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ORIGINAL RESEARCH

High-Mobility Group Box 1 Neutralization Prevents Chronic Cerebral Hypoperfusion-Induced Optic Tract Injuries in the White Matter Associated with Down-regulation of Inflammatory Responses

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Abstract
Chronic cerebral hypoperfusion (CCH)-induced white matter lesions (WMLs) are region-specific with the optic tract (OT) displaying the most severe damages and leading to visual-based behavioral impairment. Previously we have demonstrated that anti-high-mobility group box 1 (HMGB1) neutralizing antibody (Ab) prevents CCH-induced hippocampal damages via inhibition of neuroinflammation. Here we tested the protective role of the Ab on CCH-induced OT injuries. Rats were treated with permanent occlusion of common carotid arteries (2-VO) or a sham surgery, and then administered with PBS, anti-HMGB1 Ab, or paired control Ab. Pupillary light reflex examination, visual water maze, and tapered beam-walking were performed 28 days post-surgery to investigate the behavioral deficits. Meanwhile, WMLs were measured by Klüver-Barrera (KB) and H&E staining, and glial activation was further assessed to evaluate inflammatory responses in OT. Results revealed that anti-HMGB1 Ab ameliorated the morphological damages (grade scores, vacuoles, and thickness) in OT area and preserved visual abilities. Additionally, the increased levels of inflammatory responses and expressions of TLR4 and NF-κB p65 and phosphorylated NF-κB p65 (p-p65) in OT area were partly down-regulated after anti-HMGB1 treatment. Taken together, these findings suggested that HMGB1 neutralization could ease OT injuries and visual-guided behavioral deficits via suppressing inflammatory responses.

Keywords HMGB1 neutralization · Chronic cerebral hypoperfusion · Optic tract · Glial activation · NF-κB

Introduction

Chronic cerebral hypoperfusion (CCH) could cause long-lasting reduction in cerebral blood flow (CBF) and result in white matter lesions (WMLs), visual dysfunction, glial activation, and cognitive impairments (Davidson et al. 2000; Farkas et al. 2007; Back et al. 2017). Permanent occlusion of common carotid arteries (2-VO) in rats has now been widely accepted as a suitable approach to unravel the pathological changes and drug candidates for the treatment of WMLs in CCH and associated neurodegenerative diseases, such as vascular dementia (VaD), Alzheimer's disease (AD), and aging (Farkas and Luiten 2001; Akinyemi et al. 2013). In particular, substantial evidence has shown that CCH-induced WMLs are preferentially presented in regions, and the area of optic tract (OT), as a part of the visual system, displays much severer rarefaction (reduction of thickness), vacuolation, and sustained glial activation than other white matter regions (like corpus callosum, internal capsule, and caudoputamen) (Takizawa et al. 2003; Lee et al. 2006; Tian et al. 2014; Lee et al. 2015; Edrissi et al. 2016). However, previous studies have not pay sufficient attention to the pathological changes and the related functional outcomes (like visual-dependent spatial learning and motor deficits) in this area during CCH.

High-mobility group box 1 (HMGB1) can bind to receptor for advanced glycation end-products (RAGE) and toll-like receptor 4 (TLR4) and activate inflammatory responses once released into extracellular space under pathological

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conditions (Hamada et al. 2008; Pisetsky 2014). However, the role of HMGB1 in white matter injury remains controversial. On one hand, it has been reported that the activation of HMGB1 signaling results in persistent damages in the white matter in animal models of systemic inflammation and traumatic brain injury (Cardoso et al. 2015; Braun et al. 2017); on the other hand, some studies have showed that HMGB1 from astrocytes promotes the function of progenitor cells and plays as a autocrine factor in WMLs in acute ischemic models (Hayakawa et al. 2013; Choi et al. 2017). As a matter of fact, most of these studies focused on HMGB1 signaling in the acute WMLs models. However, CCH-induced chronic WMLs are usually accompanied with prolonged hyper-activation of glial cells (Choi et al. 2016), and the anti-inflammation therapies in the chronic phase of CCH via down-regulation of TLR4 and RAGE proved to be beneficial to the CCH-induced WMLs (Kim et al. 2015; Lee et al. 2015; Kim et al. 2016; Saggu et al. 2016). As the HMGB1 and its down-stream signaling TLR4/NF-κB were persistently activated in the chronic phase of CCH (Lee et al. 2015; Ashok et al. 2016; Hei et al. 2018), it is deducible that HMGB1 signaling plays a role in the pathogenesis of chronic WMLs and OT damages induced by CCH.

Previously, we have demonstrated that anti-HMGB1 neutralizing antibody (Ab) treatment suppresses glial activation, pro-inflammatory cytokine production, and oxidative stress in a rat model of CCH, which finally leads to long-time beneficial effects on the neuronal survival and cognitive ability in the chronic phase (Hei et al. 2018). Here we aimed to present the evidence that anti-HMGB1 neutralizing Ab is capable of easing OT injuries in the white matter and improving the visual-guided behavioral abilities via suppressing inflammatory responses in the chronic phase of CCH in rats.

