

Hydrogen inhibits microglial activation and regulates microglial phenotype in a mouse middle cerebral artery occlusion model

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Abstract

Microglia participate in bi-directional control of brain repair after stroke. Previous studies have demonstrated that hydrogen protects brain after ischemia/reperfusion (I/R) by inhibiting inflammation, but the specific mechanism of anti-inflammatory effect of hydrogen is poorly understood. The goal of our study is to investigate whether inhalation of high concentration hydrogen (HCH) is able to attenuate I/R-induced microglia activation. Eighty C57B/L male mice were divided into four groups: sham, I/R, I/R + HCH and I/R + N₂/O₂ groups. Assessment of animals happened in “blind” manner. I/R was induced by occlusion of middle cerebral artery for one hour). After one hour, filament was withdrawn, which induced reperfusion. Hydrogen treated I/R animals inhaled mix of 66.7% H₂ balanced with O₂ for 90 minutes, starting immediately after initiation of reperfusion. Control animals (N₂/O₂) inhaled mix in which hydrogen was replaced with N₂ for the same time (90 minutes). The brain injury, such as brain infarction and development of brain edema, as well as neurobehavioral deficits were determined 23 hours after reperfusion. Effect of HCH on microglia activation in the ischemic penumbra was investigated by immunostaining also 23 hours after reperfusion. mRNA expression of inflammation related genes was detected by PCR. Our results showed that HCH attenuated brain injury and consequently reduced neurological dysfunction after I/R. Furthermore, we demonstrated that HCH directed microglia polarization towards anti-inflammatory M2 polarization. This study indicates hydrogen may exert neuroprotective effects by inhibiting the microglial activation and regulating microglial polarization. This study was conducted in agreement with the Animal Care and Use Committee (IACUC) and Institutional Animal Care guidelines regulation (Shanghai Jiao Tong University, China (approval No. A2015-011) in November 2015.

Key words: hydrogen; ischemic stroke; reperfusion; neuroprotection; inflammation; microglial phenotype; macrophages; polarization

doi: 10.4103/2045-9912.266987

How to cite this article: Huang JL, Liu WW, Manaenko A, Sun XJ, Mei QY, Hu Q. Hydrogen inhibits microglial activation and regulates microglial phenotype in a mouse middle cerebral artery occlusion model. *Med Gas Res.* 2019;9(3):127-132.

Funding: This study was partially supported by the National Natural Science Foundation of China, No. 81671130 (to QH) and 81772015 (to WWL); and Shanghai municipal Science and Technology Commission Medical Guidance Science and Technology Support Project, No. 19411968400 (to QYM).

INTRODUCTION

Ischemic stroke is most disastrous kind of stroke with the incidence as high as 17 million worldwide.¹ Despite the intensive investigations, the therapeutic options of stroke treatment are very limited and the development of new neuroprotective agents for ischemic stroke is urgently needed.² Studies have shown that hydrogen inhalation may protect brain from injury induced by transient focal ischemia and indicate that the protection might be due to anti-inflammatory, anti-apoptotic and anti-oxidative activities of hydrogen.^{3,4} The specific mechanisms underlying hydrogen induced protection need to be adequately elucidated.

Microglial cells are the resident macrophages in brain contribute to the occurrence and development of inflammation after focal ischemia. Microglia can polarize toward different phenotypes (M1 and M2), exerting distinct effect.⁵ While M2 microglia may promote brain recovery by resolving lo-

cal inflammation and releasing a variety of trophic factors, while M1 microglia may inhibit the brain repair, deteriorate tissue injury and release factors, exerting pro-inflammatory effect. There is evidence showing that a shift from complete microglia suppression toward a subtler titration of the balance between various phenotypes may serve as an effective treatment of stroke.⁶ Although a variety of studies have shown that hydrogen can suppress the microglial activation and reduce the secretion of pro-inflammatory factors, the effects of hydrogen on the phenotypic transformation of microglia after cerebral ischemia have not yet been fully elucidated. Our previous study showed lipopolysaccharides could significantly increase the M1 macrophages, and the supernatant from oxygen and glucose deprivation-treated neurons dramatically increased the percentage of M1 microglia; hydrogen reduced that of M1 macrophages and microglia, but had little influence on the M2 macrophages and microglia.⁷ Here we investigated whether

inhalation of hydrogen rich mix (66% of hydrogen) will affect the microglia polarization and inflammatory response induced by ischemic stroke.

