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Effects of Post-Treatment Hydrogen Gas Inhalation on Uveitis Induced by Endotoxin in Rats

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Molecular hydrogen (H_2) has been widely reported to have benefiicial effects in diverse animal models and human disease through reduction of oxidative stress and inflammation. The aim of this study was to investigate whether hydrogen gas could ameliorate endotoxin-induced uveitis (EIU) in rats.

Material/Methods: Male Sprague-Dawley rats were divided into a normal group, a model group, a nitrogen-oxygen (N-O) group, and a hydrogen-oxygen (H-O) group. EIU was induced in rats of the latter 3 groups by injection of lipopolysaccharide (LPS). After that, rats in the N-O group inhaled a gas mixture of 67% N_2 and 33% O_2, while those in the H-O group inhaled a gas mixture of 67% H_2 and 33% O_2. All rats were graded according to the signs of uveitis after electroretinography (ERG) examination. Protein concentration in the aqueous humor (AqH) was measured. Furthermore, hematoxylin-eosin staining and immunostaining of anti-ionized calcium-binding adapter molecule 1 (Iba1) in the iris and ciliary body (ICB) were carried out.

Results: No statistically significant differences existed in the graded score of uveitis and the b-wave peak time in the Dark-adapted 3.0 ERG among the model, N-O, and H-O groups ($P>0.05$), while rats of the H-O group showed a lower concentration of AqH protein than that of the model or N-O group ($P<0.05$). The number of the infiltrating cells in the ICB of rats from the H-O group was not significantly different from that of the model or N-O group ($P>0.05$), while the activation of microglia cells in the H-O group was somewhat reduced ($P<0.05$).

Conclusions: Post-treatment hydrogen gas inhalation did not ameliorate the clinical signs, or reduce the infiltrating cells of EIU. However, it inhibited the elevation of protein in the AqH and reduced the microglia activation.

MeSH Keywords: Electroretinography • Lipopolysaccharides • Microglia • Uveitis

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Background

Uveitis is the most common form of intraocular inflammation and is defined as a particular type of inflammation in the uvea that can involve other ocular tissues, such as vitreous and retina [1]. The incidence of uveitis was reported to be 52.4/100 000 person-years in the USA [2], and it is the cause of approximately 10% to 15% of preventable blindness in Western countries [3]. Corticosteroids and immunosuppressive drugs are the cornerstone of treating uveitis. However, they are usually associated with systemic disorders, such as increased susceptibility to microbial infection. New and effective measures against uveitis with less adverse side effects are urgently required. Despite the complicated etiology and pathological mechanism of uveitis, oxidative stress (OS) has generally been accepted to play an important role in the progress of uveitis [4,5]. Endotoxin-induced uveitis (EIU) is a widely used animal model to mimic the pathological features and investigate potential treatments for human uveitis [6]. Its pathogenesis was deemed to be closely associated with OS [7,8], and drugs that can mitigate OS have been shown to ameliorate EIU [9,10].

Molecular hydrogen (H_2) has been found to possess antioxidant and anti-inflammatory properties, and has been established as a new kind of antioxidant reported to be beneficial in the prevention and treatment of several diseases [11,12]. Inflammation is a basic pathological process in many diseases. H_2 has been demonstrated to alleviate inflammation in the lungs, pancreas, and liver [13,14]. The H_2-induced relief of inflammation may be due to its suppression of the inflammatory mediators [15], or through its direct interaction with reactive oxygen species (ROS), which were reported to be one of the causes of inflammation [16].

Our previous study [17] found that hydrogen-rich saline (HRS) inhibited the elevation of protein in the aqueous humor (AqH), but did not obviously mitigate the clinic manifestation of EIU rats. Hydrogen gas, compared to HRS, provides a higher amount of H_2 and has also been shown to be effective against multiple diseases [18,19]. Therefore, this study was conducted to investigate whether post-treatment of hydrogen gas could somewhat ameliorate EIU in rats.