Materials and Methods

Animals

A total of 91 male Wistar rats weighing 220–230 g were used. National Institutes of Health Ethic Committee gave permission to the animal experiments, which were conducted in accordance with the National Experimental Animals Guidelines. Rats were obtained from Vital River and kept in Animal Center of the Fourth Military Medical University (Xi'an, China). Wistar rats present much severe damages in the visual system in CCH model in comparison to SD rats and are frequently used to investigate 2VO-induced WMLs (Kim et al. 2008; Lee et al. 2015; Ueno et al. 2015; Back et al. 2017). Apart from the randomly selected 18 rats in the sham group [treated with a sham surgery and phosphate-buffered saline (PBS, pH 7.4)] and six rats in the sham + anti-HMGB1 group (treated with a sham surgery and anti-HMGB1 neutralizing antibody (Ab) and used for histological assessment of WMLs only), other 67 rats were randomly selected and underwent 2VO surgery.

Model Establishment and Experimental Design

Rats underwent the surgery of permanent bilateral common carotid artery occlusion (2VO) to establish CCH as previously described (Tian et al. 2014; Back et al. 2017). Briefly, the bilateral common carotid arteries were exposed after a midline cervical incision. The bilateral nerve fibers should be gently separated. Then the bilateral arteries were permanently ligated with silk suture. Temperature was maintained at 37.5 °C using a heat pad during the surgery. Sham operation was done without ligation of the arteries. A total of 16 rats died during or after the 2VO surgery and the remained 51 rats were randomly divided into vehicle group (treated with PBS), anti-HMGB1 group (treated with anti-HMGB1 Ab) and control Ab group (treated with paired control Ab) ($n = 17$ in either group). The anti-HMGB1 Ab or the paired control Ab (1 mg/kg, dissolved in 0.9% PBS, Shino-test, Kanagawa, Japan) were injected via the tail vein after 2VO surgery for thrice. The detailed information of dose study, efficacy, and method of tail vein injection was described in our previous research (Hei et al. 2018). Specifically, as anti-HMGB1 therapy turned out good in acute WMLs (Hayakawa et al. 2013; Choi et al. 2017), we delayed the time of injection [Day 4–Day6, which is in the sub-acute phase of CCH (Farkas et al. 2007; Soria et al. 2013)] to see anti-HMGB1's effects in the chronic phase of CCH (28 days after surgery) without interfering with HMGB1's beneficial functions in the acute phase. The experimental schedule was presented in the Fig. 1a.

Klüver-Barrera (KB) and Hematoxylin–Eosin (H&E) Staining

All animals ($N = 6$ in each group) that assigned to histological analyses of CCH-induced white matter damages were intraperitoneally anesthetized with chloral hydrate (10%, 3 ml/kg) and transcardially perfused with 4% paraformaldehyde in PBS. The rat brains were processed into coronal sections (5 μm, from bregama − 2.04 to − 3.36 mm) (Wang et al. 2010) and mounted on polarized glasses. The KB staining was conducted to evaluate the general changes in white matter regions including the corpus callosum (CC), internal capsule (IC), and optic tract (OT) (assessing the grade scores and the percentage of thickness) (Tachibana et al. 2017). The tissue sections were incubated with 0.1% Luxol fast blue (Sigma, MO, USA) at 56 °C overnight and the severity of damages were graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and the disappearance of myelinated fibers (grade

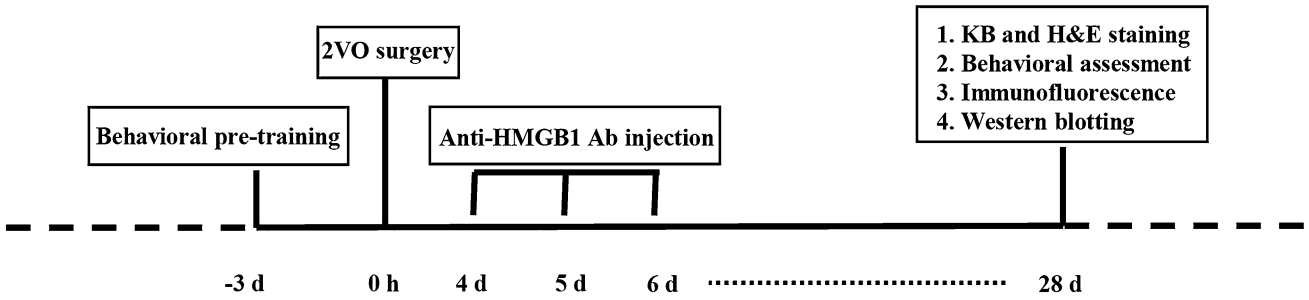

Fig. 1 The experimental schedule. Rats were per-trained for behavioral tests 3 days before 2VO surgery and pupillary light reflex (PLR), behavioral tests, histological assessment and western blotting were carried out on day 28 (which is in the chronic phase of CCH) after anti-HMGB1 treatment (day 4–day 6, which is in the sub-acute phase of CCH)

3) (Wakita et al. 1994, 2002; Tachibana et al. 2017). And the (%) of thickness within a predetermined region-of-interest (ROI) was measured by mode methods (Takizawa et al. 2003; Edrissi et al. 2016; Tachibana et al. 2017) and the value in the sham group was defined as 100% (thus the value in the sham group was presented without any error bars). Furthermore, the hematoxylin–eosin (H&E) staining was conducted to validate the vacuolation of the OT area. Briefly, sections were washed (in xylol for 10 min, ethanol (100%) for 5 min, ethanol (80%) for 5 min), incubated in hematoxylin solution (0.1%, Beyotime, Shanghai, China), washed in water for 5 min, and incubated in alcohol eosin solution (0.5%, Beyotime) and dehydrated. The % area of vacuoles was calculated in the same way as the % of thickness.