METHODS/DESIGN

Study design

Eighty mice (C57B/L, male, 7–8 weeks old and 20–24 g) were obtained from SLAC Experimental Animal Center (Shanghai, China; license No. SCXK(Hu)2012-0002), and randomly assigned into four group ($n = 20$ per group): (1) Sham group: mice received sham operation; (2) ischemia/reperfusion (I/R) group: ischemic stroke was induced by middle cerebral artery occlusion (MCAO) for 60 minutes, after it filament withdrawn, initiating reperfusion; (3) I/R + H₂/O₂ group: ischemia was induced by MCAO for 60 minutes, after it at then immediate begin the reperfusion. Animals were allowed to inhale high percent of hydrogen mixture (66.7%) balanced by oxygen for 90 minutes (4) I/R + N₂/O₂ group: hydrogen in the breathing mix was replaced with nitrogen. This study was conducted in agreement with the Animal Care and Use Committee (IACUC) and Institutional Animal Care guidelines regulation (Shanghai Jiao Tong University, China (approval No. A2015-011) in November 2015.

MCAO models

Animal model of transient MCAO was established as previously described.⁸ Briefly, animals were intraperitoneally anesthetized using 2% chloral hydrate at 0.021 mL/g, then a monofilament suture (6-0, Doccol, Sharon, MA, USA) was retrogradely inserted from ECA (external carotid artery) into ICA (internal carotid artery) and monofilament suture was removed 1 hour after brain ischemia. The body temperature of these animals was kept at $37 \pm 0.5^\circ\text{C}$ throughout the surgery and recovery period. The feedback heating system was used.

Hydrogen gas production

Both the control mixture consists of 66.7% N₂ and 33.3% O₂ and the hydrogen rich mix (66.7% H₂) was kept in cylinder, used for gas storage. Hydrogen rich mix was produced *via* electrolyzing water was provided by nebulazer (Asclepius, Shanghai, China). The hydrogen mix was delivered into a transparent box (28 cm × 18 cm × 14 cm) at 3000 mL/min, and this gas mixture was used for inhalation by the mice in a box. Before the administration of mixtures, they were filtered by the calcium hydroxide and allochroic silica gel to remove carbon dioxide and water, respectively. To replace the air, the box was flushed with mix gas for 30 minutes prior to experiment. The concentration of hydrogen was monitored with the thermal trace GC ultra-gas chromatography throughout the whole procedure (Thermo Fisher, Shanghai, China).

2,3,5-Triphenyltetrazolium chloride staining

Animals were euthanized at 24 hours after ischemia induction and the brain samples were collected. Brain infarction was evaluated by 2,3,5-triphenyltetrazolium chloride staining. In brief, 1 mm coronary brain sections were incubated for 20 minutes with TCC solution at 37°C . These brain sections were fixated then in paraformaldehyde solution (4%) and then pho-

tographed (Canon IXUS175, Aomori, Japan). The infarction area was analyzed with the Image-Pro (Media Cybernetics, Rockville, MD, USA)⁹ using the Swanson's method.¹⁰ Infarct was calculated as percentage of whole hemisphere, rather than the absolute infarct volume.

