



20-HETE Inhibition by HET0016 Decreases the Blood–Brain Barrier Permeability and Brain Edema After Traumatic Brain Injury

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Recent studies have implicated 20-HETE as a vasoconstrictive mediator in trauma, the purpose of this study was to determine whether administration of HET0016, the 20-HETE inhibitor, could protect neurons from trauma and the effect of HET0016 on the blood–brain barrier (BBB) and brain edema in experimental traumatic brain injury (TBI). Rat models with TBI were established. Brain edema was measured according to the wet and dry weight method at 3, 24, and 72 h after injury. The BBB permeability was quantified by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). Superoxide production, the activity of superoxide dismutase (SOD) and total antioxidative capability (T-AOC) in traumatic brain tissues were also measured. Western blot analysis was used to analyze the expression of the occludin, ZO-1, Matrix metalloproteinase-9 (MMP-9), and c-Jun N-terminal protein kinase (JNK) pathways. At 24 and 72 h after administration of HET0016 following TBI, the BBB permeability and brain edema decreased. The decrease in superoxide production and the increase in the activity of SOD and T-AOC were measured in this study. Western blot analysis showed that the expression of MMP-9 and JNK pathways was suppressed, but the expression of ZO-1 and occludin was increased. These results suggest that the administration of HET0016 could protect the BBB function and decrease brain edema after experimental traumatic injury by suppressing the expression of MMP-9 and activating the expression of tight junction proteins via suppressing the JNK pathway and oxidative stress.

Keywords: HET0016, blood–brain barrier, blood edema, 20-HETE, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) induces arachidonic acid (AA) release from cell membranes (Birnie et al., 2013). AA levels increase 1093% within 48 h of TBI and remain elevated for days after TBI (Yang S. et al., 2012). Cytochrome P450 (CYP) enzymes, especially four different CYP4A isozymes, can catalyze the ω -hydroxylation of AA to 20-hydroxyeicosatetraenoic acid (20-HETE) (Johnson et al., 2015). 20-HETE has long been recognized as a potent vasoconstrictor of the microvasculature (Hall et al., 2014) in cerebral circulation, and several studies suggested that 20-HETE has important biological and pathological functions in the regulation of vasculature, cerebral blood flow (CBF), cellular proliferation, and the inflammation reaction (Harder et al., 1994; Yang Z. J. et al., 2012; Fordsmann et al., 2013; Garcia et al., 2015; Zhu et al., 2015). There is one report indicating that

20-HETE increases the vascular production of reactive oxygen species (ROS), promotes NF- κ B activation in cerebrovascular endothelial cells, and consequently deteriorates inflammation (Toth et al., 2013).

Traumatic brain injury consists of two stages of pathophysiological injury: primary [e.g., brain contusion, parenchyma hemorrhage, subarachnoid hemorrhage, and diffuse axonal injury (DAI)] and secondary (e.g., edema, herniation, ischemia, and infarction) (Le and Gean, 2009; Wei et al., 2012). The mechanism of secondary injury that contributes to ongoing injury is of interest. ROS, which are considered part of the secondary injury, are implicated in the major pathology of diverse acute and chronic brain injuries (Wei et al., 2012). ROS can cause blood–brain barrier (BBB) disruption and increase the cerebral vascular permeability, leading to the formation of brain edema (Wei et al., 2012).

Several previous studies demonstrated that 20-HETE-targeted therapies after cerebral ischemia/reperfusion injury may improve neurologic outcome (Miyata et al., 2001; Renic et al., 2009, 2012). HET0016 attenuated 20-HETE levels, reduced cerebrovascular damage and improved stroke outcomes (Miyata et al., 2001; Poloyac et al., 2006; Renic et al., 2009). Thus, the administration of HET0016 after TBI may be a potentially important target for novel therapies. To the best of our knowledge, no studies have investigated whether the administration of HET0016 could protect neurons from trauma or the effect and the mechanism of HET0016 on the BBB and brain edema after TBI in experimental research.

Matrix metalloproteinase-9 (MMP-9) plays an essential role in tissue repair, cell death, morphogenesis, and outcome prediction in neurological diseases (Xu et al., 2014; Cai et al., 2015). In both animal studies and human TBI patients, MMP-9 has been demonstrated to be an integral component in the pathophysiology of brain edema. Its overexpression is associated with an increase in the BBB permeability by degrading the tight junction proteins (Kim et al., 2015; Wang G.Y. et al., 2015; Wu et al., 2015). MMP-9 is usually activated by ROS and the signaling pathways of ROS (Wang G.Y. et al., 2015). Of these pathways, the c-Jun N-terminal protein kinase (JNK) signaling pathway has been well-studied in the BBB breakdown associated with MMP-9 activation (Liu et al., 2012). The inhibition of JNK protects the BBB by suppressing MMP-9 activation (Urrutia et al., 2013). Thus, we hypothesized that HET0016 may decrease the production of ROS, increase the activity of superoxide dismutase (SOD) and total antioxidative capability (T-AOC), suppress the expression of MMP-9 and activate the expression of tight junction proteins (occludin and ZO-1) via suppressing the JNK signaling pathway, eventually protecting the BBB function. Thus, we investigated the BBB function and brain edema in experimental TBI.

RESULTS

BBB Edema

Brain water content increased as early as 3 h after injury in the TBI group compared with the sham group ($79.62 \pm 0.70\%$

vs. $78.54 \pm 0.21\%$, $P < 0.05$). In addition, our results revealed that the brain water content significantly increased at 24 and 72 h in the TBI group compared with the sham group (24 h: $81.44 \pm 0.40\%$ vs. $78.54 \pm 0.17\%$, $P < 0.01$; 72 h: $81.78 \pm 0.33\%$ vs. $78.74 \pm 0.21\%$, $P < 0.01$) (Figure 1). HET0016 administration ameliorated the change in water content at 24 h ($79.86 \pm 0.36\%$, $P < 0.01$) and 72 h ($79.78 \pm 0.45\%$, $P < 0.01$) but not at 3 h ($79.52 \pm 0.75\%$, $P > 0.05$), compared with the vehicle-treated group (Figure 1).

