

# Cerebral Hemorrhage, Brain Edema, and Heme Oxygenase-1 Expression After Experimental Traumatic Brain Injury

Shuichi Okubo, Guohua Xi, Richard F. Keep, Karin M. Muraszko, and Ya Hua

**Abstract** Intracranial bleeding is a common and serious consequence of traumatic brain injury (TBI). In the present study, we investigated cerebral hematoma occurrence, brain edema formation, blood–brain barrier (BBB) disruption, and heme oxygenase-1 (HO-1) expression after TBI. Moderate severity (1.8–2.2 atmospheres [ATM]) TBI was induced by lateral fluid percussion in male adult Sprague–Dawley rats. Sham rats underwent only a craniotomy. Rats were euthanized 24 h later for brain histology and immunoblotting analysis. We found TBI-induced cerebral hematomas and iron deposition in the ipsilateral hemisphere in all rats. TBI also caused marked BBB disruption ( $p < 0.05$ ) and brain swelling ( $p < 0.05$ ). HO-1, a key enzyme for heme degradation, was upregulated significantly after TBI ( $419 \pm 89$  vs  $194 \pm 59$  pixels in the sham,  $p < 0.05$ ). These results suggest that cerebral hematomas might play a role in brain injury after TBI. Future studies should determine the role of iron released from the cerebral hematoma in TBI.

**Keywords** Brain edema • Cerebral hemorrhage • Iron • Traumatic brain injury

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S. Okubo  
Department of Neurosurgery,  
University of Michigan Medical School,  
R5018 Biomedical Science Research Building,  
109 Zina Pitcher Place, Ann Arbor, MI, 48109-2200, USA

Department of Neurosurgery,  
Kagawa Medical University,  
Kagawa, Japan

G. Xi, MD, R.F. Keep, K. M. Muraszko, and Y. Hua, MD (✉)  
Department of Neurosurgery,  
University of Michigan Medical School,  
R5018 Biomedical Science Research Building,  
109 Zina Pitcher Place, Ann Arbor, MI, 48109-2200, USA  
e-mail: yahua@umich.edu

## Introduction

Each year in the United States, approximately 1.6 million people suffer a traumatic brain injury (TBI) and there are more than 5 million Americans currently living with TBI-related disabilities [10]. There are currently no effective therapies for TBI patients. Intracranial bleeding is a common and serious consequence of TBI. MRI has shown a preponderance of hemorrhagic lesions and few ischemic lesions in the acute phase and 56 % of TBI patients were reported to have at least one intracranial bleed [12]. The release of iron from the breakdown of hemoglobin during hematoma resolution may result in a build-up of non-heme iron in brain tissue and brain damage.

In the present study we established a TBI model with cerebral hematoma. The lateral fluid percussion (LFP) model produces both focal and diffuse injury with vascular disruption, neuronal cell death and glial proliferation [1, 2, 7, 14, 15, 17, 20].

## Materials and Methods

### *Animal Preparation and Experimental Group*

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals at the University of Michigan. Adult male Sprague–Dawley rats (Charles River Laboratories, Portage, MI, USA) weighing 260–340 g were used in this study. Animals were euthanized at 24 h post-TBI or sham operation for brain histology and Western blot analysis.

## Lateral Fluid Percussion Injury

Traumatic brain injury was induced by lateral fluid percussion injury, as described previously [3, 15]. In brief, rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and rectal temperature was maintained at 37.5 °C by using a feedback-controlled heating pad. The animals were placed in a stereotaxic frame, and the scalp and temporal muscle were reflected. A 4.8-mm diameter hole was drilled between the bregma and lambda on the right convexity (anterior edge 2.0 mm posterior to the bregma; lateral edge adjacent to the left lateral ridge), and a hollow female Luer–Lock fitting secured with dental cement. Lateral fluid percussion injury was induced by a transient (21–23 ms) fluid pulse impact against the exposed dura by using a fluid-percussion device (AmScien Instruments, Richmond, VA, USA). The impact pressure was measured by an extra-cranial transducer and controlled to 1.8–2.2 atmospheres (ATM). After impact, the dura was checked to ensure that it had remained intact. Sham-operated control animals underwent all surgical procedures, except the fluid-percussion impact. Animals that did not survive the injury were predetermined to be excluded from the study.