Material and Methods

Animals and LPS administration

Male Sprague-Dawley (SD) rats (6 to 8 weeks old) were obtained from the Laboratory Animal Center of the Fourth Military Medical University (License number: 2014270138S, Xi'an, China), and kept under dim cyclic light (5 lux, 12 h on/off, 6 a.m.–6 p.m.), with food and water available ad libitum. All experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Care and Use of Laboratory Animals.

The rats were randomly divided into a normal group, a model group, a nitrogen-oxygen (N-O) group, and a hydrogen-oxygen (H-O) group. Lipopolysaccharide (LPS, #L6511, Sigma, USA) was stored in the dark at 4°C upon arrival and was dissolved in saline to a concentration of 2 mg/ml before use [20]. In the rats of the latter 3 groups, we induced uveitis by subcutaneous injection of 1/8 mg/kg LPS into their footpads. Twenty-four rats (6 for each group) were subjected for evaluation of the clinical manifestation in EIU under slit-lamp and electroretinography (ERG) examination, as these 2 indicators were obtained while the rats were alive. Another 36 rats were subjected for protein concentration measurement, histological examination with hematoxylin & eosin (HE) staining, and immunostaining of microglia cells. To collect these data, 12 rats were included in the other three groups except the normal group (6 rats for each group at each time point). This was aimed to minimize the number of animals according to the 3Rs principles of animal experiments.

Hydrogen gas intervention

Rats in the N-O group inhaled a gas mixture of 67% N_2 and 33% O_2 (the N_2-O_2 mixed gas, product code #10021120, Shaanxi XingHua Chemical Co. LTD., Xi'an, China) at a flow rate of 3 L/min for 1 h at 0 and 1 h after LPS injection and once a day for 3 weeks afterwards. Rats in the H-O group inhaled a gas mixture of 67% H_2 and 33% O_2 at a flow rate of 3 L/min at the same period. As previously reported [21], the H_2-O_2 mixed gas was produced using an AMS-H-01 hydrogen-oxygen nebulizer (Shanghai Asclepius Meditec Co., Ltd., China), which can extract 67% H_2 and 33% O_2 from water. During the period of gas inhalation, the rats were awake and freely moving in a special gas chamber consisting of a transparent closed box. The concentration of H_2 in the box was monitored using thermal trace GC ultra-gas chromatography (Thermo Fisher, MA, USA), and was confirmed to be 67% throughout the study. Rats in the normal and model groups did not receive any treatment.

Clinical evaluation of EIU

Slit-lamp examination was conducted under euthanization at 12, 24, 48, 72, and 96 h after LPS injection. Pictures of the rat eyes with the slit on the middle of the cornea were acquired. Clinical scoring of uveitis was graded as previously reported [22]: iris hyperemia (0–2), flare (0–2), hypopyon (0–2), and miosis (0–2). The maximum possible score was 8.

ERG recording

ERG was carried out 1, 4, 7, 14, and 21 d after inducing EIU, as previously reported [23]. All ERG indicators were recorded and

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analyzed according to the ISCEV guidelines [24] by a technician blinded to the animal grouping and treatment.

Protein concentration measurement in the AqH

AqH was collected by anterior chamber puncture with an insulin syringe immediately after euthanization of rats at 1 and 4 d after LPS administration. Total protein concentration in the AqH was measured by a bicinchoninic acid (BCA) assay (#W041, Jiancheng, Nanjing, China) according to the manufacturer's instructions.

HE staining of ICB

Eyes of rats from all groups were enucleated rapidly after an intraperitoneal injection of a lethal dose of sodium pentobarbital (#P3761,Sigma-Aldrich, USA) at 1 and 4 d after LPS administration, and were stored in 4% paraformaldehyde solution at 4°C overnight. The 4-μm sagittal sections were cut along the pupil-optic nerve axis. For each eye, 3 sections that included the optic nerve were stained with HE. Images of ICB were taken using a digital imaging system (DP71, Olympus, Japan) and analyzed by counting the infiltrating cells in the ICB at high magnification (×400).