Changes in K^{trans}

An immediate and sustained increase in K^{trans} (min^{-1}) was observed post-injury in the TBI group compared with the sham group. As is shown in Figure 2, K^{trans} was increased at 3, 24, and 72 h (3 h: 0.024 ± 0.002 vs. 0.003 ± 0.001 , $P < 0.01$; 24 h: 0.034 ± 0.004 vs. 0.003 ± 0.001 , $P < 0.01$; 72 h: 0.056 ± 0.003 vs. 0.003 ± 0.003 , $P < 0.01$) (Figure 2). HET0016 administration decreased the K^{trans} value at 24 h (0.025 ± 0.004 , $P < 0.05$) and 72 h (0.038 ± 0.001 , $P < 0.01$) but not 3 h (0.025 ± 0.004 , $P > 0.05$) compared with the value in the vehicle-treated group (Figure 2).

Analyses for MDA, SOD, and T-AOC Activity

Malondialdehyde (MDA) increased at 3, 24, and 72 h in the TBI group, compared with that of the sham group (3 h: 6.04 ± 0.11 vs. 3.02 ± 0.13 , $P < 0.01$; 24 h: 7.38 ± 0.15 vs. 3.02 ± 0.20 , $P < 0.01$; 72 h: 9.32 ± 0.08 vs. 3.02 ± 0.08 , $P < 0.01$) (Figure 3A). HET0016 significantly reduced the MDA production at 24 h (5.94 ± 0.21 , $P < 0.01$) and 72 h (7.25 ± 2.63 , $P < 0.01$) but not 3 h (5.84 ± 0.09 , $P > 0.05$) compared with production in the vehicle-treated group (Figure 3A).

Our results also revealed that the activity of SOD decreased at 3, 24, and 72 h in the TBI group, compared with activity in the sham group (3 h: 53.84 ± 2.12 vs. 91.68 ± 0.99 , $P < 0.01$; 24 h: 42.22 ± 0.85 vs. 91.67 ± 0.98 , $P < 0.01$; 72 h: 29.89 ± 0.53 vs. 91.64 ± 1.09 , $P < 0.01$) (Figure 3B). HET0016 significantly increased the activity of SOD at 24 h (72.62 ± 1.01 , $P < 0.01$) and 72 h (7.25 ± 2.63 , $P < 0.01$) but not 3 h (82.22 ± 1.41 , $P > 0.05$) compared with activity in the vehicle-treated group (Figure 3B).

Total antioxidative capability (T-AOC) was observed to decrease at 3, 24, and 72 h in the TBI group, compared with that of the sham group (3 h: 6.74 ± 0.15 vs. 2.14 ± 0.11 , $P < 0.01$; 24 h: 5.78 ± 0.08 vs. 2.12 ± 0.13 , $P < 0.01$; 72 h: 4.48 ± 0.08 vs. 2.12 ± 0.24 , $P < 0.01$) (Figure 3C). HET0016 significantly increased T-AOC at 24 h (6.8 ± 0.07 , $P < 0.01$) and 72 h (8.0 ± 0.37 , $P < 0.01$) but not at 3 h (5.82 ± 0.08 , $P > 0.05$) compared with that of the vehicle-treated group (Figure 3C).

Expression of JNK and c-Jun

A Western blot of rat brain homogenates was performed with antibodies specific for the phosphorylated active forms of JNK and c-jun. The expression of phospho-JNK (p-JNK) (Figures 4A,B) and phospho-c-jun (p-c-jun) (Figures 4C,D) were significantly increased in the TBI group at 3, 24, and 72 h after injury ($P < 0.05$). Interestingly, p-JNK and p-c-jun

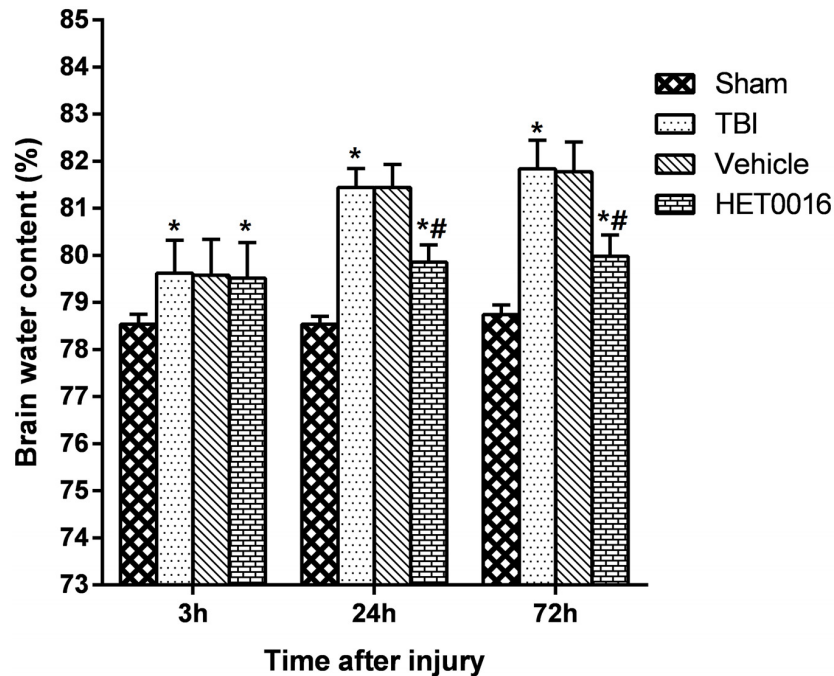


FIGURE 1 | The effect of HET0016 on brain edema. Water content of traumatic brain tissues in the sham, traumatic brain injury (TBI), vehicle and HET0016-treated groups at 3, 24, and 72 h after injury. Data are shown as the mean \pm SD. * $P < 0.05$ compared with the corresponding sham group at the same time point; # $P < 0.05$ compared with the vehicle-treated group.