Brain water content

At the same time point (24 hours) brain water content was calculated. Animals were euthanized under deep anesthesia. The brain samples were instantly collected and dissected into four parts: right and left hemisphere, cerebellum as well as brain stem. The samples was weighed immediately (wet weight), then the samples was dried for 48 hours in an infrared oven (WS70-1; Hu Yue Ming Scientific Instrument Corporation, Shanghai, China) and weighed again (dry wet). For the calculation of the brain water content following formula was used: brain water content = [(wet weight – dry weight) / wet weight] × 100%.¹¹

Assessment of neurological functions

Neurological functions were assessed in a blind manner 24 hours after stroke induction as previously described.¹² Following scale system was used: 0, No apparent deficits; 1, Flexion of contralateral forelimb; 2, While tail pulled, weak grip of the contralateral forelimb; 3, Spontaneous movement in all directions or contralateral circling only if pulled by tail; and 4, Spontaneous contralateral circling.

Quantitative real-time PCR

Twenty-four hours after MCAO, the total RNA was extracted from ischemic hemiencephalon using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at -80°C for real-time PCR. Reverse transcription of mRNA into cDNA was done with a 20- μL mixture containing 1 μg of total RNA using the FastQuant RT Kit with gDNase (Tiangen, Beijing, China). Then, mRNA expression of inflammatory mediators (interleukin [IL]-1 β , IL-6, tumor necrosis factor [TNF]- α and IL-10) was detected using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA), and the glyceraldehyde 3-phosphate dehydrogenase served as a reference gene. The NM numbers for all genes were as follows: IL-1 β , NM_008361.4; IL-6, NM_001314054.1; TNF- α , NM_001278601.1; IL-10, NM_010548.2; glyceraldehyde 3-phosphate dehydrogenase, NM_008084.3. The primers used for PCR were as follows: IL-1 β , 5'-TGT CTG AAG CAG CTA TGG CAA-3' (forward) and 5'-GAC AGC CCA GGT CAA AGG TT-3' (reverse); IL-6, 5'-GAT GGA TGC TAC CAA ACT GG-3' (forward) and 5'-TGA AGG ACT CTG GCT TTG TC-3' (reverse); TNF- α , 5'-TAG CCC ACG TCG TAG CAA AC-3' (forward) and 5'-ACA AGG TAC AAC CCA TCG GC-3' (reverse); IL-10, 5'-ATG CTG CCT GCT CTT ACT GAC TG-3' (forward) and 5'-CCC AAG TAA CCC TTA AAG TCC TGC-3' (reverse); glyceraldehyde 3-phosphate dehydrogenase, 5'-CCT CGT CCC GTA GAC AAA ATG GT-3' (forward) and 5'-TTG AGG TCA ATG AAG GGG TCG T-3' (reverse). Primers were synthesized in Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The results were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.

All experiments were carried out in triplicate.

Immunofluorescence staining

Twenty-four hours after ischemia, the brain was sectioned and samples were placed onto slides and then processed for immunofluorescence staining. Sections were incubated with 0.3% Triton X-100 in PBS (30 minutes at room temperature). The antigen retrieval (citrate solution at 95°C for 20 minutes) followed. After passive cooling down to the room temperature, sections were incubated for 2 hours with 10% goat serum. After overnight incubation with Iba-1 primary antibody (rabbit; 1:1000; ab153696; Abcam, Cambridge, UK) at 4°C, sections were rinsed with PBS thrice. A 2 hours incubation with secondary antibodies at room temperature followed. After rinsing with PBS, visualization was done with fluorescence microscope (AXIO SCOPE A1, ZEISS, Stockholm, Sweden). At least three sections from each mouse were randomly selected for analysis.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay was done to detect the contents of inflammation related mediators (IL-6, IL-10, TNF- α and IL-1 β) in the brain according to the provided protocol (R&D Systems, MN, USA).

