apoptosis, we also found that HBO therapy significantly reduced the activated inflammation, which occurred in the cortex during TBI. The infarction volume of brain tissue correlated with functional recovery, which was improved by HBO. Taken together, these results suggest that HBO may improve the neuropathologic and functional outcomes of TBI, at least in part, by inhibiting activated inflammation, especially TNF-α production. Second, treatment with HBO 8 h after TBI still had anti-inflammatory and neuroprotective effects on the TBI rats. This result demonstrated that the therapeutic window of HBO therapy could be expanded to 8 h after TBI, which may be explored in the clinical setting.

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