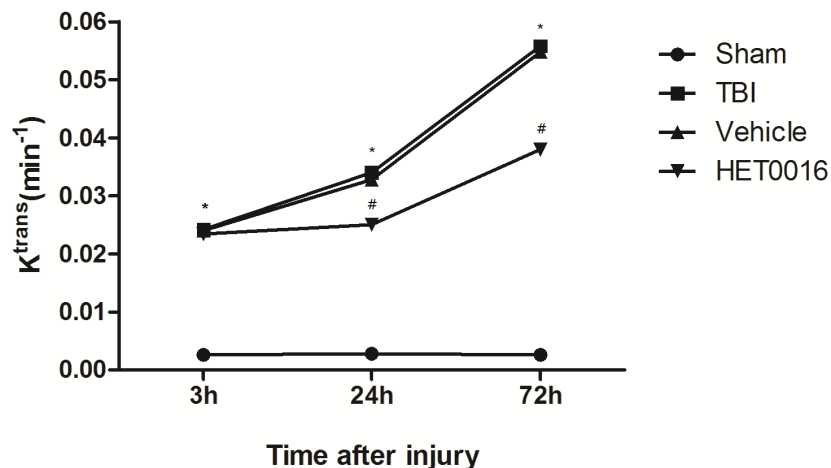


FIGURE 2 | The effect of HET0016 on the blood–brain barrier permeability. In the focal area, K^{trans} in the sham, TBI, vehicle and HET0016-treated groups at 3, 24, and 72 h after injury. Data are represented as the mean \pm SD. * $P < 0.05$ compared with the corresponding sham group at the same time point; # $P < 0.05$ compared with the vehicle-treated group.

expression were decreased in HET0016-treated group, compared with expression in the vehicle-treated group at 24 and 72 h after injury ($P < 0.05$).

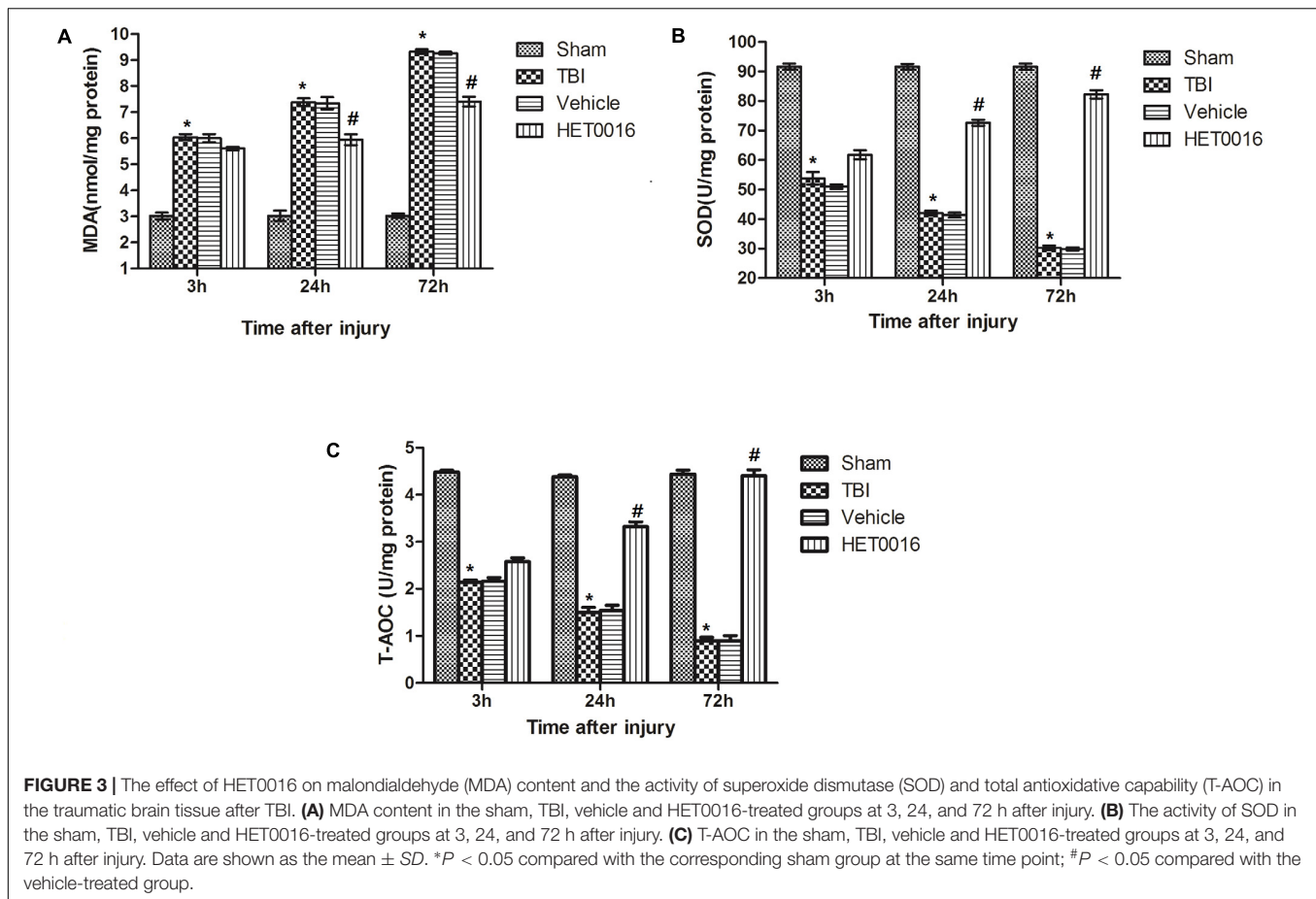
Expression of ZO-1 and Occludin

Western blot analysis also revealed that the expression of ZO-1 (Figures 5A,B) and occludin (Figures 5C,D) markedly diminished in the TBI group compared with the sham group at