Statistical analysis

Statistical analysis was performed with using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). All the data are expressed as the mean \pm standard error (SEM), and one-way analysis of variance followed by Bonferroni's multiple comparison test were used to compare the differences among groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Hydrogen decreased I/R induced infarction

For infarct volume calculation 2,3,5-triphenyltetrazolium chloride staining was performed. Sham animal had no in-

farct. After I/R, the average infarct ratio was $38.23 \pm 2.68\%$ of the hemisphere. In I/R + N₂/O₂ group, the infarct ratio was decreased to that in I/R group, but high concentration hydrogen (HCH) markedly decreased the infarct ratio ($P < 0.001$; **Figure 1A & B**). The survival rate of all animals was monitored during 24 hours after surgery. No sham operated animal died during this time. In other group survival rate of 80% without significant differences between experimental groups was observed ($P > 0.05$; **Figure 1C**).

Hydrogen alleviated brain edema after cerebral I/R

I/R induced significant increase of brain water content in the ipsilateral hemisphere. The brain water content was investigated at 24 hours and compared to the sham group, which was observed in sham-operated animals ($P < 0.01$; **Figure 1D**). HCH significantly reduced this parameter ($P < 0.05$), but the water content in I/R + N₂/O₂ group was similar to that in I/R group ($P > 0.05$; **Figure 1D**).

Hydrogen improved neurobehavioral deficits after cerebral I/R

The neurological functions were evaluated as previously reported.¹² Cerebral I/R caused significant neurological deficit. HCH significantly improved the neurological function of I/R animals ($P < 0.05$). No effects of N₂/O₂ inhalation was observed ($P > 0.05$; **Figure 1E**).

Hydrogen inhibited the activation of microglia after cerebral I/R injury

The number and morphology of microglia in the ischemic penumbra were examined by immunofluorescence staining at 24 hours after MCAO. Consistent with morphological criteria described before, microglia were distinguished into three phenotypes: ramified, intermediate and amoeboid/round in the ischemic penumbra after MCAO.¹³ The different morphological phenotypes are considered to represent different functional states of microglia. While ramified cells represent resting state, amoeboid/round are typically signifying the activated states.¹⁴ The most of microglia in our study were

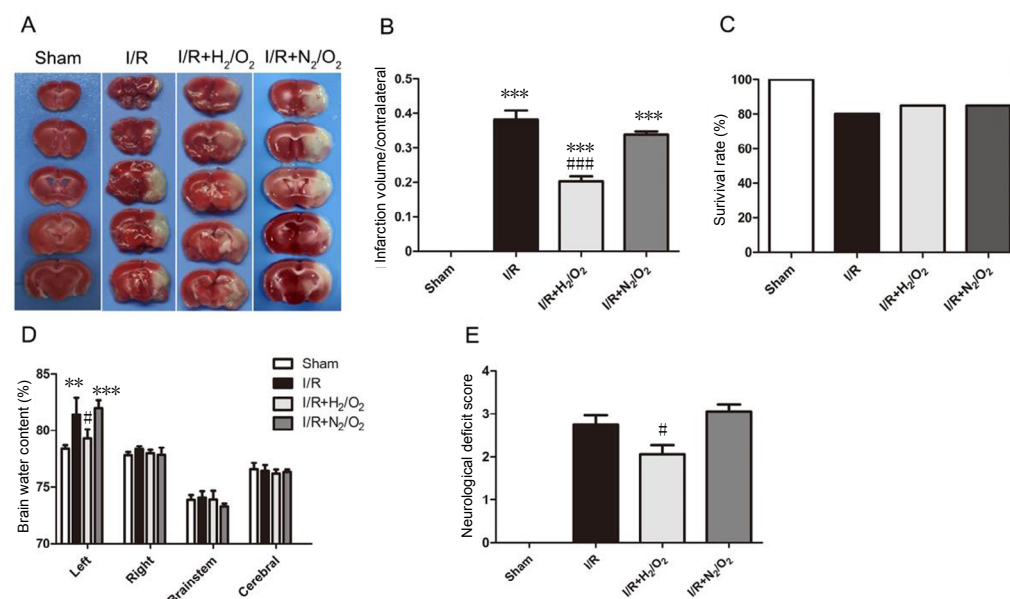


Figure 1: Hydrogen reduced infarct volume, alleviated brain edema and improved neurobehavioral deficits at 24 hours after middle cerebral artery occlusion in mice.