## Histological and Immunohistochemical Study

Rats were reanesthetized with pentobarbital (60 mg/kg, i.p.), followed by intracardiac perfusion with 4 % paraformaldehyde in 0.1 mol/L phosphate-buffered saline, pH 7.4. Brains were removed, kept in 4 % paraformaldehyde for 24 h, and immersed in 25 % sucrose for 3–4 days at 4 °C. The brains were then embedded in an optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA, USA) and sectioned on a cryostat (18 µm). Brain swelling was assessed morphometrically. Coronal sections from 1 mm posterior to the anterior edge of the craniotomy site were stained with hematoxylin and eosin. On the microscopic images, the area of the ipsilateral or contralateral hemisphere was measured using ImageJ software. Brain swelling was estimated by the ratio of the volume of (ipsilateral – contralateral)/contralateral hemisphere. Immunohistochemical staining was performed using avidin–biotin complex technique. The primary antibody was polyclonal rabbit anti-rat HO-1 IgG (1:200 dilution, StressGene) and the secondary antibody was biotinylated goat anti-rabbit IgG (1:500 dilution, Vector Laboratories). Normal rabbit IgG was used as negative control. Brain iron deposition was determined by enhanced Perls' reaction [22].

## Western Blot Analysis

Animals were anesthetized before undergoing transcardiac perfusion with 0.1 mol/L phosphate-buffered saline. The brains were removed and a 4-mm thick coronal brain slice was cut approximately 5 mm from the frontal pole. The slice was separated into ipsilateral or contralateral cortex and basal ganglia. Western blot analysis was performed as previously described [24]. The primary antibodies were polyclonal rabbit anti-rat HO-1 IgG antibody (1:2,500 dilution; StressGen, Farmingdale, NY, USA) and polyclonal sheep anti-rat albumin antibody (1:20,000 dilution; Bethyl Lab, Montgomery, USA). The secondary antibodies were peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories, Hercules, CA, USA) and rabbit anti-sheep antibody (Millipore, Billerica, MA, USA).

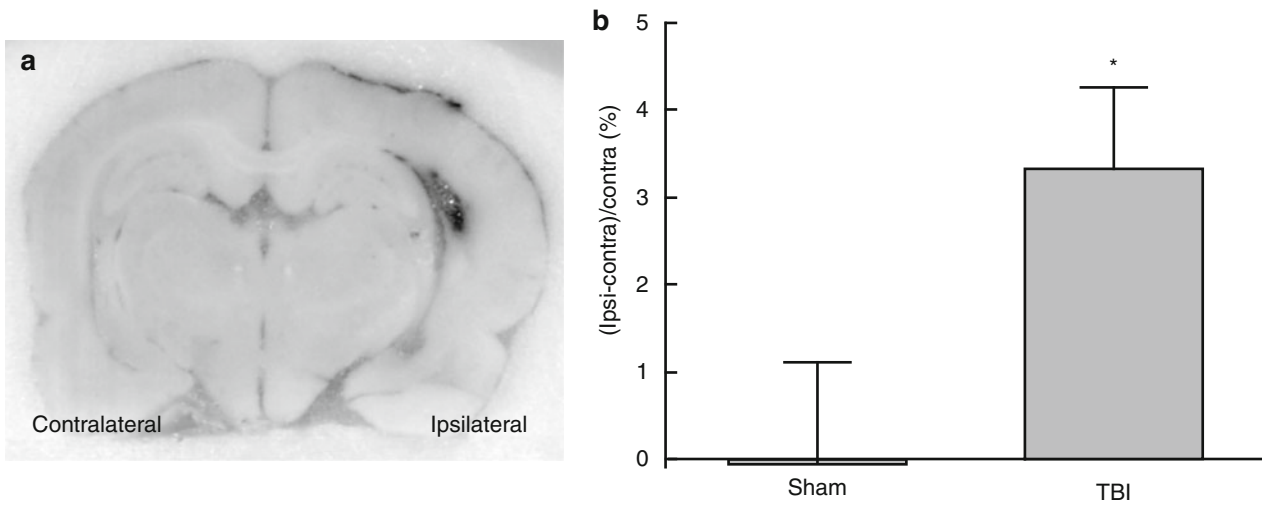
## Statistical Analysis

All data in this study are presented as mean ± standard deviation. Data were analyzed using Student's *t* test or Kruskal–Wallis test. Statistical significance was set at  $p < 0.05$ .