Immunostaining

Vertical sections including the optic nerve in the paraffin-embebbed eye samples collected at 24 h after LPS administration were submerged in xylene and then rehydrated with ethanol gradient washes. Before further use, slides were washed 3 times in phosphate-buffered saline (PBS) for 5 min. The sections were then heated in a microwave oven for antigen recovery, washed in PBS, and treated with 10% normal goat serum (#AR0009, Boster, Wuhan, China) for 1 h at room temperature. The slides were subjected to immunostaining at 4°C overnight with the primary antibody against microglia cells: polyclonal rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1, #019-19741, Wako Chemicals, Japan) at 1: 500 dilution. After washing in PBS, the secondary antibody (goat anti-rabbit IgG conjugated to Alexa Fluor 594, #ZF-0516, ZSGB-BIO, Beijing, China) at a 1: 500 dilution was used. The sections were washed again in PBS and then counterstained with 4',6-diamidino-2-phenylindole (DAPI, #C1005, Beyotime, Nantong, China). Images of ICB in the sections were obtained using a confocal microscope (LSM510, Zeiss, Oberkochen, Germany). The activation state of microglia cells was measured by calculating the ratio of Iba1-positive cell number to DAPI-postive cell number in 3 sections for each eye and averaged.

Statistical analysis

All data were shown as mean ± standard error (S.E.) and analyzed by one-way analysis of variance (ANOVA) followed by contrast analysis (LSD-t test when equal variances were assumed and Dunnett's T3 test when equal variances were not assumed) using SPSS software (version 16.0, Chicago, USA). Differences were considered statistically significant at $P<0.05$.

Results

Effects of hydrogen gas on clinical manifestation and retinal function of EIU

Rats in the normal group had no signs of uveitis, while rats of the other 3 groups manifested typical signs of uveitis, such as iris hyperemia and hypopyon after injection of LPS. From 12 to 74 h after LPS administration, the graded score of uveitis signs in the model, N-O, or H-O group was higher than that of the normal group ($P<0.05$), while there were no significant differences among the 3 groups ($P>0.05$). At 96 h, the uveitis of the model, N-O, and H-O groups disappeared (Figure 1A, 1B).

From 1 to 7 d after LPS injection, the b-wave peak time in the Dark Adaptation 3.0 ERG of the model, N-O or H-O group was longer than that of the normal group ($P<0.05$), while there were no statistical differences among the 3 groups ($P>0.05$). At 10 d, the b-wave peak time of the model or N-O group was statistically longer than that of the normal group ($P<0.05$), while the peak time of the H-O group was not significantly different from that of the normal group ($P>0.05$). However, no significant differences were found among the model, N-O, and H-O groups. At 14 and 21 d, no significant differences were found among the 4 groups ($P>0.05$) (Figure 1C).

Effects of hydrogen gas on AqH protein concentration in EIU

One day after LPS injection, protein concentrations in the AqH of the normal, model, N-O, and H-O groups were 1.65±0.09, 4.47±0.12, 4.28±0.08, and 3.34±0.46 mg/ml, respectively. The AqH protein concentration in the model, N-O or H-O group was higher than that of the normal group, with differences being statistically significant ($P=0.002$, the model group *vs.* the normal group; $P=0.003$, the N-O group *vs.* the normal group; $P=0.006$, the H-O group *vs.* the normal group). The protein concentration of the H-O group was significantly lower than that of the model or the N-O group ($P=0.011$, the H-O group *vs.* the model group; $P=0.013$, the H-O group *vs.* the N-O group). At 4 d, protein concentration returned to a normal level in the model, N-O, and H-O groups, and no significant differences were found among the 4 groups ($P>0.05$) (Figure 2).