Note: (A) 2,3,5-Triphenyltetrazolium chloride staining (representative sections). (B) Quantification of 2,3,5-triphenyltetrazolium chloride staining ($n = 5-7$ /group). (C) Quantitative result of postoperative survival rate ($n = 20$ per group). (D) Evaluation of brain edema development ($n = 4-5$ per group). (E) Neurological deficit score ($n = 16-20$ per group). Data are presented as the mean \pm SEM. ** $P < 0.01$, *** $P < 0.001$, vs. sham group; # $P < 0.05$, ### $P < 0.001$, vs. I/R group (one-way analysis of variance followed by Bonferroni's multiple comparison test). I/R: Ischemia/reperfusion.

ramified and the rest cells were intermediate and amoeboid/round. Results showed the ramified microglia reduced from 93% to 2% at 24 hours after MCAO (**Figure 2**). On the contrary, the percentage of amoeboid/round microglia dramatically increased up to 58%, and that of intermediate microglia increased to 40%. While HCH dramatically decreased the number of amoeboid/round ($P < 0.01$), it also increased the intermediate microglia. The proportion of ramified microglia was low (around 2%) in both I/R and I/R + H₂/O₂ group. N₂ inhalation (I/R + N₂O₂ group) had no influence on the microglia of different morphological forms. Furthermore, the total microglia (Iba-1+ cells) were counted in each group. MCAO increased the number of microglia in the ischemic area, but neither H₂/O₂ nor N₂O₂ significantly affected the number of microglia after MCAO (**Figure 2**), and HCH decreased the number of amoeboid/round microglia after cerebral I/R injury.

Hydrogen promoted microglial phenotype transformation after cerebral I/R injury

In this study, mRNA expression of inflammation related genes (IL-1 β , IL-6, TNF- α and IL-10) were detected by quantitative real-time PCR at 24 hours after MCAO, aiming to assess the microglial phenotype. Compared to sham group,

MCAO dramatically increased the mRNA expression of pro-inflammatory factors (IL-1 β , IL-6, TNF- α), but had no effect on the production of anti-inflammatory factors (IL-10). HCH inhibit the mRNA expression of pro-inflammatory mediators (IL-1 β , IL-6 and TNF- α) after MCAO, while promoting the production of anti-inflammatory factors (IL-10) (**Figure 3**). Enzyme-linked immunosorbent assay showed similar changes in the contents of IL-10, IL-6, TNF- α , and IL-1 β .

DISCUSSION

This study indicated HCH could attenuate the cerebral I/R-induced brain injury *via* inhibiting microglial activation and regulating microglial phenotype transformation. Our results showed HCH reduced the infarct volume, alleviated the brain edema and attenuated the neurobehavioral deficits at 24 hours in a mouse MCAO model. We also observed that while HCH reduced the number of amoeboid/round microglia, it increased the number of the intermediate microglia, consequently decreasing the production of the pro-inflammatory and increasing production of the anti-inflammatory mediators. Our results describe a novel pathway, underlying the anti-inflammatory property of hydrogen after the ischemic stroke.