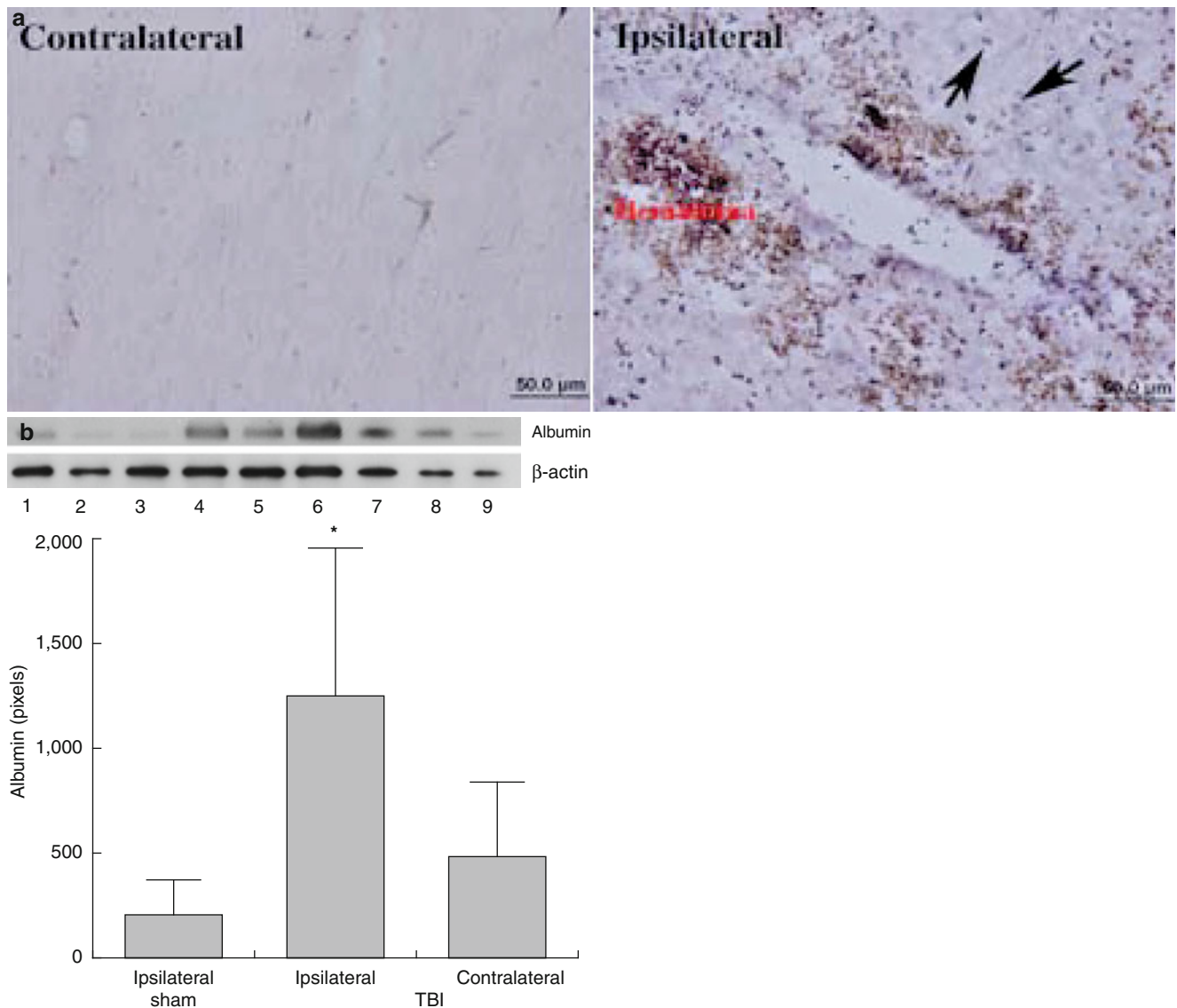
## Results

All animals undergoing lateral fluid percussion had intracerebral hematomas in the ipsilateral cortex and caudate at 24 h (Fig. 1a). TBI caused ipsilateral brain swelling ( $3.3 \pm 0.9$  % vs  $-0.04 \pm 1.2$  % in the sham group,  $p < 0.05$ , Fig. 1b). Release of iron from the degradation of heme occurred around clots 24 h after brain trauma, as indicated by Perls' staining. Iron-positive cells were found in the ipsilateral hemisphere, especially around hematomas (Fig. 2a). TBI also resulted in blood–brain barrier disruption. Albumin levels were increased in the ipsilateral hemisphere of TBI rats ( $1,248 \pm 710$  vs  $204 \pm 171$  pixels in the sham group,  $p < 0.05$ , Fig. 2b).