Behavioral Assessment

Rats ($N=6$ in the sham group; $N=5$ in the vehicle group, the anti-HMGB1 group and the control Ab group) underwent behavioral assessment including the pupillary light reflex (PLR) examination (at 9:00 a.m. before the beam-walking test), tapered beam-walking test (after the PLR), and visual water maze (at 3:00 p.m.) 28 days after 2VO surgery.

First of all, the PLR was examined by two investigators that are blind to the groups (Lavinsky et al. 2006). Briefly, rats were allowed to adapt to darkness for 1 min prior to the examination. Then a light from an otoscope was directed to the right eye for evaluating the direct PLR. Subsequently, the beam quickly moved to the left eye to assess the indirect reflex. The same procedure was conducted on the other eye with time interval of 1 min. PLR loss was finally defined as the failure of pupillary constriction within a 10 s light exposure. For tapered beam-walking (Edrissi et al. 2016), the test was performed in a quiet room by two investigators that are blind to the groups.

The beam was 1.65 m in length, tapering from 6 cm to 1.5 cm in width with a ledge extending beneath the beam; 1 m above the floor. The time to traverse the beam and the number of foot faults (slip off the beam) were counted in 2 m-distance under a monitor (Smart, Shanghai, China). Afterwards, visual water maze test with a visible platform was performed. The platform was put 1 cm above the surface of the water (could be seen clearly) located in quadrant I. Rats were given maximum 60 s to locate the platform and repeated for 3 times. After finding the platform, the rats were allowed to stay on the platform for additional 20 s. Rats in the four groups were trained for the tapered beam-walking test and visual water maze test 3-day before the 2VO surgery for three times.

Immunofluorescence

The preparation of the brain sections from rats ($N=6$ in each group) were the same as above. After washing with PBS, sections were incubated with Triton x-100 (0.1%) and 5% BSA for 30 min. Then the primary antibodies were added including rabbit anti-Iba1 (1:100, Abcam, CA, USA) and mouse anti-GFAP (1:100, Abcam, CA, USA) at 4 °C overnight. The secondary antibodies (1:1000, Invitrogen, CA, USA) were added accordingly and incubated for 4 h at room temperature. Besides, 0.0001% 4′,6-diamidino-2-phenylindole (DAPI) staining (Beyotime) was conducted to stain cell nuclei for 10 min. The images were obtained using confocal laser scanning (Olympus, Japan) (magnification, ×200) and analyzed in gray scale by Image pro 6.0 with a fixed exposure.

Western Blotting

Following the behavioral tests, rats ($N=6$ in each group) were decapitated after anesthesia (10% chloral hydrate, 3 ml/kg, i.p.). Tissues of the OT area were carefully separated from the rat brain on ice under microscope and the protein were extracted using BioRad protein assay kit (Hercules, DE, USA). But for the detection of p65 (nuclear expression) and p-p65, the nuclear and cytosolic proteins were obtained using Nuclear & Cytoplasmic Protein Extraction Kit (Beyotime). As previously described (Zhang et al. 2011; Ouyang

et al. 2016), the homogenized protein samples (50 μg) were added to SDS-PAGE. Then the bands of the proteins were transferred to 0.22 μm NC membranes (Whatman, Germany). The primary antibodies of TLR4 (1:1000, Abcam, CO, USA), β-actin and Histone H3 antibody (1:2000, Abcam, CO, USA), and NF-κB p65 and phosphorylated NF-κB p65 (p-p65) (1:1000; Cell Signaling Technology, MA, USA) (known as the important pro-inflammatory molecular signaling that could be regulated by HMGB1), were added to the membranes in blocking solution. After incubation overnight at 4 °C, the membranes were washed and incubated with appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1.5 h and the final electrochemiluminescence was conducted with an IPP 6.0 system. Densities were normalized to β-actin and the value of the sham group was defined as 100%.

Statistical Analysis

Data are expressed as mean ± s.e.m. Multiple comparisons in behavioral tests and morphological changes in the OT area were analyzed using one-way ANOVA followed by post hoc Bonferroni's test using SPSS 22.0.0 and GraphPad Prism 7.0.1 (GraphPad software). The $P < 0.05$ were considered to be statistically significant.

Results

Anti-HMGB1 Ab Ameliorated the CCH-Induced WMLs Especially in the OT Subarea in the Chronic Phase

As the KB staining was frequently used to show the WMLs (expressed as grade scores and thickness), firstly we performed such experiment 28 days after 2VO surgery to show the damages in the CC, IC, and OT regions (Fig. 2). Compared with the vehicle group, the anti-HMGB1 group showed a decrease in the grade scores ($F_{(4,25)} = 73.51$, $P < 0.0001$) and an increase of thickness ($F_{(4,25)} = 96.77$, $P < 0.001$) in the OT area, and showed only a decrease of grade scores ($F_{(4,25)} = 67.8$, $P < 0.0001$) in the CC area, and showed no significant difference in the IC area ($P > 0.05$). It should be noted that the sham + anti-HMGB1 group showed no significant alteration either in the grade scores or thickness in the KB staining in comparison to the sham group ($P > 0.05$), indicating that the anti-HMGB1 treatment alone has no visible influence on white matter. We therefore focused on the investigation of CCH-induced OT injuries within the other four groups and further used H&E staining to verify the results (Fig. 3). We found that there were significant increases in the grade scores ($F_{(3,20)} = 56.06$, $P < 0.0001$) and vacuoles ($F_{(3,20)} = 49.75$, $P < 0.0001$) and there was a decrease of thickness ($F_{(3,20)} = 87.35$, $P < 0.001$) in the vehicle group in comparison with the sham group. Anti-HMGB1 group presented dramatically reduced damages (grade score: $P < 0.05$; thickness: $P < 0.05$; vacuoles: $P < 0.001$ vs. the vehicle group), indicating that anti-HMGB1 Ab significantly ameliorated the OT injuries in the chronic phase of CCH. Meanwhile, no significant difference in the above parameters was found between the vehicle group and the control Ab group.