Effects of hydrogen gas on the cells infiltration in EIU

There was an obvious sign of cells infiltration in the ICB of rats from the model, N-O, and H-O groups at 1 d after LPS

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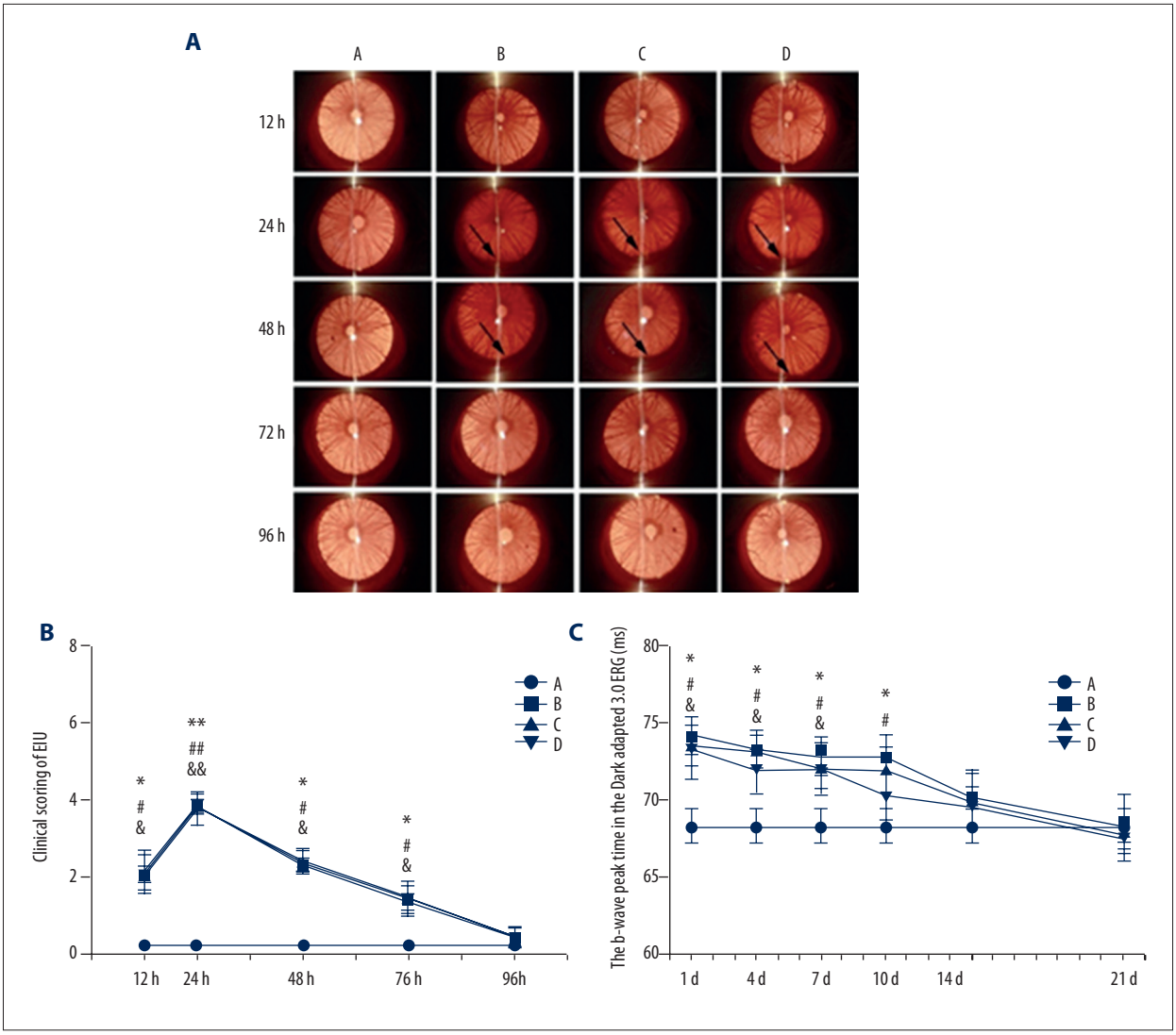

Figure 1. Clinical manifestation (**A**) and graded scoring (**B**) of uveitis under slit-lamp within 96 h after LPS injection, and the b-wave peak time in the Dark-adapted 3.0 ERG within 21 d after LPS administration (**C**). Arrow: hypopyon in the anterior chamber. A – normal group; B – model group; C – nitrogen-oxygen (N-O) group; D – hydrogen-oxygen (H-O) group; *, #, & (**, ##, &&) $P<0.05$ ($P<0.01$): model, N-O, or H-O *vs.* normal group, respectively.