It has been confirmed that inflammation is involved in the

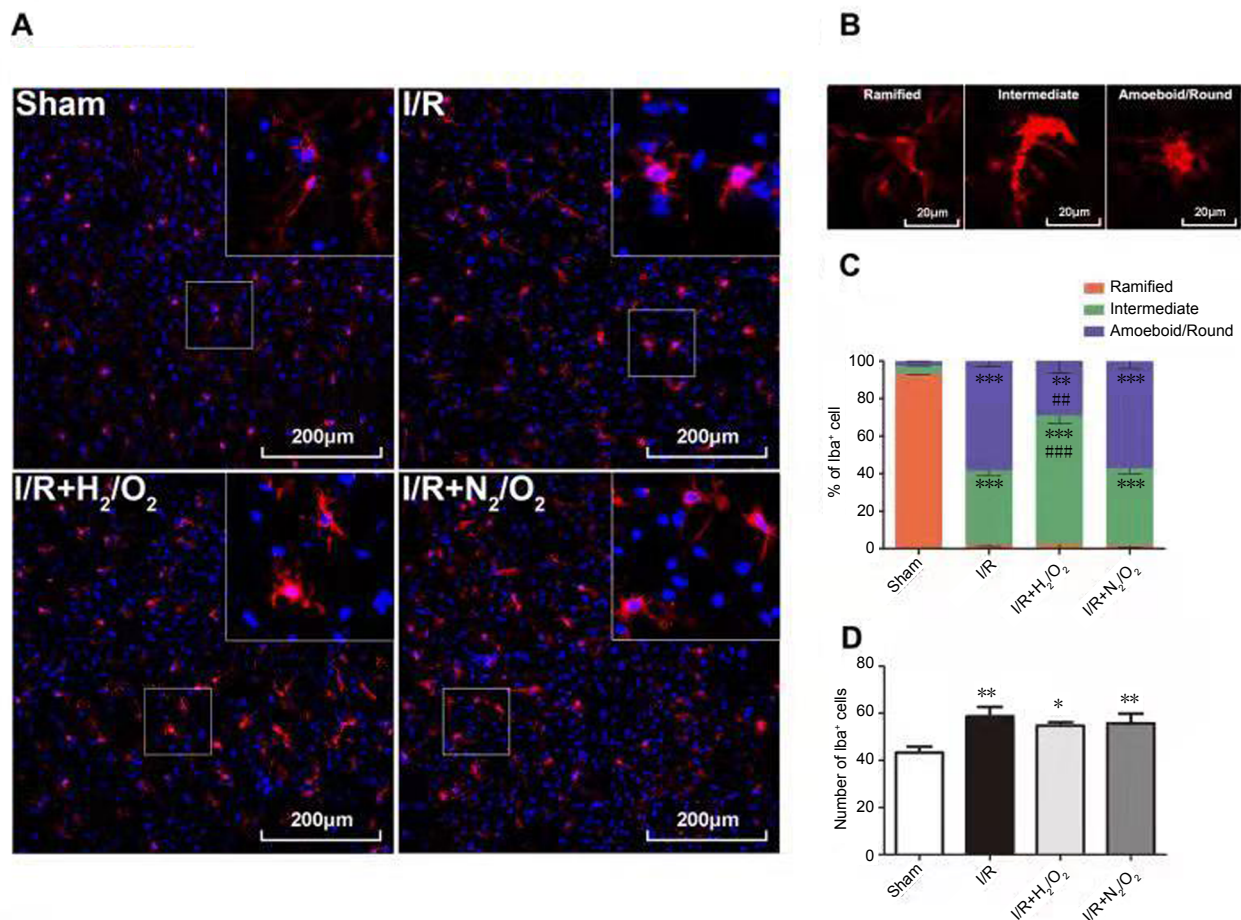


Figure 2: The effect of hydrogen on number and morphological change of microglia in brain at 24 hours after ischemic stroke.

Note: (A) The morphological change of microglia in ischemic brain tissues (immunofluorescence staining). Blue: DAPI; Red: tetramethylrhodamine isothiocyanate (TRATC). Scale bars: 200 μ m. (B) Photomicrographs showing examples of microglia classified into three different phenotypes: ramified, intermediate and amoeboid/round. Scale bars: 20 μ m. (C) Proportion of microglia with different morphological phenotypes of Iba-1⁺ cells. (D) Quantitative result of total number of microglia (Iba-1⁺ cells). Data are expressed as the mean \pm SEM of three brain sections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. sham group; ## $P < 0.01$, ### $P < 0.001$, vs. I/R group (one-way analysis of variance followed by Bonferroni's multiple comparison test). I/R: Ischemia/reperfusion.