Anti-HMGB1 Ab Improved Behavioral Performances in the Visual Water Maze and Tapered Beam-Walking Tests in the 2VO Model

First of all, the pupillary light reflex (PLR) examination was conducted (Fig. 4a). Nearly 50% rats in the vehicle group lost the PLR ($F_{(3,17)} = 64.12$, $P < 0.001$ vs. the sham group), but the anti-HMGB1 treatment reduced the PLR loss ($P < 0.05$) in comparison with the vehicle group. In the visual water maze tests (Fig. 4b), the vehicle group presented reduced percentage of platform findings compared with the sham group ($F_{(3,17)} = 49.72$, $P < 0.01$). And again, anti-HMGB1 group showed better performances compared to the vehicle group ($P < 0.05$). Besides, we further investigated the visual-based motor ability using the beam-walking test (Fig. 4c, d). Similarly, the vehicle group showed longer time latencies ($F_{(3,17)} = 19.52$, $P < 0.001$) and foot faults ($F_{(3,17)} = 11.66$, $P < 0.001$) in comparison to the sham group and rats in the anti-HMGB1 group performed better compared to the vehicle group (time latency: $P < 0.01$; foot faults: $P < 0.05$). In addition, rats in the control Ab group performed worse than the ones in the sham group, but no significant difference in the above parameters was found between the vehicle group and the control Ab group.

Anti-HMGB1 Ab Suppressed Inflammatory Responses Characterized by the Activation of Glial Cells and TLR4/NF-κB Signaling

Meanwhile, to further evaluate the level of glial activation in the OT area, we performed Iba1 and GFAP immunostaining (Fig. 5). The results showed that the intensity of Iba1 and GFAP staining was significantly up-regulated in the vehicle group in comparison to the sham group. However, after anti-HMGB1 treatment, these two parameters were relatively down-regulated compared with the vehicle group. To further clarify the molecular mechanisms associated with inflammatory responses in the OT region, western blotting analysis was performed 28 days after surgery. As shown in Fig. 6, the relative protein expressions of TLR4 ($F_{(3,20)} = 20.11$, $P < 0.05$), p65 ($F_{(3,20)} = 35.12$, $P < 0.05$), and p-p65 ($F_{(3,20)} = 22.41$, $P < 0.05$) significantly increased in comparison with the sham group. However, anti-HMGB1 treatment partly suppressed the activation of the TLR4