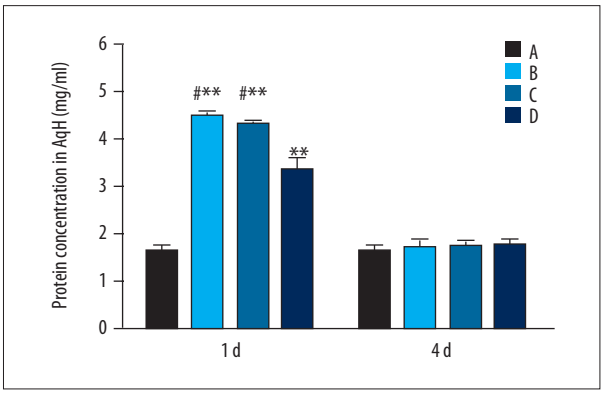

Figure 2. Protein concentration in the AqH 1 and 4 d after LPS injection. A – normal group; B – model group; C – nitrogen-oxygen (N-O) group; D – hydrogen-oxygen (H-O) group; ** $P<0.01$ *vs.* normal group; # $P<0.05$ *vs.* H-O group.

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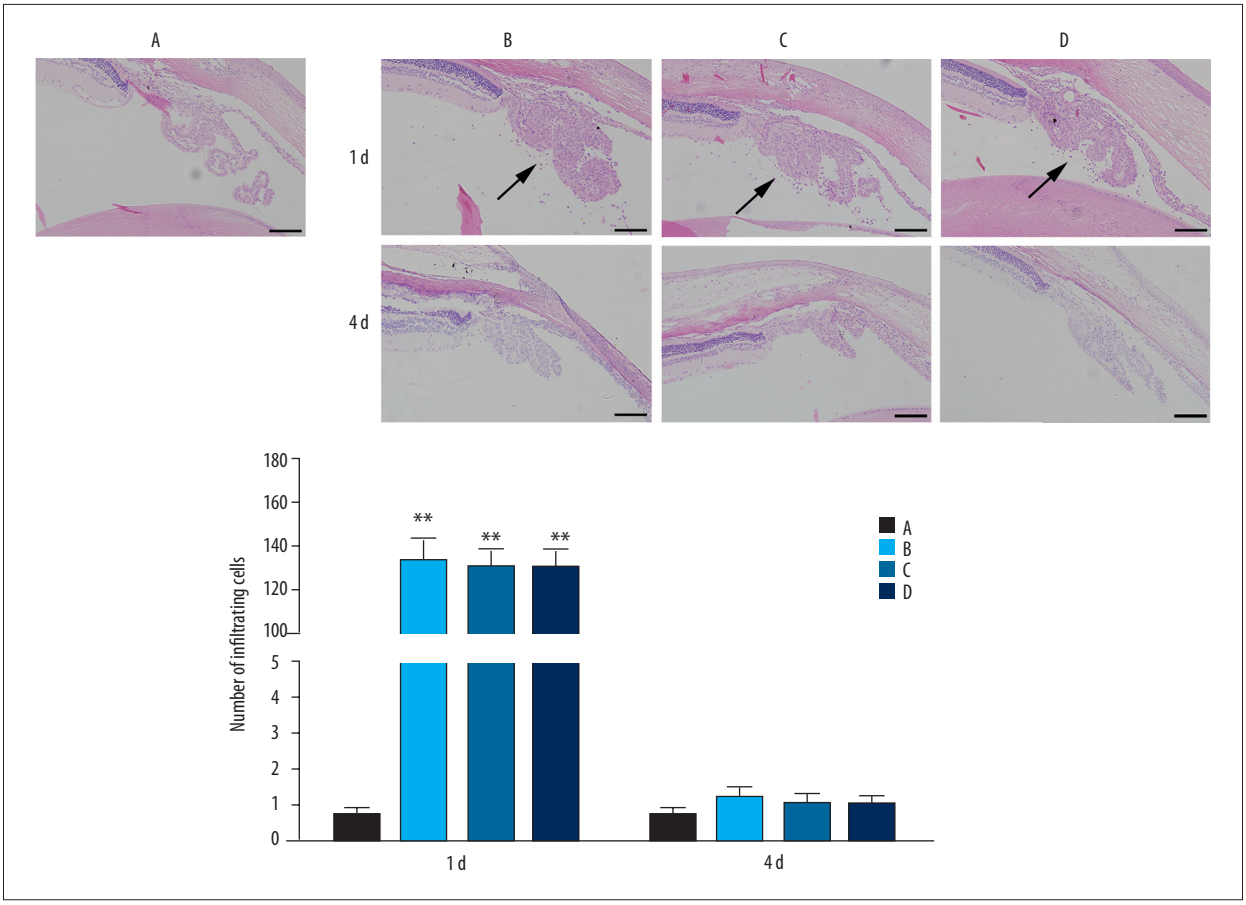