3, 24, and 72 h after injury ($P < 0.05$). The administration of HET0016 significantly increased the expression levels of ZO-1 and occludin at 24 and 72 h after injury ($P < 0.05$).

Expression of MMP-9

As shown in Figures 6A,B, the expression of MMP-9 was upregulated in the TBI group compared with expression in the sham group at 3, 24, and 72 h after injury ($P < 0.05$). Meanwhile,



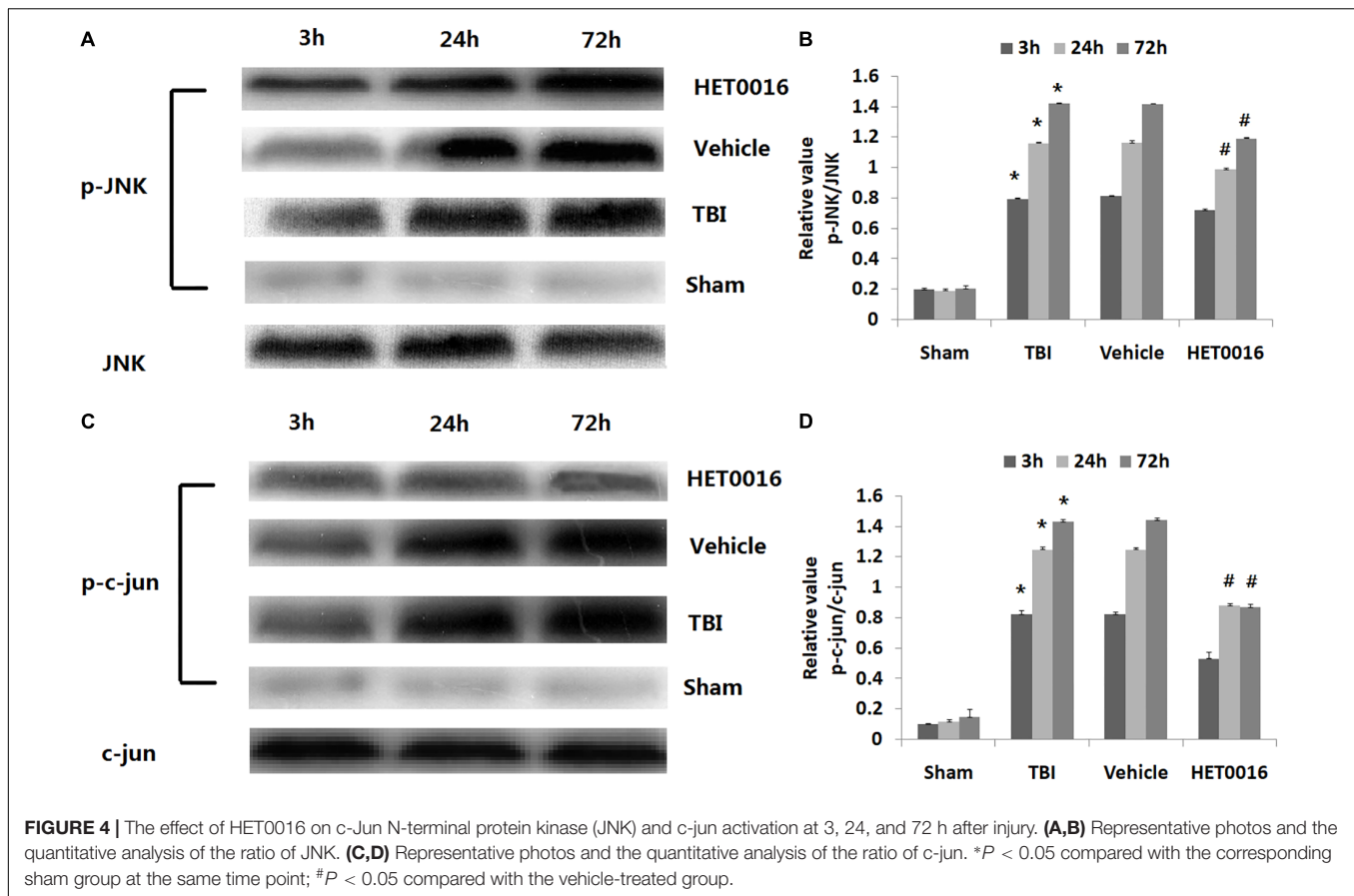
HET0016 administration significantly decreased the expression of MMP-9 compared with expression in the vehicle-treated group ($P < 0.05$) at 24 and 72 h after injury.