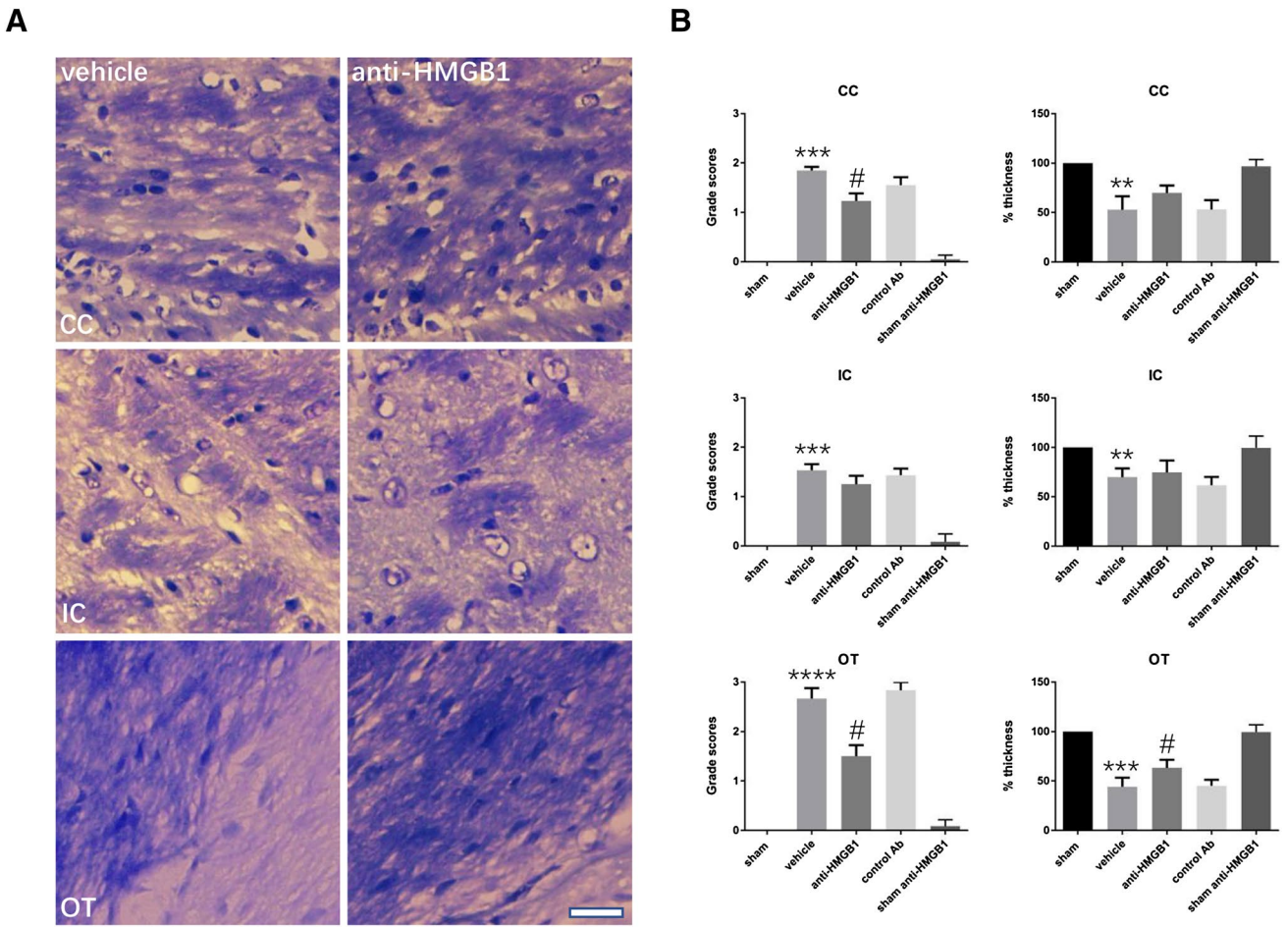

Fig. 2 The influence of anti-HMGB1 Ab on the CCH-induced white matter injuries. **a** Representative images of the KB staining in white matter areas including the corpus callosum (CC), internal capsule (IC), and optic tract (OT) in the vehicle group (left) and anti-HMGB1 group (right). **b** The quantification of the injuries (including the grade score and the percentage (%) of thickness) among the five groups in different subareas in the white matter. Bar = 50 μm. Data are expressed as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus sham group; $^{\#}P < 0.05$ versus vehicle group

($P < 0.05$), p65 ($P < 0.05$), p-p65 ($P < 0.05$) as compared to the vehicle group. Besides, no significant difference was found between the vehicle group and the control Ab group, indicating that the control Ab has no visible influence on the expressions of TLR4/NF-κB signaling.

Discussion

CCH causes progressive WMLs, which appear 5 days after 2VO surgery and are persistent in the chronic phase (Davidson et al. 2000; Tian et al. 2014). Specifically, the white matter, which is predominantly consisted of glia and myelinated axons, presents remarkable demyelination, vacuolation, axonal damage, and glial activation during CCH, and the OT region is selectively damaged; leading to visually guided behavioral deficits (Wakita et al. 1994; Edrissi et al. 2016). Previously we have used anti-HMGB1 neutralizing antibody to treat CCH-induced hippocampal damages via inhibition of neuroinflammation. However, it is still lack of reports about the therapeutic role of HMGB1 neutralization on the chronic OT injuries in CCH.

The white matter areas display varying degrees of vulnerabilities to CCH, and given the fact that the OT region is dependent on the direct blood supply of the internal carotid artery, damage in this specific optic structure comes heavier in comparison with other white matter regions during CCH (Kim et al. 2009; Zhang et al. 2011). Consistently, in the present study we found increased level of WMLs (especially in the OT area) and the anti-HMGB1 treatment seems to gain much more benefits in this area (using KB and H&E staining). Besides, we also tested the effects of the Ab on sham-operated rats (sham + anti-HMGB1 group) and found that the Ab did not alter the morphological characteristics in the CCH model, which was also in line with others (Festoff et al. 2016; Kigerl et al. 2017). Thus, we focused on the OT