Figure 3. HE staining of the ICB (×200) and number of total infiltrating cells in the ICB 1 and 4 d after LPS injection. Arrow: infiltrating cells; Scale: 100 μm. A – normal group; B – model group; C – nitrogen-oxygen (N-O) group; D – hydrogen-oxygen (H-O) group; ** $P<0.01$ *vs.* normal group.

injection; the cell numbers were 133.02±10.5, 125.30±9.23, and 130.00±8.73, respectively. The number of infiltrating cells in the model, N-O or H-O group was significantly different from that of the normal group (0.67±0.20) ($P<0.01$), while there were no significant differences among the model, N-O and H-O groups ($P>0.05$). At 4 d, cells infiltration in the ICB disappeared in the model, N-O, and H-O groups, with no significant differences among the 4 groups ($P>0.05$) (Figure 3).

Effects of hydrogen gas on microglia activation in EIU

To visualize the presence of microglia cells, we used the Iba1 marker [25]. At 24 h after LPS administration, there was a substantial increase of Iba1-positive cells in the model and N-O groups, with the ratio of Iba1-positive cell number to DAPI-positive cell number significantly reduced compared to that in the normal group ($P=0.013$, the model group *vs.* the normal group; $P=0.019$, the N-O group *vs.* the normal group). The ratio of Iba1-positive cell number to DAPI-positive cell number in the H-O group was markedly lower than that of the model or the N-O group with statistically significant differences ($P=0.012$, the H-O group *vs.* the model group; $P=0.018$, the H-O group *vs.* the N-O group) (Figure 4).

Discussion

Inhalation of hydrogen gas, compared to HRS administration, provides a much higher amount of H_2, keeps a higher peak concentration in the body and maintains it for a longer time [26]. Therefore, to some extent, hydrogen gas inhalation might yield a better result in disease control and prevention. It has been reported that hydrogen gas ameliorates several diseases [27]. As early as 1975, Dole et al. [28] showed that a high concentration of hydrogen gas (97.5% H_2 and 2.5% O_2) somewhat reduced the area of squamous cell carcinoma in albino mice. Xie et al. [29] discovered that hydrogen gas ameliorated the lung inflammation in mice with acute lung injury, which was induced by bronchial suction of LPS. By using a rat model of cardiac arrest, Hayashida et al. [30] found that hydrogen gas inhalation during cardiopulmonary resuscitation (CPR) improved heart and brain function.