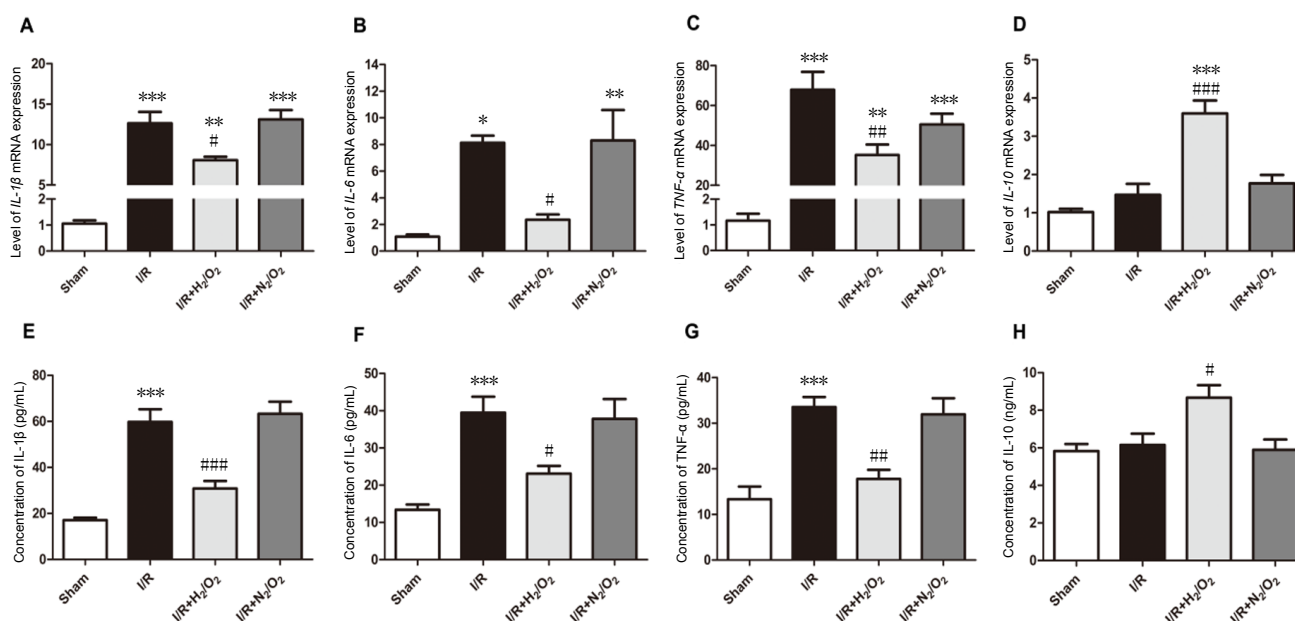


Figure 3: The effect of hydrogen on mRNA and protein expression of inflammation related genes in brain at 24 hours after middle cerebral artery occlusion. Note: (A–D) mRNA expression: interleukin (IL)-1 β (A), IL-6 (B), tumor necrosis factor (TNF)- α (C) and IL-10 (D) (real-time PCR); (E–H) protein expression: IL-1 β (E), IL-6 (F), TNF- α (G) and IL-10 (H) (enzyme-linked immunosorbent assay). Data are expressed as the mean \pm SEM ($n = 4-5$ per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. I/R group (one-way analysis of variance followed by Bonferroni's multiple comparison test). I/R: Ischemia/reperfusion.

pathogenesis of I/R injury.^{15,16} Microglia contributes to ischemic stroke induced inflammation.^{17,18} It has been confirmed that the morphological phenotypes of microglia represent different steps of microglial activation, which is related to distinct functional states.¹⁹ In addition, there is evidence that the repair of ischemic brain includes M1 macroglia related harmful inflammation responses and M2 macroglia related beneficial inflammation responses.^{5,6} Therefore, understanding of the mechanisms of inflammatory responses after stroke is helpful for the development of neuroprotective agents.²⁰