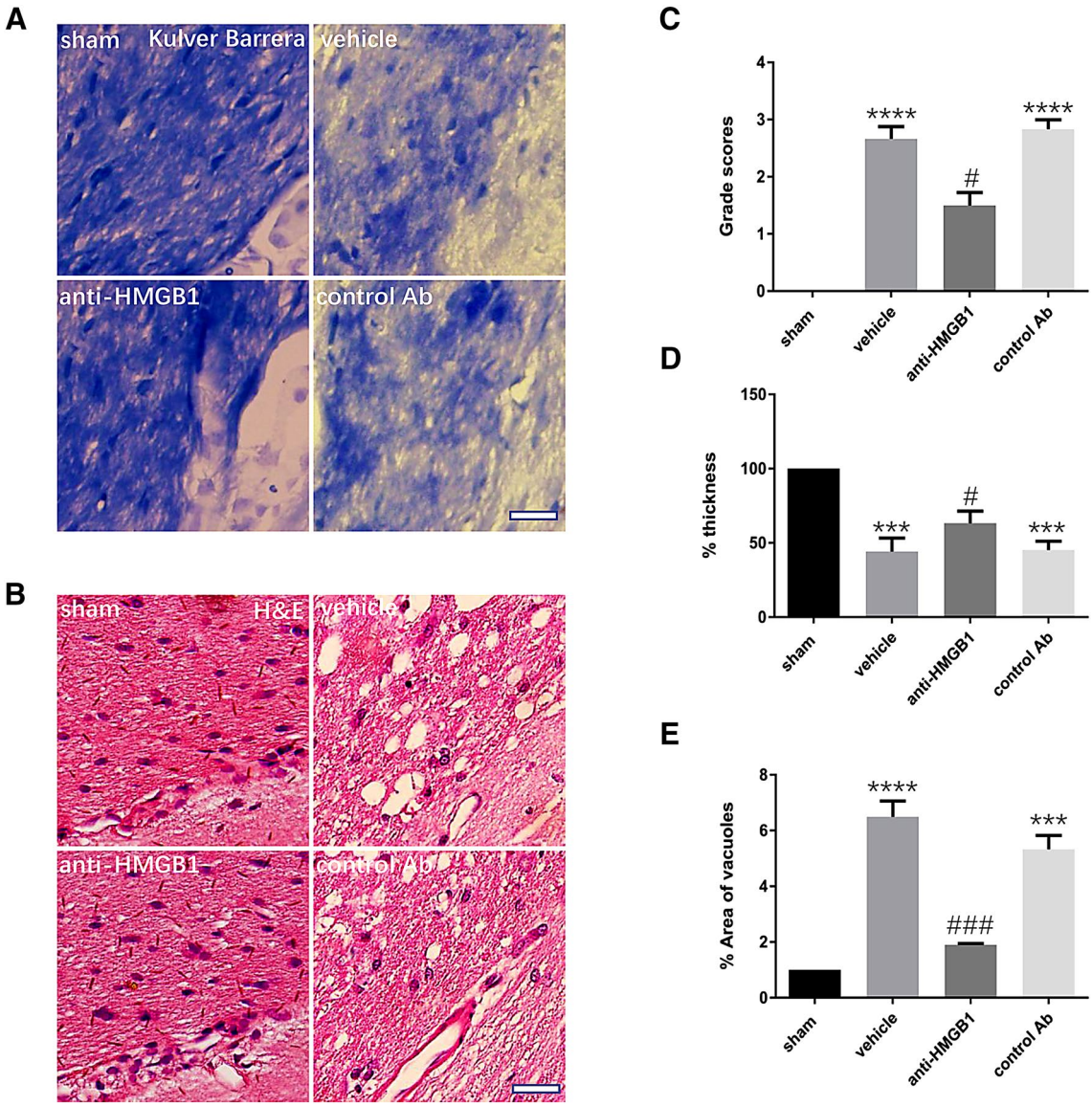

Fig. 3 The influence of anti-HMGB1 Ab on the CCH-induced OT injures assessed by KB and H&E staining. **a**, **c**, **d** The KB staining in OT area and the quantification of the injuries (including the grade score and the percentage (%) of thickness). **b**, **e** The H&E staining and the quantification of the percentage (%) area of vacuoles. Bar = 50 μm. Data are expressed as mean ± s.e.m. $^{***}P < 0.001$, $^{****}P < 0.0001$ versus sham group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus vehicle group

damages to perform the further study using the four groups (sham group, vehicle group, anti-HMGB1 group, and control Ab group).

The presence of dilated pupils and impaired performances in visually guided tasks sufficiently suggested the existence of damages in the visual system. In addition, we further found significant correlations between the existence of PLR and the behavioral ability (both for visual water maze and beam-walking tests) (data not shown). Interestingly, previous studies have shown that while 2VO surgery affects PLR within 5 days, the morphological changes in retinas cannot be assessed until 3 months later (Davidson et al. 2000; Stevens et al. 2002). Therefore, CCH-induced OT injuries come earlier and heavier in the visual system, indicating that 2VO surgery rapidly damages the optic nerve and causes loss of PLR.

Substantial evidence has demonstrated that inflammatory responses in 2VO rats has spatial preference in the white matter with OT area presenting most severe and prolonged damages during the course of disease (Tian et al. 2014; Kim et al. 2015). In accordance with these findings, we have also found excessive glial activation (including activated microglia and astrocytes) in OT area together with increased degenerative changes, such as rarefaction and vacuolation in