DISCUSSION

In the present study, the water content of brains was observed to increase as early as 3 h after injury (Figure 1), which is consistent with the previous reports showing that brain edema occurs 3 h after the onset of injury (Wei et al., 2012; Jungner et al., 2015). Importantly, we found that brain water content decreased at 24 and 72 h after TBI in the HET0016-treated group, compared with the vehicle-treated group, suggesting that HET0016 administration plays a protective role in traumatic brain edema (Figure 1). Several methods are currently available that can evaluate the BBB integrity, including Evans Blue (Lahoud-Rahme et al., 2009), histology (Lescot et al., 2010), cerebrospinal fluid serum albumin index (CSFAI) (Song et al., 2011) and magnetic resonance imaging (MRI) (Wei et al., 2012). Of these tools, only MRI allows for the repeated sampling within the same subjects over time in a non-invasive manner. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and its volume transfer coefficient (K^{trans}) can be used to quantitatively analyze the BBB permeability and have been

applied widely to monitor tumor angiogenesis (Haris et al., 2008; Song et al., 2011). DCE-MRI was used to assess the BBB permeability of traumatic brain tissue in this study. We suggested that HET0016 administration might protect the integrity of the BBB and decrease the permeability of the BBB in TBI. Here, we showed for the first time, that HET0016 could decrease the BBB permeability and brain edema in a traumatic rat model. One explanation may be based on the pathology of edema. Traumatic brain edema consists of cytotoxic edema and vasogenic edema (Wei et al., 2012). It is generally agreed that cytotoxic edema occurs within 30 min after an injury, which is attributed to the increased inflammatory and oxidative stress on the BBB, along with enzymatic degradation of the extracellular matrix (Jungner et al., 2015; Hanrahan and Campbell, 2016). The vasogenic edema, however, appears at least several hours after a TBI (Jungner et al., 2015; Hanrahan and Campbell, 2016). HET0016 reduces the brain water content after injury, probably because the effect of HET0016 administration on brain edema is mainly due to its role in decreasing the cytotoxic and vasogenic edema.

To further explore the underlying mechanisms of the effect of HET0016 administration on brain edema in this study, the ROS generation in rat brains was measured. The results showed that ROS markedly increased in the TBI group compared with the sham group, and excessive ROS contribute to the weakening of

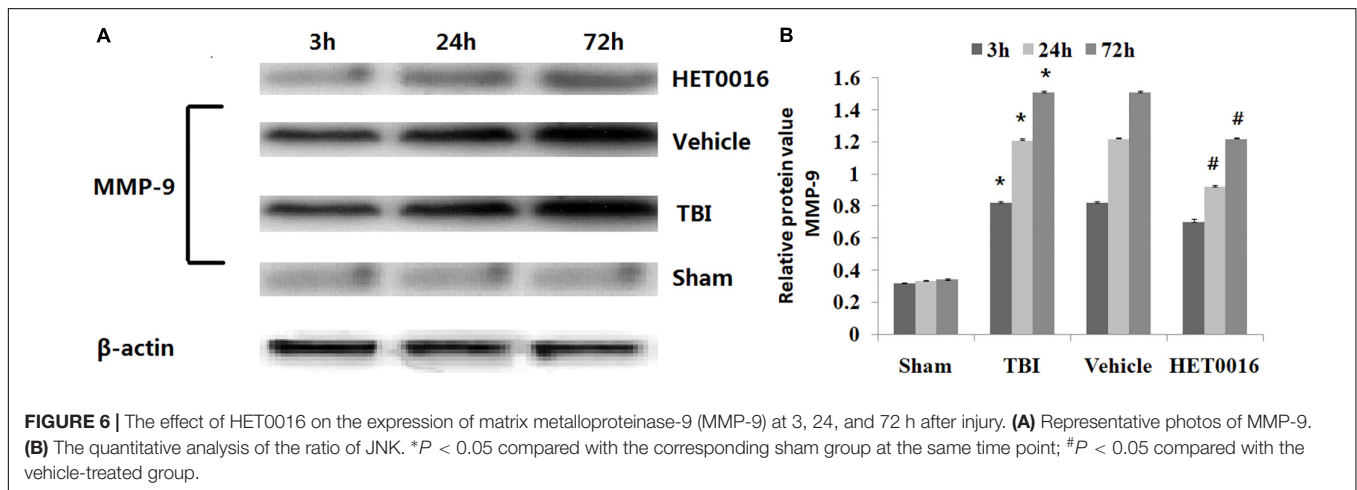
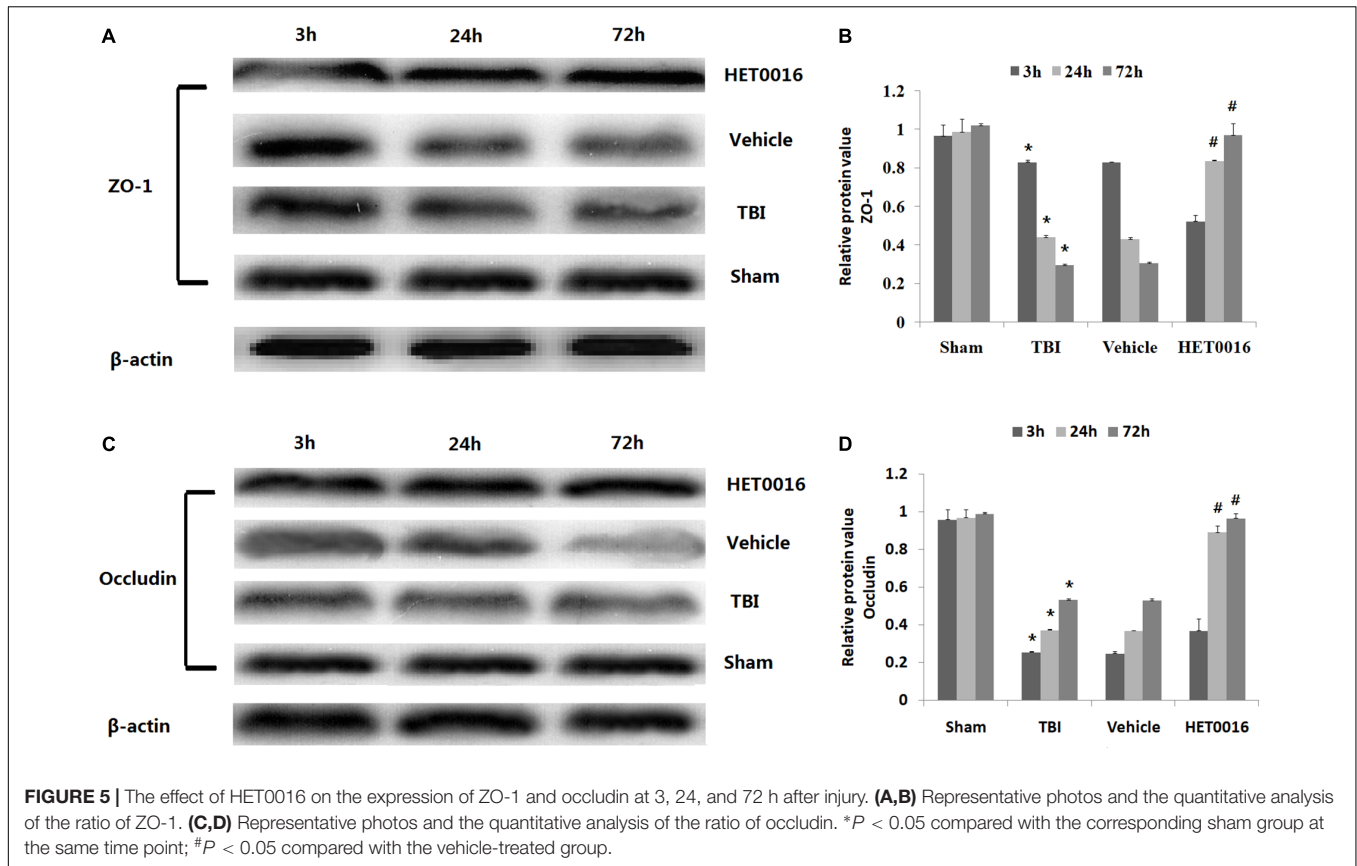