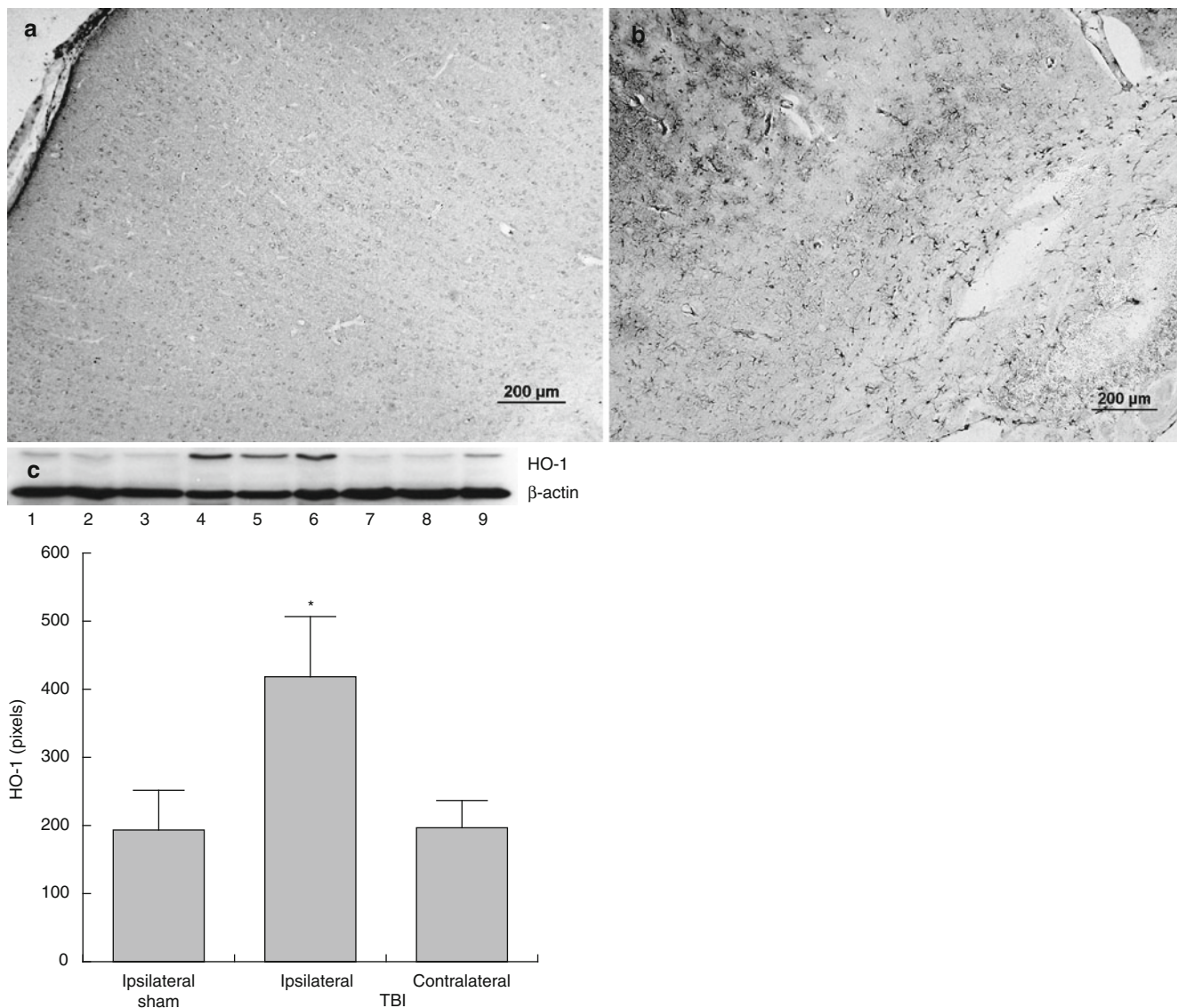
Immunoreactivity of HO-1 was increased markedly in the ipsilateral cortex and caudate of TBI rats (Fig. 3a, b). Most HO-1 positive cells were microglia-like cells. HO-1 protein levels were determined by Western blot analysis and we found that HO-1 levels in the ipsilateral cortex were significantly higher in TBI rats ( $419 \pm 89$  vs  $194 \pm 59$  pixels in the sham,  $p < 0.05$ , Fig. 3c).