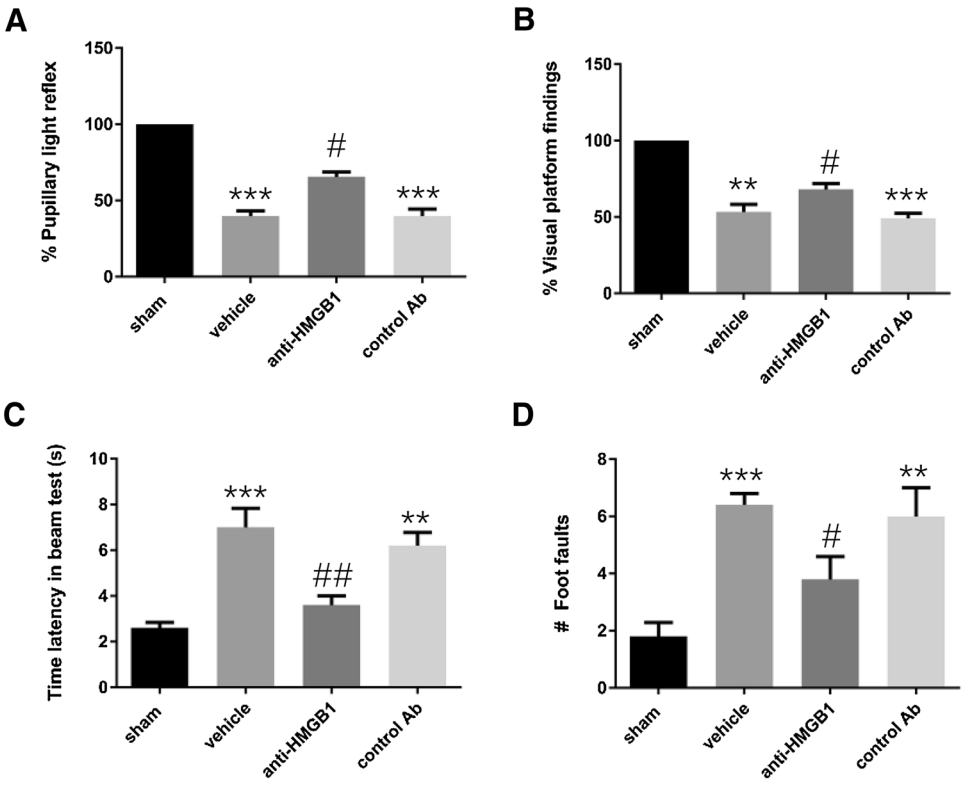

Fig. 4 The influence of anti-HMGB1 Ab on visual-based behavioral tests. **a** The percentage (%) of PLR. **b** The percentage (%) of visual platform findings in the visual water maze tests. **c**, **d** Time latency and the number of foot faults in the tapered beam-walking tests. Data are expressed as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$ versus sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle group

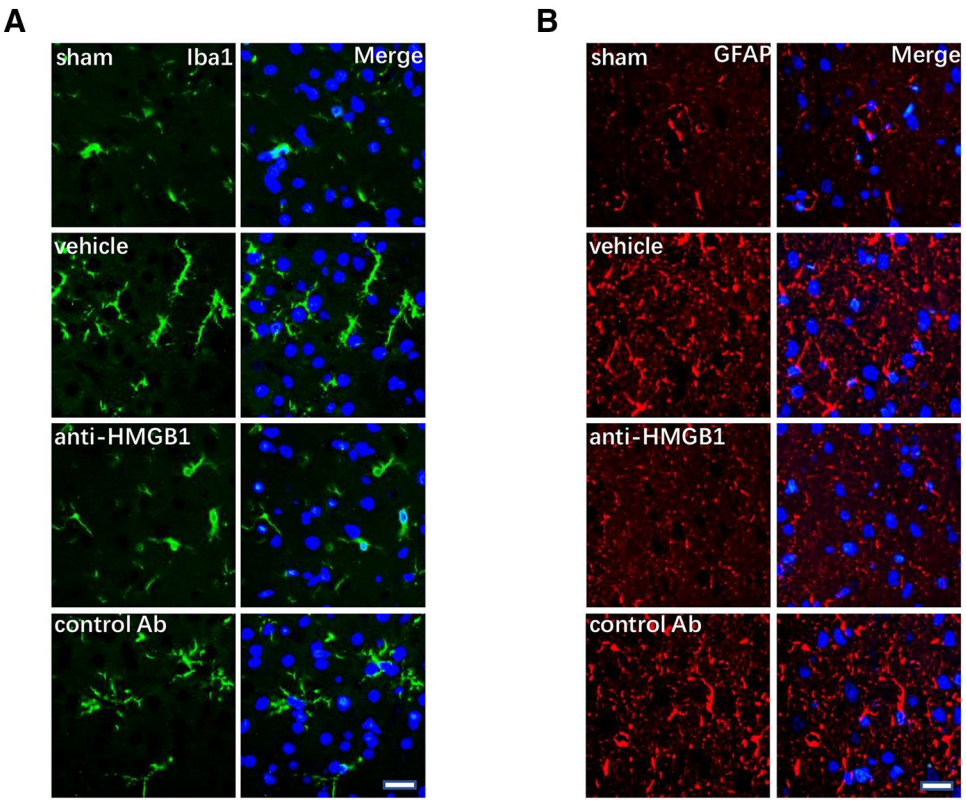

Fig. 5 The influence of anti-HMGB1 Ab on the CCH-induced glial activation in the OT area. **a** Representative images of Iba1 immunostaining. **b** Representative images of GFAP immunostaining. Bar = 50 μm

the chronic phase of CCH. Some studies have demonstrated that in the early stage of CCH the activated astrocytes could release neurotrophic factors (which help axonal regrowth) and to the contrary, the excessive gliosis hinders axonal