the BBB. The present study showed that the production of ROS decreased after TBI in the HET0016-treated group compared with the vehicle-treated group, which suggested that the effect of HET0016 on the BBB opening and brain edema may due to the decrease in oxidative stress. Several studies have demonstrated that oxidative stress is strongly correlated with MMP-9 in the pathophysiology of BBB damage in TBI (Lapchak et al., 2000; Gasche et al., 2001). Oxidative stress can trigger numerous cellular and molecular cascades that mediate the activation of MMP-9 (Gu et al., 2013), leading to the degradation of tight junction proteins (ZO-1 and occludin) and increasing the BBB permeability in traumatic brain tissues (Wang Z.G. et al., 2015). Our results showed that the expression of MMP-9 was increased after TBI, which is in line with the results of previous studies (Gu et al., 2013; Wang Z.G. et al., 2015). In this study, the expression of tight junction proteins (ZO-1 and occludin) also changed following TBI. These results suggest that the effect of HET0016 administration on the BBB permeability and brain edema in TBI may due to the regulation of MMP-9 expression and of tight junction proteins of the BBB. Furthermore, both our study and other previous studies have shown that the JNK signaling pathway is involved in neuronal injury triggered by TBI (Liu et al., 2014). A recent study revealed that the JNK pathway has an important effect on the regulation of tight junction proteins and the BBB integrity and that SP600125 (JNK inhibitor) protects the BBB integrity by suppressing the activation

of MMP-9 (Urrutia et al., 2013). The present study also showed that the expression of JNK and its downstream transcription factor c-jun induced by TBI is more likely to be attenuated by HET0016 administration, suggesting that the effect of HET0016 administration on MMP-9 may be inhibited by the JNK signaling pathway. There is a strong relationship between 20-HETE and the expression of MMP-9 via the PI3K and ERK1/2 pathways in human non-small cell lung cancer cells (Yu et al., 2011). The underlying mechanisms by which HET0016 administration affects the expression of MMP-9 might be different in different species and cell types. Our finding also revealed that the potential mechanism of the effect of HET0016 administration on MMP-9 may be partially based on the decrease in ROS production and the increase in activity of SOD and T-AOC. Fordsmann et al. (2013) demonstrated that HET0016 blocked the reduction in CBF in a rat model. Therefore, the protective effect of HET0016 on the BBB and brain edema formation may also partly be due to the restoration of CBF.

CONCLUSION

HET0016 administration plays an important role in protecting the BBB integrity and reducing brain edema after TBI. The mechanism of this protective role is more likely to be associated with the decrease of oxidative stress and the increase of SOD and



T-AOC activity, which regulate the expression of MMP-9 and tight junction proteins (ZO-1 and occludin) by the inhibition of the JNK pathway.

MATERIALS AND METHODS

Animals and Materials

Four to six-week-old male Sprague-Dawley (SD) rats (body weight, 180–260 g) (Shanghai SLAC Laboratory Animal

Corporation, Shanghai, China) were studied. Rats were housed in a 12/12 h light/dark cycle environment and with provided food and water. This study was approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Biomedical Engineering. Antibodies against MMP-9, occludin, ZO-1, and anti-phospho-JNK rat polyclonal antibody and anti-phospho c-Jun rat polyclonal antibody were purchased from Abcam, Inc. (Abcam, England). HET0016 was purchased from Cayman Chemical Company (Ann Arbor, MI, United States).