**Fig. 1** (a) Cerebral hematomas in the coronal brain section of a rat. The rat had lateral fluid percussion-induced traumatic brain injury (TBI). (b) Brain swelling was expressed as the volume of (ipsilateral – contralateral)/contralateral hemisphere. Values are mean ± SD, *n*=4–5, \**p*<0.05 vs sham



**Fig. 2** (a) Perls' staining showing iron deposition in the ipsilateral hemisphere 24 h after TBI, scale bar=50 μm; (b) Albumin levels in the ipsilateral hemisphere of sham rats (lanes 1–3) or in the ipsilateral hemisphere (lanes 4–6) or in the contralateral hemisphere (lanes 7–9) of TBI rats. Values are mean ± SD, *n*=3, \**p*<0.05 vs sham. Arrows indicate perls' positive cells



**Fig. 3** HO-1 immunoreactivity in the ipsilateral cortex of sham rat (a) or TBI rat (b). Scale bar=200 μm. HO-1 protein levels (c) in the ipsilateral hemisphere of sham-operated rats (lanes 1–3) and in the ipsilateral

hemisphere (lanes 4–6) and the contralateral hemisphere (lanes 7–9) of TBI rats at 24 h. Values are mean ± SD,  $n=3$ ,  $*p<0.05$  vs the other groups

## Conclusion

In this study, we found that TBI resulted in cerebral hematomas, brain swelling, and BBB disruption. HO-1 upregulation and brain iron deposition were also found in the ipsilateral hemisphere 24 h after TBI.

Intracranial bleeding is a common and serious consequence of TBI and contributes to the injury. The primary brain injury after trauma may manifest as diffuse axonal injury, intraparenchymal contusions, intracranial hematomas, diffused axonal injury, and skull fractures [9, 12]. Brain edema after TBI is a common and serious consequence of

severe traumatic brain injury [6]. BBB disruption was reported previously after TBI [4, 19]. A brain contusion results in immediate cell death in a limited region of the brain. But secondary cell loss and chronic atrophy can occur at sites other than at or near the primary injury [13]. It may be related to regional metabolic distress that was mediated by accumulated iron-induced oxidative stress and metabolic dysfunction [25].

Iron deposition occurs in TBI brain, as indicated in this study using Perls' staining. We have gathered substantial evidence that iron plays a major role in brain injury after intracerebral hemorrhage and subarachnoid hemorrhage. The release



of iron from the breakdown of hemoglobin during hematoma resolution in the rat results in a build-up in the levels of non-heme iron in brain tissue. The high level of non-heme iron remains in the brain for at least 28 days [22]. Through enhanced Perls' reaction, iron-positive cells were found in the perihematomal zone as early as the 1st day [22], as found in our study with TBI. Increases in brain iron levels cause brain edema, oxidative stress, BBB disruption, brain atrophy, and neurological deficits following intracranial hemorrhage (ICH) [8, 16, 21, 23]. Our previous studies showed that free iron levels in cerebrospinal fluid (CSF) increase almost 14-fold on the third day after ICH, and remain high for at least 28 days after experimental ICH [18]. The duration of iron overload after lateral fluid percussion remains to be elucidated.

Brain HO-1 levels are increased after TBI. HO-1, also called heat shock protein 32, is an enzyme for heme degradation into iron, carbon monoxide, and biliverdin. It can be induced in the brain by heat shock, heme and hemoglobin, a variety of oxidants [23], and TBI [5]. In the rat, marked HO-1 upregulation at day 3 accompanied by the elevation of nonheme iron, transferrin, transferrin receptor, and ferritin levels after subarachnoid hemorrhage [11].

In conclusion, TBI induced cerebral hematomas, brain edema formation, BBB disruption, and iron deposition. Cerebral hematomas and brain iron overload could be therapeutic targets for TBI.

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**Conflict of Interest** We declare that we have no conflict of interest.

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