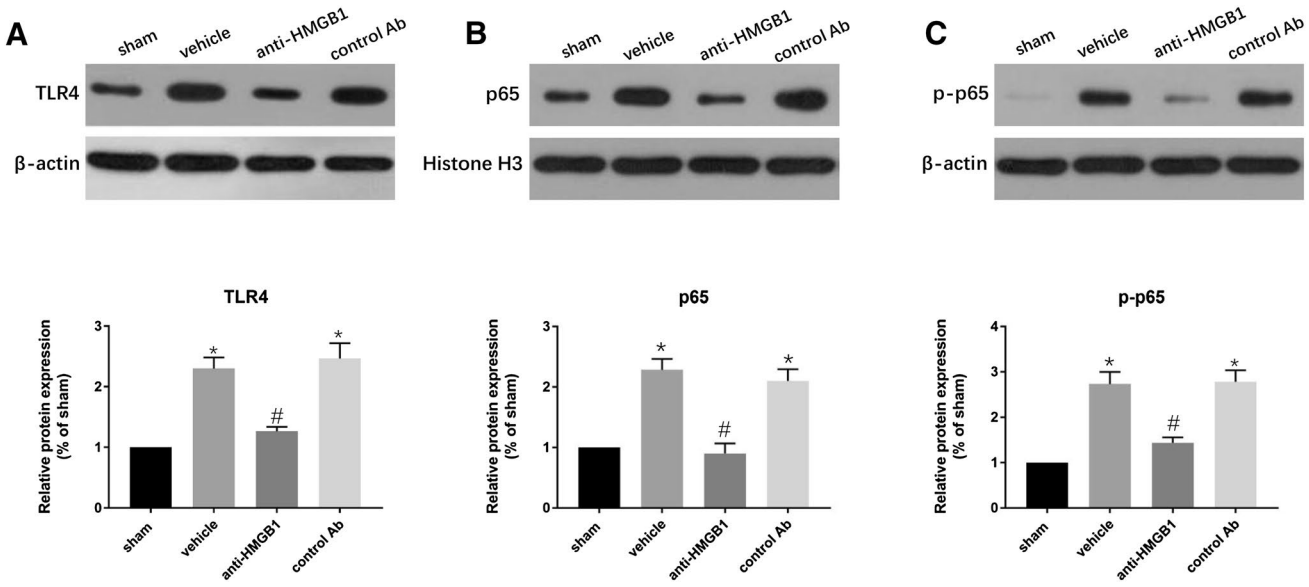

Fig. 6 The influence of anti-HMGB1 Ab on suppression of the activation of TLR4/NF-κB signaling in the OT area. **a**, **c** The protein bands and the corresponding quantification of TLR4 (**a**), NF-κB p65 (**b**) and phosphorylated NF-κB p65 (p-p65) (**c**). The value of the sham group was defined as 100%. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$ versus sham group; $^{\#}P < 0.05$ versus vehicle group

growth and starts its glial-scar formation process in the chronic phase of CCH and in other neurodegenerative diseases (Maragakis and Rothstein 2006; Phatnani and Maniatis 2015). Thus, it stands a good chance that inflammatory responses plays a core role in the chronic phase of CCH-induced WMLs (rather than in the acute phase), the anti-HMGB1 treatment was done early in the sub-acute phase in consideration of the BBB penetration of the neutralizing Ab and the early activation of HMGB1 signaling during CCH (Lee et al. 2015; Hei et al. 2018). After all, these findings strongly suggested that inhibition of CCH-induced inflammatory responses probably lead to beneficial outcomes in the chronic phase.

HMGB1, one damage-associated molecular patterns (DAMPs) molecule, has been well-established to exert its pro-inflammatory cytokine-like effects once activated in various diseases (mainly through the pro-inflammatory receptors RAGE and TLR4) (Liu et al. 2007; Terrando et al. 2016; Wang et al. 2017). Both we and others have shown the therapeutic role of anti-HMGB1 Ab in behavioral and biochemical improvement in various inflammatory conditions in CNS (Festoff et al. 2016; Kigerl et al. 2017). Although recent studies have shown that HMGB1 is capable of promoting oligodendrocytes survival, re-myelination of the axons and the migration of EPCs after acute WMLs (Hayakawa et al. 2013; Choi et al. 2017), the chronic WMLs show difference in the pattern of glial activation (as described above) and hypoxia-related gene expressions (Bang et al. 2013; Yang et al. 2013). Specifically, hypoxia inducible factor (HIF)-1α and the associated pro-survival factors (like glucose transport 1 and endothelial growth factor), which could induce HMGB1 relocation (Andrassy et al. 2008), significantly decreased in the chronic phase of CCH (Bang et al. 2013; Yang et al. 2013). Therefore, the function of HMGB1 signaling (both the pattern of HMGB1 mobilization and the final effects on glial activation) probably changes accordingly. In agreement with these findings, we revealed that HMGB1 neutralization is capable of suppressing inflammatory responses in the chronic phase of CCH, suggesting that excessive HMGB1 signaling in the OT area probably leads to detrimental results. Previous studies have also used anti-inflammatory methods to treat CCH-induced WMLs and demonstrated that the inhibition of HMGB1 signaling (such as TLR4/NF-κB) results in various beneficial effects such as the up-regulation of myelin basic protein (MBP) and cholinergic transmission and down-regulation of glial activation and pro-inflammatory cytokine production (Kim et al. 2015; Lee et al. 2015; Kim et al. 2016; Saggu et al. 2016; Hei et al. 2018), which are in line with ours.

In conclusion, this study demonstrated that anti-HMGB1 neutralizing Ab treatment eases CCH-induced optic tract injuries in the white matter associated with down-regulation of inflammatory responses characterized by the activation of glial cells and HMGB1's down-stream TLR4/NF-κB signaling. This study further elucidates the significance of inhibition of HMGB1 signaling and glial activation in the chronic phase of CCH, which may provide new targets for

development of molecular therapies to treat CCH-induced WMLs in related vascular disorders and neurodegenerative diseases.

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Author Contributions YH and XZ performed the experiments, RC and YZ wrote the manuscript, DG and WL designed the experiments, and WL is responsible for the final version. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article.

Compliance with Ethical Standards

Conflict of interest No conflict of interest was declared.

Ethical Approval National Institutes of Health Ethic Committee gave permission to the animal experiments, which were conducted in accordance with the National Experimental Animals Guidelines.

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