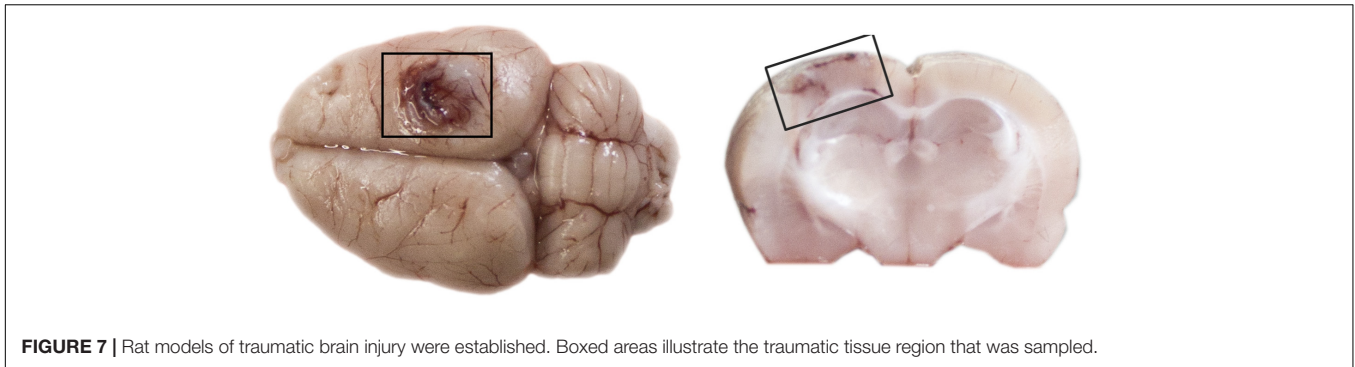


FIGURE 7 | Rat models of traumatic brain injury were established. Boxed areas illustrate the traumatic tissue region that was sampled.

Animal Model

Sprague-Dawley rats were randomly divided into four groups: (1) TBI group (**Figure 7**): The rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg), and mounted in a stereotaxic frame (Stoelting, Wood Dale, IL, United States). A 15-mm long midline scalp incision was made and a craniotomy (6 mm in diameter) was then performed over the central aspect of the right parietal cortex, 2 mm lateral to the sagittal suture. Care was taken to keep the dura intact when it was exposed. A controlled cortical injury model of TBI was established by using an impact device (PinPoint Precision Cortical Impactor PCI3000; Hatteras Instruments Inc., Cary, NC, United States) with a 2.5-mm, rounded, steel impactor tip. A moderate injury was produced using a right parietal cortex deformation depth of 2.5 mm, a velocity of 1.5 m/s and duration time of 85 ms, and then, the incision was closed. After recovering from the injury, the rats were returned to their home cages and allowed free access to water and food. (2) Sham group: The sham injury group underwent the same surgery as the injured rats but received no impact. (3) HET0016-treated group: The HET0016-treated group underwent the same surgery as the injured rats and was administered intravenously, just before injury, 1.2 mg/kg HET0016 dissolved in 15% hydroxypropyl- β -cyclodextrin (1 mg/ml), according to a previous study and our preliminary study. (4) Vehicle-treated group: The vehicle-treated group underwent the same surgery as the injured rats and was administered an equal volume of solvent. Rats were excluded from the study if the dural integrity was breached. There were five rats in each group.

BBB Edema

Brain edema was measured by using the wet-dry weight method, as described previously. Briefly, the wet weight of a 3-mm coronal tissue section of the ipsilateral cortex, centered on the impact site were measured quickly after the mice were sacrificed at 3, 24, and 72 h of injury. The tissue samples were then dried in an oven at 100°C for 48 h to obtain the dry weight. Tissue water content of each time was calculated as follows: (wet weight–dry weight)/wet weight \times 100%.

BBB Permeability

BBB permeability was investigated at 3, 24, and 72 h following TBI by DCE-MRI (Fordsmann et al., 2013). All rats

underwent MRI using a clinical 3.0 T MRI scanner with an eight-channel head coil (Intera Achieva SMI-2.1; Philips Medical Systems, Netherlands). The MRI sequences were as follows: T2W image, T2 map, post-T1W image, and DCE-MRI. Image processing software (CINEtool, GE Healthcare) was used to obtain the quantitative parameters (K^{trans}) of DCE-MRI. K^{trans} was calculated based on a modified two-compartment model (**Figure 8A**) (Lu et al., 2018). In this model, K^{trans} was determined by the Gd-DTPA influx from the intravascular space into the extravascular extracellular space. The K^{trans} was calculated by manually outlining the regions of interests (ROIs). Three ROIs at all time points were manually drawn on a 2-mm single slice of the T2W scan at 72 h, as shown in **Figure 8B** (Lu et al., 2018). ROIs for the sham and TBI groups included the focal lesion areas and the contralateral brain area. First, we outlined the hyperintensity area of the T2W images (**Figure 8C**) (Lu et al., 2018) after TBI by manually using the signal intensity difference of T2W (the difference threshold was 300) to define the border between the hyperintensity area and the healthy-appearing tissue. Then, additional T2 maps (scaling of 0–150 ms) were used to define the border between hyperintense and healthy-appearing tissue when the border was ill-defined on the T2W images. The focal lesion area refers to this outlined hyperintensity area, which may include cortex, hippocampal tissues, etc. This procedure was performed by two radiologists with more than 5 years of experience.

Measurement of MDA, SOD, and T-AOC Activity

The levels of MDA, oxidative stress-SOD, and T-AOC were measured using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, after TBI, the left brain hemispheres of mice were removed and homogenized in ice-cold PBS. The samples were centrifuged at 2000 g for 10 min at 4°C. The supernatant was used for the measurement of MDA, SOD, and T-AOC activity, according to the manufacturers' instructions.