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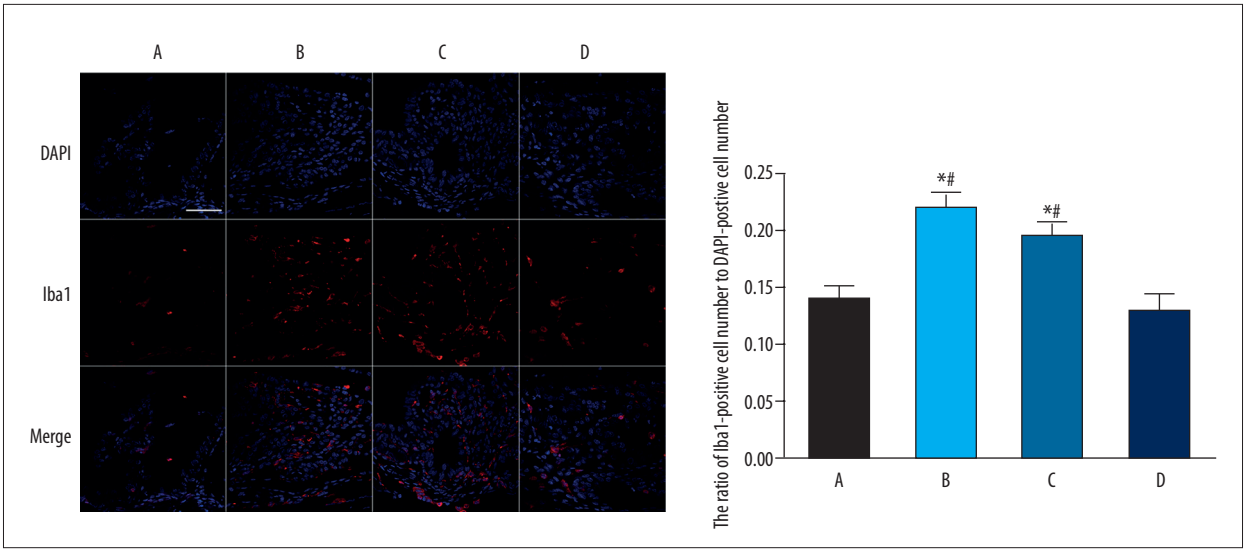

Figure 4. Representative photomicrographs of Iba1-positive microglia cells in the ICB and quantification of ratio of Iba1-positive cell number to DAPI-postive cell number 24 h after LPS injection. Scale: 50 μm; A – normal group; B – model group; C – nitrogen-oxygen (N-O) group; D – hydrogen-oxygen (H-O) group; * $P<0.05$ *vs.* normal group; # $P<0.05$ *vs.* H-O group.

In the current study, we applied a relatively high concentration of hydrogen gas (67% H_2 and 33% O_2) as an intervention for EIU in rats, with the control rats inhaling the N_2-O_2 mixed gas (67% N_2 and 33% O_2). The method of hydrogen gas inhalation is portable, easy to administer, and safe. The gas mixture used to prevent decompression sickness contains up to 49% of H_2 [31]. Besides, the gas mixture containing up to 90% of H_2 did not affect the cell viability in normally cultured RAW 264.7 macrophages [32]. Throughout our study, the H_2-O_2 or N_2-O_2 mixed gas did not influence the mental state, daily activities, diet, sleep, or body weight of rats.

EIU is an animal model of human uveitis induced by LPS, a component of the outer membranes of gram-negative bacteria [33]. A major pathological characteristic of EIU is the ocular infiltration of inflammatory cells, which manifests as hypopyon under slit-lamp and cells infiltrating in the ICB in histological examination. The majority of the infiltrating cells were polymorphonuclear leucocytes, namely neutrophils, which have been recognized as a key element in the pathogenesis of EIU [34,35]. Due to the production of proteolytic enzymes and toxic intermediates, neutrophils are essential for innate immunity in evading infection and attacking a foreign agent [36]. However, these defense processes, which occur through destroying and digesting extraneous matter, are potentially deleterious to the body tissue [37]. Thus, it is vital that neutrophils are cleared in a timely manner following the completion of their physiological function. In this study, we found that the hypopyon and the infiltration of inflammatory cells in EIU were not obviously alleviated by post-treatment hydrogen gas inhalation. In the model of EIU, the inflammatory mediator that elicited neutrophils infiltration was reported to be leukotriene B4 (LTB4) [38]. An elevated level of LTB4 in isoproterenol (ISO)-induced heart failure was shown to be reduced by hydrogen sulfide (H_2S) [39], an antioxidant gasotransmitter. However, to our knowledge, whether H_2 could reduce the LTB4 level has not been illustrated before. As we previously suggested [17], LTB4 may not be influenced by H_2 in the form of HRS, or even in the form of a high concentration of hydrogen gas. Therefore, the signs of hypopyon and cells infiltration in EIU were not relieved by H_2 intervention.