In recent years, hydrogen gas has been used in the treatment of some medical diseases. It has been confirmed that hydrogen possesses anti-inflammatory, anti-oxidative, and anti-apoptotic properties.²¹ Injury such as I/R injury can induce the production of excess reactive oxygen species, causing the imbalance between anti-oxidation and oxidation. Hydrogen can selectively scavenge hydroxyl radicals ($\bullet\text{OH}$) and peroxynitrite (ONOO^-) and improve the imbalance of the redox state. In addition, hydrogen is also able to down-regulate the expression of pro-inflammatory mediators and the release of pro-inflammatory mediators *via* many signaling pathways.²² However, a further in-depth study is needed to investigate the mechanism underlying the anti-inflammatory effects of hydrogen following cerebral I/R. Currently, little is known about the effect of hydrogen on microglia activation. This study investigated the amount and morphology of microglia in the ischemic penumbra after MCAO in a mouse model. Three different phenotypes of Microglia can be found in the ischemic penumbra after MCAO: ramified, intermediate and amoeboid/round.¹³ Our results showed hydrogen dramatically reversed the proportion of amoeboid/round microglia after MCAO, rather than the number of microglia. Hydrogen ameliorated cerebral

I/R injury, which has been confirmed in both mouse and rat models.^{12,23} It has been shown that hydrogen affects anti-inflammation through reducing several inflammatory mediators (IL-1 β , IL-6 and TNF- α).²³ Consistent with these findings a more recent study confirms hydrogen can reduce the IL-1 β , IL-6, TNF- α , high mobility group protein B1, interferon- γ and inducible nitric oxide synthase in different animal models.²² However, in above mentioned studies, only the pro-inflammatory factors (factors related to M1 microglia) are detected. In the present study, both anti-inflammatory and pro-inflammatory factors were detected at mRNA level in the brain after MCAO. Our results indicated HCH not only reduced the pro-inflammatory mediators (IL-1 β , IL-6, inducible nitric oxide synthase and TNF- α), but increased the anti-inflammatory factors (insulin-like growth factor-1, IL-10, and vascular endothelial growth factor) after MCAO. Our study for the first time suggests that the anti-inflammatory effects of hydrogen are related to the phenotype transformation of microglia in the brain. Of course, the inhibition of microglial phenotype may be one of mechanisms underlying the neuroprotective effects of hydrogen, and other mechanisms have also been reported.²²

There were still limitations in this study. First, the phenotype of microglia should be clarified by immunohistochemistry to confirm above findings; second, the underlying mechanism by which HCH regulates the phenotype transformation of microglia after MCAO was not further investigated.

Taken together, our results show ischemic stroke may cause the microglial activation, and the neuroprotection of HCH may be related to the regulation of phenotype transformation of microglia. More studies are needed to warrant our findings *in vivo* and *in vitro*.



Acknowledgements

The authors thank Jian-Fei Lu (Shanghai Jiao Tong University School of Medicine) for his excellent technical assistance.

Author contributions

JLH and WWL performed experiments and drafted the paper; AM and XJS revised this paper. QH and QYM conceived, designed and supervised this project. All authors approved the final version of the manuscript for publication.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Financial support

This study was partially supported by the National Natural Science Foundation of China (No. No. 81671130 to QH and 81772015 to WWL) and Shanghai municipal Science and Technology Commission Medical Guidance Science and Technology Support Project (No. 19411968400 to QYM).

Institutional review board statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, Shanghai, China (approval No. A2015-011) in November 2015 and all the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of Shanghai Jiao Tong University, China.

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Received: April 2, 2019

Reviewed: April 16, 2019

Accepted: July 23, 2019