Western Blot Analysis

Brains were removed and cut into two hemispheres. The focal lesion areas were homogenized in a buffer containing 50 mM

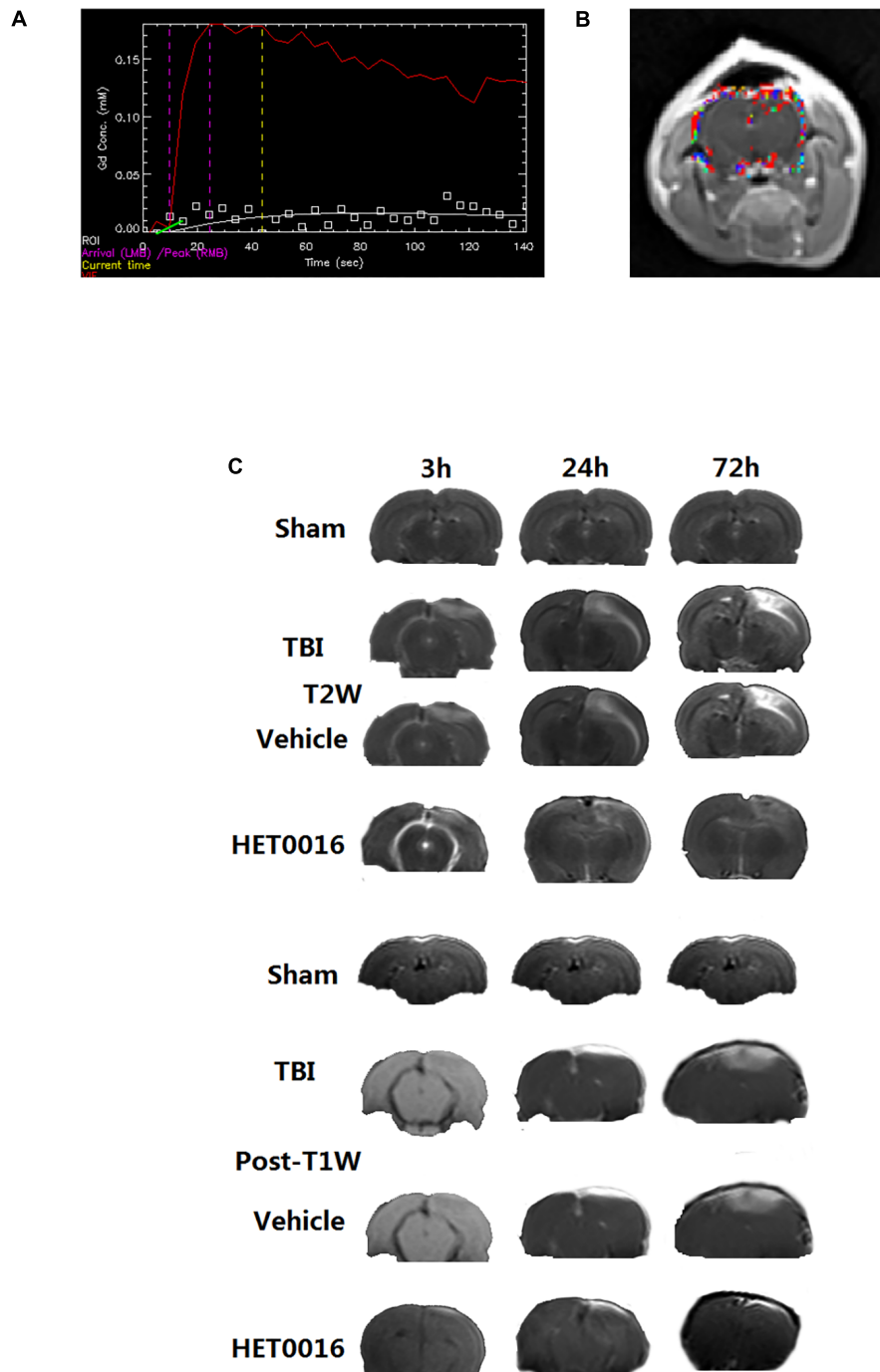


FIGURE 8 | Magnetic resonance imaging (MRIs) from a representative animal. **(A)** Quantitative analysis of a typical animal brain dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) with post-processing software (CINEtool; GE Healthcare) 72 h after TBI. The white line represents the lesioned area, and the red line indicates the middle cerebral artery. **(B)** K^{trans} map of the same animal. The blood-brain barrier permeability is significantly increased at the site of the lesion, and HET0016 administration significantly decreased the blood-brain barrier permeability. **(C)** T2-WI images show the focal lesion, while the post-contrast T1-WI (post-T1-WI) images show the Gd-DTPA enhancement in the focal lesion area at different time points (3, 24, and 72 h after TBI).

Tris (pH = 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 2 mM PMSF. The brain extracts were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were used for experiments. The protein concentration of the supernatant

was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL, United States) with bovine serum albumin as a standard. Protein (50 µg) was subjected to 12% SDS-PAGE and then was transferred to nitrocellulose membranes. Western

blot analysis was carried out with antibodies against occludin, ZO-1, MMP-9, p-JNK, and p-c-jun. Immunoreactive bands were identified, and a densitometric analysis was performed with an enhanced chemiluminescence detection system (Amersham, United States).

Statistical Analysis

All data are expressed as the mean \pm SD. Differences between groups were compared by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test (SNK) in the case of a significant difference between groups. A *P* less than 0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS

LL and MW conceived and designed the experiments. LL, MW, and XW performed the experiments and analyzed the data. LL and WL prepared the first draft of the manuscript. All authors reviewed and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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