The retina is easily involved in the process of uveitis, and decreased retinal function has been reported in EIU [40]. In our study, ERG results showed that the reduced retinal function of EIU rats was not significantly improved by hydrogen gas inhalation. Studies on the animal model of light-induced retinopathy and the MNU-indcued retinal degeneration suggested an amelioration of retinal function reduction by a combination of H_2-pre-treatment and H_2-post-treatment [41,42]. Similarly, inclusion of hydrogen gas inhalation prior to LPS injection may be beneficial to the relief of EIU in our study.

Under normal circumstances, the protein concentration in the AqH is relatively low. Elevation of protein in the AqH suggests the breakdown of blood-AqH barrier, which is a typical sign of uveitis [43]. In our study, the AqH protein concentration in the H-O group was reduced compared to that of the model or N-O group, indicating that hydrogen gas somewhat inhibited the elevation of the AqH protein in EIU rats. Prostaglandin E_2 was shown to be elevated in EIU rats and was recognized the inflammatory mediator responsible for the exudation of protein into the AqH [44]. The reduction of AqH protein by

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hydrogen gas might be attributed to the fact that hydrogen gas, as HRS [17], reduces the level of PGE2 in EIU.

Microglia cells are the main form of adaptive immune response in the central nervous system. In response to insults, microglia cells undergo a number of changes [45], such as processes retraction, and cell body enlargement, leading to the amoeboid shape. Overactivation and prolonged activation of microglia cells have been closely linked to various neurological diseases [46,47] and retinal degeneration [48]. The activated microglia cells are often highly mobile. The activation and migration of these cells is also regarded as a non-specific early stress response in inflammation [49]. Uveitis, as a particular type of inflammation in the uvea, might involve the activation of microglia cells [50]. Our results showed that microglia cells were actively involved in EIU rats, and the activation state of microglia cells was somewhat mitigated by hydrogen gas. Monocyte chemoattractant protein-1 (MCP-1), a chemoattractant and an inflammatory cytokine, plays a crucial role in microglia migration/infiltration and neuroinflammatory disorders [51,52]. Interleukin (IL)-6 and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) are 2 other important inflammatory factors associated with microglia activation [53,54]. The reduction of MCP-1 and IL-6 by H_2 has been reported in the animal models of spontaneous hypertensive and nephrotoxicity [55,56], while H_2 has no significant effect on the level of RANTES [57]. Elevation of MCP-1 and IL-6 has also been confirmed in the model of EIU [58,59]. Furthermore, suppression of microglia activation by H_2 has also been demonstrated by other investigators [60,61]. Thus, the hydrogen gas-induced amelioration of microglia activation in EIU rats might due to the inhibition of MCP-1 and IL-6.

Conclusions

This study found that post-treatment hydrogen gas inhalation somewhat suppressed the elevation of AqH protein and the activation of microglia cells in EIU rats, while it did not obviously ameliorate the cells infiltrating in the ICB and reduced retinal function of EIU. The discrepant effects of molecular hydrogen on different indicators of EIU might be due to its selective suppression on some inflammatory mediators. Further investigations are needed to clarify the above hypothesis. Besides, hydrogen gas treatment prior to LPS injection might be beneficial, and would be worth investigating in the future.

Conflicts of interest

None.

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